C1–Membrane ATPases and channels

C1-001
Aquaporin Water Channels: From Atomic Structure to Clinical Medicine

P. Agre
Johns Hopkins School of Medicine, Baltimore, MD, USA.
E-mail: pagre@jhmi.edu

The high water permeability of certain biological membranes is due to the presence of aquaporin water channel proteins. AQP1 was discovered in human red cells. AQP1 has been thoroughly characterized biophysically, and the atomic structure of AQP1 has been elucidated. Ten homologs have been identified in humans. These are selectively permeated by water (aquaporins) or water plus glycerol (aquaglyceroporins). The sites of expression predict the clinical phenotypes in humans. Individuals lacking Colton blood group antigens have mutations in the AQP1 gene. When deprived of water, AQP1-null individuals exhibit a defect in urine concentration and a marked reduction in fluid exchange between capillary and interstitium in lung. AQP1 is expressed in multiple tissues where physiologically important fluid secretion is known to occur including choroid plexus and anterior chamber of eye. AQP0 is expressed in lens fiber cells and mutations result in familial cataracts. AQP2 is expressed in renal collecting duct principal cells where membrane trafficking is regulated by vasopressin. Mutations in the human AQP2 gene result in nephrogenic diabetes insipidus, but underexpression is found in clinical disorders with reduced urinary concentration (e.g. lithium therapy and nocturnal enuresis) and overexpression is found in disorders with fluid retention (e.g. congestive heart failure and pregnancy). AQP5 is expressed in the apical membranes of salivary and lacrimal gland acini, and mistargeting has been identified in some patients with Sjogren's syndrome. Involvement of aquaporins is expected in other human clinical disorders such as brain edema and muscular dystrophy (AQP4), anhidrosis (AQP5) renal tubular acidosis (AQP6), conversion of glycerol to glucose during starvation (AQP7 and AQP9), and cystic fibrosis (several aquaporins). Aquaporins are known to protect micro-organisms from freezing and osmotic shock. Plant aquaporins are involved in numerous processes including the uptake of water by rootlets and carbon dioxide by leaves. The physiological roles of aquaporin homologs are being pursued by multiple laboratories worldwide.

C1-002
The cardiac glycoside binding site of the Na,K-ATPase plays a role in blood pressure regulation

J. Lingrel, I. Dostanic[1], J. Neumann[1], J. Lorenz[2] and J. Van Huysse[3]
[1]*Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, Ohio, United States of America,* [2]*Department of Molecular and Cellular Physiology, University of Cincinnati, Cincinnati, Ohio, United States of America,* [3]*Department of Medicine and Biochemistry, Hypertension Unit, University of Ottawa, Heart Institute, Ottawa, Ontario, Canada. E-mail: jerry.lingrel@uc.edu*

Na,K-ATPase transports sodium ions out of cells and potassium ions in utilizing ATP as the driving force. The gradients formed by this enzyme are coupled to a variety of physiological processes and because there are multiple isoforms of both the á and â subunits, it is possible that individual isoforms play specific biological functions. In order to address this question, we have developed mice that lack the α1 or α2 isoform genes, as well as animals where the role of each of these two α isoforms can be examined individually. Our studies demonstrate that both the α1 and α2 isoforms play a similar function in cardiovascular physiology. In addition, we are addressing whether the highly conserved cardiac glycoside binding site of the Na,K-ATPase plays a functional role *in vivo*. To accomplish this, we have used gene-targeting procedures to develop mice where the α2 isoform, which is naturally sensitive to ouabain, is relatively resistant to this compound. These animals develop normally and have normal baseline cardiovascular hemodynamics indicating that the cardiac glycoside binding to Na,K-ATPase does not play a significant role under normal conditions. However, when these targeted animals are exposed to conditions, which are known to increase blood pressure, they fail to develop hypertension, in contrast to wild type mice, which express the cardiac glycoside sensitive α2 isoform. These studies suggest that cardiac glycoside binding of the α2 isoform of the Na,K-ATPase plays a physiological role *in vivo*. As endogenous cardiac glycosides increase along with blood pressure, they represent potential ligands for the α2 isoform receptor.

C1-003
TRP channels, mediators of sensory signaling

C. Montell
Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD, United States of America. E-mail: cmontell@jhmi.edu

TRP channels comprise a large family of cations, which are conserved from worms to humans. We identified the original member of this superfamily as a channel required for *Drosophila* phototransduction. This channel, TRP, functions as part of a supermolecular signaling complex, which includes the INAD scaffold protein, protein kinase C, rhodopsin, myosin III and other proteins. The complex is required for normal localization of TRP and other signaling proteins and for rapid signaling. The TRP channel is critical for calcium entry in photoreceptor cells. However, the extrusion of calcium is equally important for signaling. Nevertheless, the protein functioning in calcium extrusion in fly photoreceptor cells has been elusive. We will present our recent findings that a sodium/calcium exchanger co-localizes with TRP in photoreceptor cells and is critical for many aspects of visual transduction. An emerging theme is that many members of the *Drosophila* and mammalian TRP superfamily function in sensory signaling. These include roles in thermosensation, mechanosensation and chemosensation. We have recently identified a *Drosophila* TRP channel, referred to as TRPA2, which is required for both taste and smell. Our recent analyses of TRPA2 will be presented, along with related studies concerned with uncovering the roles of other proteins functioning in *Drosophila* taste. We will also present our recent analyses of TRP channels, which function in other processes in flies and mammals. These include a member of the TRP superfamily that functions in male fertility.

C1-004
SPCA: the secretory pathway Ca^{2+} transport ATPase isoforms1 and 2

F. Wuytack, J. Vanoevelen[1], K. Van Baelen[1], L. Dode[1], R. J. Fairclough[2], L. Missiaen[1] and L. Raeymaekers[1]
[1]*Physiology, Molecular Cell Biology, K.U.Leuven, Leuven, Belgium,* [2]*Centre for Diabetes, Endocrine Unit, Oxford, Oxford, United Kingdom. E-mail: frank.wuytack@med.kuleuven.ac.be*

The cellular secretory pathway is comprised of an ordered series of subcellular membrane-enclosed compartments characterized by a high luminal Ca^{2+} concentration. This luminal Ca^{2+} can represent a store of activator Ca^{2+} triggering a plethora of cytosolic processes upon its release, but it is (together with Mn^{2+}) also an indispensable cofactor for the majority of the luminal secretory maturation processes. Whereas the endoplasmic reticulum (ER) acquires the Ca^{2+} through the action of the thapsigargin-sensitive SERCA2b pump, the Golgi and possibly more distal parts of the secretory pathway rely on the thapsigargin-insensitive SPCA pumps. The early Golgi compartment in addition appears to contain SERCA pumps and like the ER can still function as an IP_3-releasable store. Mutations in the SPCA1-encoding gene (*ATP2C1*) result in Hailey-Hailey skin disease. Four different splice variants SPCA1a–d were described. Of these SPCA1c refers to a transport-defective truncated form. The other isoforms are mainly targeted to the Golgi were they catalyse the accumulation of one Ca^{2+} or Mn^{2+}/ATP with submicromolar ion affinity (like in PMCA also in SPCA only ion-transport site II of SERCA is conserved). Whereas SPCA1 is expressed in fungi (yeast orthologue pmr1) and in invertebrate and vertebrate animals, *ATP2C2* with the same exon/intron layout as *ATP2C1* is found only in birds and mammals so far, but apparently is absent in invertebrates and teleost fish. *ATP2C2* encodes the SPCA2 pump which also transports Ca^{2+} or Mn^{2+} into the Golgi but its expression is limited to epithelia of the digestive and respiratory tract, mammary gland and keratinocytes.

C1-005
Molecular determinants of receptor-mediated regulation of the $2PK^+$ potassium channel, TRESK

P. Enyedi and G. Czirják
Department of Physiology, Semmelweis University, Budapest, Hungary. E-mail: enyedi@puskin.sote.hu

TRESK, the recently discovered two pore domain (2P) potassium channel is efficiently activated by stimulation of Gq coupled receptors. The effect is mediated by the cytoplasmic Ca^{2+}-signal. Microinjection of the calcium chelator, EGTA, prevents the effect of the receptor stimulation which, on the other hand, can be mimicked by the calcium ionophore, ionomycin or by intracellular microinjection of saturated Ca^{2+}-buffer. Ca^{2+} does not influence the channel directly; application of the ion to inside-out membrane patches failed to alter TRESK single channel activity. Calcineurin (the Ca^{2+}/calmodulin-dependent phosphatase 2B) was identified as the link between the Ca^{2+}-signal and TRESK activation. Inhibitors of the enzyme, cyclosporine A and FK506, blocked the ionomycin-evoked TRESK activation. In oocytes

expressing TRESK, coexpression or microinjection of a constitutively active form of calcineurin, activated the channel also in the absence of Ca^{2+}-signal. To examine whether the channel itself was phosphorylated/dephosphorylated, 17 serine or threonine residues in the intracellular domains of the channel were mutated to alanine. Three serine residues, all in the intracellular loop between the 2nd and 3rd transmembrane segments were identified as possible sites of phosphorylation. The S276A mutant showed the most striking alteration expressing high basal activity with minor further sensitivity to Ca^{2+} changes. The S276C mutant behaved similarly to S276A, while S276E mutation resulted in low basal activity, again with negligible responsiveness to Ca^{2+}. These results support the role of direct phosphorylation/dephosphorylation of TRESK, however, the putative kinase involved in the regulation has yet to be identified.

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C1-006
Functional properties of PMCA2 and its splice variants

L. Coletto[1], **A. Lelli**, C. Ortega[1], M. Brini[2], F. Mammano[1,3] and E. Carafoli[1]
[1]Venetian Institute of Molecular Medicine, Padova, Italy, [2]Department of Biochemistry, University of Padova, Padova, Italy, [3]Department of Physics "G. Galilei", University of Padova, Padova, Italy. E-mail: andrea.lelli@unipd.it

The plasma membrane Ca^{2+} pump (PMCA) is encoded by four genes. Additional variants are generated by alternative splicing at site A, located in the first cytosolic loop next to a domain sensitive to acidic phospholipids, and at site C, in the C-terminal calmodulin binding domain. As the splice variants are tissue- and development-specific, they may respond to specific Ca^{2+} demands. We have studied the properties of the PMCA2 variants: a full length pump (z/b or AI/CI), a C-terminally truncated variant (z/a or AI/CII), a variant with three exons inserted in splice site A (w/b or AIII/CI), and one with the three site A inserted exons and a C-terminal truncation (w/a or AIII/CII). These variants have been overexpressed in CHO cells and their effects on Ca^{2+} homeostasis monitored using recombinant aequorins targeted to the cytoplasm, to the reticulum and to mitochondria. Of the four PMCA2s the variant w/a (AIII/CII) was by far the least effective in restoring basal cytoplasmatic and mitochondrial [Ca^{2+}] after the transient induced by an InsP3 generating agonist. As this variant lacks half of the calmodulin binding domain that also binds phospholipids (PL), and has an insert next to the N-terminal PL binding domain, the regulation of the four splice variants by acidic PL (PIP2) was studied. CHO cells were transfected with the PMCA2 variants and loaded with FURA-2 and the AM ester form of caged Ca^{2+}. Uncaging of Ca^{2+} confirmed that the w/a (AIII/CII) variant was the least active. That the lack of activation by acidic PL was responsible for the poor activity of this variant was indirectly supported by the reduction of the activity of all other variants upon PIP2 depletion obtained by inhibiting phosphatidylinositol 4-kinase.

C1-007P
Formate hydrogen lyase – a versatile protein in converting energy

M. Hakobyan, R. Avanesyan and **K. Bagramyan**
Department of Biophysics, Yerevan State University, Yerevan, Armenia. E-mail: kbaghramyan@ysu.am

Proton translocation coupled to formate oxidation and hydrogen evolution was studied in anaerobically grown fermenting *Escherichia coli* JW136 carrying formate hydrogen lyase subunits, hydrogenase 1 (hya) and hydrogenase 2 (hyb)-double deletions. Rapid acidification of the medium by EDTA-treated anaerobic suspension of the whole cells or its alkalization by inverted membranes was observed in response of application of formate. The formate-dependent proton translocation and proton–potassium exchange coupled to hydrogen evolution were sensitive to the uncoupler, carbonylcyanide-*m*-chlorophenilhydrazone (CCCP) and to copper ions, inhibitors of hydrogenases. No pH changes were observed in a suspension of formate-pulsed aerobically grown ("respiring") cells. The apparent proton/formate ratio of 1.3 was obtained in cells oxidizing formate. The *N*,*N*(-dicyclohexylcarbodiimide (DCCD)-sensitive ion fluxes (proton and potassium exchange) does take place in JW136 cell suspension. Hydrogen formation from formate by cell suspensions of *E. coli* JW136 resulted in the formation of a membrane potential (delta psi) across the cytoplasmic membrane of –130 mV (inside negative). This was abolished in the presence of copper ions although had little effect on the value of membrane potential generated by *E. coli* under respiration. We conclude that the hydrogen production by hydrogenase 3 is coupled to formate-dependent proton pumping that regulates proton–potassium exchange (stoichiometric ratio is two protons per one potassium) in fermenting bacteria.

C1-008P
Function and trafficking of the choline-transporter like protein CTL1

Z. Yuan, M. D. Fullerton, L. Wagner and **M. Bakovic**
Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada. E-mail: mbakovic@uoguelph.ca

The objective of the present study is to further elucidate the role of the choline-transporter like protein CTL1 in choline transport. Human CTL1 is different from neuronal choline transporters it is located on chromosome 9.q31.2, ubiquitously expressed and alternatively spliced at its C-terminal region. Comparison of several transporter expression levels reveals that CTL1 is the only significant choline transporter in human THP-1 monocytes. The bulk of choline uptake is associated with changes in surface expression of the CTL1 protein, as demonstrated by flow cytometry and protein fractionation using a highly specific CTL1 monoclonal antibody. The surface expression of CTL1 is without changes in total protein or mRNA levels, supporting the role of reduced CTL1 trafficking to the cell surface as the main inhibitor of transport activity in differentiating macrophages. We suggest that the hCTL1 protein is a unique choline transporter, ubiquitously expressed and regulated at transcriptional and post-transcriptional levels and by protein trafficking. Altogether, our work demonstrate a complex control of CTL1 expression in association with specific physiological demands that will advance our knowledge of the choline transport phenomena, once largely unrecognized regulatory aspect of choline metabolism.

C1-009P
Mitochondria and calcium signalling in ureteric smooth muscle

L. A. Borisova S. Wray and T. Burdyga
Department of Physiology, The University of Liverpool, Liverpool, United Kingdom. E-mail: l.borysova@liv.ac.uk

A possible role for mitochondria in control of calcium signalling in ureteric myocytes was examined using the mitochondrial Ca^{2+} uptake inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), in the presence of oligomycin B – a blocker of mitochondrial ATP synthase to prevent cellular ATP depletion. The

experiments were performed on isolated voltage clamped and non-voltage clamped myocytes as well as the intact preparations. CCCP (10 μM) caused a small but significant increase in the resting intracellular concentration of Ca^{2+}. The effect of CCCP on Ca^{2+} sparks was biphasic, there was an initial transient increase in their frequency followed by a gradual inhibition. In voltage-clamped rat ureteric cells held at –80 mV elevation of the resting baseline level of intracellular Ca^{2+} was associated with the generation of inward current. There was also a significant decrease in the rate of restoration of the Ca^{2+} transients induced by depolarizing voltage steps. Calcium transients and Ca^{2+} activated Cl^- current induced by 10 μM carbachol were also reduced in the presence of CCCP. In voltage-clamped (–40 mV) ureteric cells, CCCP in the presence of oligomycin also caused time-dependent inhibition of STOCs. These results suggest that mitochondrial depolarization inhibits Ca^{2+} sparks and STOCs in ureteric myocytes via a mechanism that does not involve a decrease of cytosolic ATP. CCCP did not affect Ca^{2+} transients evoked by high-K^+(120 mM) in the intact preparations. These results indicate that inhibition of mitochondrial Ca^{2+} uptake alters the duration and propagation of Ca^{2+} signals within cells, suggesting that mitochondria play a physiological role in the regulation of intracellular signals and excitability of the ureteric cells.

C1-010P
Nucleotide regulation of mitochondrial ATP-regulated potassium channel

P. Bednarczyk[1,2], K. Dołowy[1] and A. Szewczyk[2]
[1]Departmeny of Biophysics, Agricultural University SGGW, Warsaw, Poland, [2]Laboratory of Intracellular Ion Channels, Nencki Institute of Experimental Biology, Warsaw, Poland.
E-mail: bednar@delta.sggw.waw.pl

The mitochondrial ATP-regulated potassium (mitoKATP) channel was identified in the inner membrane of liver, heart, brain and skeletal muscle mitochondria. However, molecular properties and regulation by endogenous effectors of the mitoKATP channel remain unclear. In our study, inner mitochondrial membranes from bovine heart were reconstituted using planar lipid bilayer. After incorporation, a potassium-selective current was observed. The mean conductance was about 103 pS at symmetrical solution 150/150 mM KCl. The effect of different nucleotide on single channel activity were examined. The channel activity was inhibited by ATP/Mg^{2+} and activated by GDP or GTP. Detailed analysis of regulation of the mitoKATP channel by ATP-PNP/Mg^{2+} and ATP-g-S/Mg^{2+} was performed. We did not observe inhibition of mitoKATP channel activity by non-hydrolysable ATP analogue. Additionally we observed ''run down'' of mitoKATP channel activity. Efficacious way for activation of mitoKATP channel was transient/perfusion with ATP/Mg^{2+} complex. We conclude that ATP/Mg^{2+} regulates activity of the cardiac mitoKATP channel probably by protein phosphorylation.

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C1-011P
Interaction of SR Ca^{2+}-ATPase with drugs

G. Bartolommei F. Tadini-Buoninsegni, M. R. Moncelli and R. Guidelli
Department of Chemistry, University of Florence, Florence, Italy.
E-mail: gianluca.bartolommei@unifi.it

Sarcoplasmic Reticulum (SR) Ca^{2+}-ATPase is an integral membrane protein with a central role in cellular calcium homeostasis. It is found in the SR of muscle cells and it pumps two calcium ions, against their electrochemical gradient, from the cytoplasm into the lumen of the SR, using the energy released after the hydrolysis of an ATP molecule. In such way SR Ca^{2+}-ATPase promotes muscle relaxation. Failure in the functioning of this protein can generate relevant diseases. Drugs can correct such failures, restoring the normal calcium pumping by the protein. We are currently investigating the interaction of some drugs with Ca^{2+}-ATPase: *clotrimazole* and *miconazole*, two antimycotic drugs, and *curcumin*, a molecule with antioxidant and antitumoral properties. We are making use of a rapid solution exchange technique: the protein is first adsorbed on a modified gold surface (the SSM: Solid Supported Membrane) and then it is activated by a rapid concentration jump of an appropriate substrate (e.g. ATP, calcium, etc.). If at least one electrogenic step is involved in the reactions following such activation, a transient current can be measured in the external electrical circuit. The acquisition of such type of signals under different experimental conditions, together with their subsequent elaboration, can give important kinetic information about protein functioning and its modulation by drugs. In our case, while clotrimazole seems to be a pure blocker of the pump, binding to it before ATP in the enzymatic cycle, the others two molecules show a more complicate behaviour, affecting both calcium binding and general pumping kinetic.

C1-012P
Structure–activity relationship models for cardiac glycoside binding and inhibition of Na,K-ATPase activity

W. J. Ball Jr., M. Tabet and S. Paula
Department of Pharmacology, University of Cincinnati, College of Medicine, Cincinnati, Ohio, United States of America.
E-mail: william.ball@uc.edu

The Na,K-ATPase is a membrane bound enzyme that transports sodium and potassium ions in opposite directions across the membrane and serves as the physiological receptor for digitalis. Through inhibition of the enzyme and a subsequent raising of myocardial Ca^{2+} levels, the cardioactive steroids digoxin/digitoxin remain an important component of the treatment of congestive heart failure and some arrhythmias. The molecular mechanisms of drug action are complex in that drug/enzyme interactions vary as the enzyme proceeds through the catalytic cycle. In this work we have used 3-D structure–activity relationship (3D-QSAR) analysis techniques to develop structural models to identify structural elements of the cardioactive steroids that may differentially distinguish between high affinity binding and ATPase inhibiton. In testing the actions of ~37 compounds we found that the relative contributions of steric, electrostatic, hydrophobic and H-bonding interactions to drug binding and activity inhibition were relatively similar but specific differences in the two QSAR models were identified. In particular, bufadienolides with a six-membered lactone ring vs. the five-member ring of the cardinolides were the most potent inhibitors of ATPase activity but they did not have the highest binding affinities. Further, while the glycoside moiety generally had little influence on inhibitory potency the α-sugar enhanced drug affinity. The largest effect noted was with ouabain/ouabagenin where removal of α-rhamnose had little effect on inhibitory potency but caused a 300-fold decrease in affinity. The ultimate goal of such studies is to determine how a safer, less toxic cardioactive agent may be developed.

C1-013P
Spectroscopic studies of phospholamban variants in phospholipid bilayers.

J. C. Clayton, E. Hughes and D. A. Middleton
Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom. E-mail: j.clayton@postgrad.manchester.ac.uk

Muscle relaxation is triggered by the rapid removal of Ca^{2+} from the cytoplasm to the sarcoplasmic reticulum (SR). Removal to the SR is facilitated by sarco(endo)plasmic reticulum Ca^{2+}ATPases (SERCA). Phospholamban (PLB) is a small protein that regulates calcium transport by SERCA2a in cardiac myocytes. This regulation forms part of the contraction/relaxation cycle. The structure, membrane topology and oligomeric state of PLB are all important properties that influence how SERCA enzymes are regulated. It has been suggested that the cytoplasmic domain of PLB undergoes an orientational rearrangement that allows it to make contact with the inhibitory sites within SERCA. A number of peptides corresponding to different sections of PLB have been synthesized in order to study the membrane topology and oligomeric state in detail. Peptides corresponding to the regulatory N-terminal cytoplasmic domain (residues 1–23) of PLB were synthesized, with S16 in both phosphorylated and unphosphorylated form. A range of spectroscopic techniques including circular dichroism (CD), fluorescence (FS) and nuclear magnetic resonance (NMR) were used in order to examine how the regulatory cytoplasmic domain is orientated relative to the surface of cell membranes. In addition, peptides corresponding to a full-length null-cysteine PLB (C36A, C41A, C46A) and transmembrane domain (residues 29–52) PLB (TM-PLB) have been prepared to examine the oligomeric state of PLB in phospholipid bilayers by measuring rotational diffusion rates using solid-state NMR techniques. We have shown that the cytoplasmic domain binds as a helix to the surface of phospholipid membranes, and that TM-PLB and full-length null-cysteine PLB form oligomers in lipid bilayers through contacts within the transmembrane region.

C1-014P
Structural changes in the MscL mechanosensitive ion channel measured using FRET spectroscopy

B. Corry, B. Martinac[2] and P. Rigby[3]
[1]*Chemistry, The University of Western Australia, Crawley, WA, Australia,* [2]*Pharmacology, The University of Western Australia, Crawley, WA, Australia,* [3]*Biomedical Imaging and Analysis Facility, The University of Western Australia, Crawley, WA, Australia. E-mail: ben@theochem.uwa.edu.au*

The MscL channel acts as a safety valve in bacterial cells. When the membrane incorporating these channels is placed under tension, such as when it is in osmotic stress, these channels change their structure to open a wide pore that can quickly expel some of the cell contents before the cell bursts. Here, we determine the structural change involved in channel gating by labelling specific sites in the protein with fluorescent markers. These are then incorporated into proteoliposomes and the distance between sites is determined using resonance energy transfer and a confocal microscope. The state of the channel can be controlled by the addition of different phospholipids. We find that the radius of the MscL protein increases by more than 15 Å upon channel opening.

C1-015P
Ca^{2+} signaling in HEK-293 and skeletal muscle cells expressing recombinant ryanodine receptors harbouring malignant hyperthermia and central core disease mutations

M. Brini[1,2], S. Manni[1,2], N. Pierobon[1,2], G. G. Du[3], P. Sharma[3], D. H. MacLennan[3] and **E. Carafoli**[1,2]
[1]*Department of Biochemistry, University of Padova, Padova, Italy,* [2]*Venetian Institute of Molecular Medicine, Padova, Italy,* [3]*Banting and Best Department of Medical Research, Charles H. Best Institute, University of Toronto, Toronto, Ontario, Canada. E-mail: ernesto.carafoli@unipd.it*

Malignant hyperthermia (MH) and central core disease (CCD) are caused by mutations in the RYR1 gene encoding the skeletal muscle isoform of the Ca^{2+} release channel ryanodine receptor. RyR1 mutant cDNAs carrying mutations that cause MH and CCD were expressed in HEK-293 cells, which do not express endogenous RyR, and in primary cultures of rat skeletal muscle, which express RyR1. Analysis of intracellular Ca^{2+} pools was performed using aequorin probes targeted to the lumen of the endo/sarcoplasmic reticulum (ER/SR), to the mitochondrial matrix, or to the cytosol. Mutations associated with MH caused alterations in intracellular Ca^{2+} homeostasis different from those associated with CCD. Measurements of lumenal ER/SR Ca^{2+} revealed that the mutations generated leaky channels in all cases, but the leak was more pronounced in CCD mutants. In particular, the analysis of cultured muscle cells revealed that the reduction in ER/SR Ca^{2+} level was confined to the terminal cisternae, the portion of the SR from which Ca^{2+} is released through RyR1 to trigger muscle contraction. This suggests that localized differences in Ca^{2+} handling of the ER/SR are sufficient for the generation of the pathological phenotype, and that the severity of the disease could be related to the degree of Ca^{2+} store depletion and/or to the depletion of a specialized portion of the Ca^{2+} store. Cytosolic and mitochondrial Ca^{2+} transients induced by caffeine stimulation were drastically augmented in the MH mutant, slightly reduced in one CCD mutant (Y523S) and completely abolished in another (I4898T). This suggests that local Ca^{2+} derangements of different degrees account for the specific cellular phenotypes of the two disorders.

C1-016P
Structural investigations on the V-ATPase of *Saccharomyces cerevisiae*

M. Diepholz, D. Venzke, S. Kronenberg and B. Böttcher
Department of Structural and Computational Biology, European Molecular Biology Laboratory, Heidelberg, Germany. E-mail: diepholz@embl.de

Vacuolar H^+ translocating ATPases (V-ATPases) are fundamentally important proteins. They pump protons into the interior of most cellular endomembrane compartments at the expense of ATP, thus controlling the activity of many associated enzymes and processes. V-ATPases are large membrane bound protein complexes of at least 13 different subunits (ca. 900 kDa), which are comprised of a membrane bound part, V_0 and a soluble part V_1. In contrast to the related F-ATPase only the overall shape is known (Domgall et al., 2002) and very little information at atomic level is available to date. The proton channel is build by a ring of subunits c (and subunit a) and the catalytic head part (V_1) is a hetero hexamer of subunits A and B. But the localization of the subunits C to H, which are ascribed to the connecting region between V_1 and V_0 remains unknown. Knowledge of the organization of the region is not only important to understand

the mechanism and regulation of the complex but also to comprehend the interactions to other proteins which were mainly found for subunits C to H. The goal of this work is a structure of the V-ATPases from yeast at high resolution from electron-cryomicroscopy and following single particle analysis. Two tags were genetically added to different subunits of the protein complex which allowed the purification of only intact complexes. Here we show a preliminary low resolution 3D structure of the yeast V-ATPase from single particles in vitreous ice. Additionally we present classes of V-ATPase complexes from negative stain which carry a GFP label on different subunits. The globular structure of GFP can be easily detected in single particle classes and allows us to determine the position of the labelled subunit.

C1-017P
Phosphorylated intermediate of the Na-ATPase associated with Second Sodium pump

J. R. del Castillo, F. J. Romero, L. E. Thomas and L. Cariani
Fisiología Gastrointestinal, Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas (IVIC), Caracas, Miranda, Venezuela. E-mail: jdelcas@ivic.ve

Intestinal transepithelial Na^+ transport is mediated by Na^+ passive entry across luminal membrane and exit through basolateral membrane by two actives mechanisms, the Na^+/K^+ pump and the Second Sodium pump (BBA,812:402,1985). These processes have been associated to the ouabain-sensitive Na^+/K^+ ATPase and the ouabain-insensitive, furosemide-inhibitable Na^+ ATPase, respectively (BBA,812:413,1985). Pumps and ATPases constitute two different biochemical entities. Na^+ ATPase is Mg^{2+}-dependent, vanadate-sensitive and can be phosphorylated from 32Pi (ABB,419:190,2003), suggesting that this enzyme could be a type P ATPase. In this report, we characterized the phosphorylated intermediate formed from [32P]-ATP, using purified Na^+ ATPase from enterocyte. Phosphorylation was Mg^{2+}-dependent, vanadate-sensitive and stimulated by Na^+ with two different K_m (0.66 and 15 mM). Stimulatory effect was specific for Na^+ and independent of anions. K_m for ATP was 48 ± 9.1 μM and optimal pH was 7.2. Phosphorylated intermediate was insensitive to ouabain but stimulated by furosemide with an EC50 of 1.8 ± 0.54 μM. In addition, 0.5 mM ADP partially (50%) inhibited it. Phosphorylated enzyme was sensitive to alkaline pH and hydroxylamine, suggesting an acyl-phosphate bond, which was associated with the 100 kDa polypeptide of the enzyme. These results permit suggest a reaction cycle for Na^+ ATPase, where the enzyme has a E1 form that can be phosphorylated from ATP in the presence of Mg^{2+} and Na^+, producing E1.P.Na form, sensitive to ADP. Furosemide stabilized E1.P.Na form of the enzyme. The enzyme would change to E2.P.Na form, insensitive to ADP, which is susceptible to dephosphorylation. Conformational change would induce Na^+ translocation through the membrane.

C1-018P
Classical molecular dynamics simulation of the ADP/ATP carrier in presence and absence of the carboxyatractyloside inhibitor

M. Falconi[1], G. Chillemi[2], M. Ceruso[3], D. Dimarino[1], I. D'Annessa[1], B. Morozzo della Rocca[1] and **A. Desideri**[1]
[1]Department of Biology, University of Roma Tor Vergata, Rome, Italy, [2]CASPUR Consortium for Supercomputing Applications, Rome, Italy, [3]Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, NY, United States of America. E-mail: desideri@uniroma2.it

The transport of various metabolites across the mitochondrial membranes is essential for eukaryotic metabolism. Specific transport through the inner mitochondrial membrane is achieved by nuclear encoded carriers which form a large transport family, the mitochondrial carrier family. The structure of the ADP/ATP carrier in complex with its inhibitor carboxyatractyloside (CATR) has been recently solved by X-ray crystallography providing for the first time an insight into one conformation of the protein. In order to shed light on the possible conformation sampled by the protein and on the effect of CTR on constraining a definite configuration we have carried out two 10 ns molecular dynamics simulation of the protein embedded in a lipid bilayer of palmitoyloleoylphosphatidylcholine (POPC) with and without its co-crystallized inhibitor CATR. The RMSF calculated on the trajectories and averaged over each residue well reproduces the crystallographic B-factors but reveals a different behaviour of selected protein loops that exhibit larger or lower fluctuations in the presence or in the absence of CATR, respectively. The transmembrane helices in the simulations are characterized by RMSF values lower than the corresponding crystallographic B-factors, likely because of a stabilizing effect induced by the POPC bilayer that is absent in the crystal. The number and the strength of the salt bridge is strikingly difference in the two system and permits to suggest the opening and closing mechanism of the transporter. The volume present in the internal protein channel is constant in the inhibited protein while in the CATR-free carrier the main cavity initially present tends to decrease and a new one is appearing localized in the matricial side of the carrier.

C1-019P
Effects of decreasing mitochondrial volume on the regulation of the permeability transition pore

N. Véronique[1], D. Anne[2], W. Ludivine[1], R. Michel[2], L. Xavier[1] and **F. Eric**[1]
[1]LBFA, Joseph Fourier, Grenoble, France, [2]IBCG, Bordeaux 2, Bordeaux, France. E-mail: eric.fontaine@ujf-grenoble.fr

The permeability transition pore (PTP) is a Ca^{2+}-sensitive mitochondrial inner membrane channel involved in several models of cell death. Because the matrix concentration of PTP regulatory factors depends on matrix volume, we have investigated the role of the mitochondrial volume in PTP regulation. By incubating rat liver mitochondria in media of different osmolarity, we found that the Ca^{2+} threshold required for PTP opening dramatically increased when mitochondrial volume decreased relative to the standard condition. This shrinkage-induced PTP inhibition was not related to the observed changes in proton motive force, or pyridine nucleotide redox state and persisted when mitochondria were depleted of adenine nucleotides. On the other hand, mitochondrial volume did not affect PTP regulation when mitochondria were depleted of Mg^{2+}. By studying the effects of Mg^{2+}, cyclosporin A (CsA) and ubiquinone 0 (Ub0) on PTP regulation, we found that mitochondrial shrinkage increased the efficacy of Mg^{2+} and Ub0 at PTP inhibition, whereas it decreased that of CsA. The ability of mitochondrial volume to alter the activity of several PTP regulators represents a hitherto unrecognized characteristic of the pore that might lead to a new approach for its pharmacological modulation.

C1-020P
Role of Fps1 hydrophilic extensions and identification of residues controlling glycerol transport.

C. Filipsson[1], K. Hedfalk[2,3], S. Karlgren[2], J. G. Mullins[4], R. M. Bill[5], S. Hohmann[2] and J. Rydström[1]
[1]*Department of Chemistry/Biochemistry, Göteborg University, Göteborg, Sweden,* [2]*Department of Cell and Molecular Biology, Göteborg University, Göteborg, Sweden,* [3]*Department of Chemistry and Bioscience/Molecular Biotechnology, Chalmers University of Technology, Göteborg, Sweden,* [4]*Swansea Clinical School, University of Wales Swansea, Swansea, United Kingdom,* [5]*School of Life and Health Science, Aston University, Birmingham, United Kingdom. E-mail: caroline.filipsson@chem.gu.se*

The controlled export of solutes is of fundamental importance for cells to survive and adapt to hypotonic conditions. Fps1, a glycerol facilitator from the yeast *Saccharomyces cerevisiae*, is an integral membrane protein belonging to the aquaporin family. Fps1 is located in the plasma membrane where it mediates efflux of the compatible solute glycerol in the adaptation to lower osmolarity. Fps1 is an unusual aquaglyceroporin due to its long hydrophilic extensions at both termini. In addition to crystallization studies on the full length protein, structural studies on the hydrophilic domains are being carried out separately. The Fps1 N- and C-termini are produced and purified in both *Escherichia coli* and *Pichia pastoris*. Based on the theory that the regulatory stretches dip into the membrane, we are currently investigating Fps1 variants where the hydrophilic extensions are anchored to the membrane via the closest transmembrane helix. The goal is to achieve reliable 3D models of the hydrophilic extensions, which together with the membrane anchors can reveal the putative interactions between the membrane spanning parts and the regulatory stretches. In order to learn more about the mechanisms that control Fps1, we have set up a genetic screen for hyperactive Fps1. In this screen we have isolated mutations in fourteen distinct residues, all facing the inside of the cell. Our findings provide a framework for further genetic and structural analysis to better understand the mechanism that controls Fps1 function by osmotic changes.

C1-021P
Cerebrocrast acts as an H^+/Cl^- symport and as a fluidizing agent in rat liver mitochondria

M. A. S. Fernandes[1], A. S. Jurado[2], R. A. Videira[3], M. S. Santos[1], A. J. M. Moreno[1], A. Velena[4], G. Duburs[4], C. R. Oliveira[5] and J. A. F. Vicente[6]
[1]*Departamento de Zoologia, Universidade de Coimbra, Coimbra, Portugal,* [2]*Departamento de Bioquímica, Universidade de Coimbra, Coimbra, Portugal,* [3]*Instituto Politécnico de Viseu, Escola Superior de Tecnlogia de Viseu, Viseu, Portugal,* [4]*Latvian Institute of Organic Synthesis, Riga, Latvia,* [5]*Serviço de Bioquímica, Faculdade de Medicina, Universidade de Coimbra, Coimbra, Portugal,* [6]*Departamento de Botânica, Universidade de Coimbra, Coimbra, Portugal. E-mail: mfer@ci.uc.pt*

The mechanism underlying the uncoupler-like activity of cerebrocrast was assessed on non-respiring rat liver mitochondria by osmotic swelling in K-acetate and NH_4-chloride. The partition coefficient of cerebrocrast in mitochondrial membranes, and its ability to act as a membrane-active compound disturbing membrane lipid organization were evaluated by spectrofluorimetry and by differential scanning calorimetry of DMPC membrane bilayers, respectively. Cerebrocrast did not permeabilize the inner membrane to protons by itself, but did it in association with chloride (H^+/Cl^- symport). Cerebrocrast showed a strong incorporation into mitochondrial membranes with a partition coefficient (Kpm/w) of 2.7 (± 0.1) $\times 10^5$. Cerebrocrast also reduced, in a concentration dependent manner, the phase transition temperature, the cooperative unit size, and the enthalpy associated with the phase transition temperature of DMPC membrane bilayers. It was concluded that cerebrocrast is not a protonophore; instead its uncoupler-like activity is due to the co-transport of protons with chloride through the inner membrane. The uncoupler-like activity of the compound may be potentiated by its ability to disturb membrane lipid organization.

C1-022P
Connection between gap junction communication and myoblast fusion

A. Gorbe[1], D. L. Becker[2], L. Dux[1], Z. Tiszlavicz[1] and T. Krenacs[3]
[1]*Faculty of Medicine, Biochemistry, University of Szeged, Szeged, Hungary,* [2]*BIRU, Anatomy and Developmental Biology, University College of London, London, United Kingdom,* [3]*Bay Zoltan Foundation for Applied Research Institute for Biotechnology, Szeged, Hungary. E-mail: aniko@biochem.szote.u-szeged.hu*

Direct cell–cell communication plays an important role in information exchange between neighboring cells. Membrane associated gap junction channels (GJ) formed by connexins (Cx) share ions, metabolites and second messengers and have important function in different tissues. GJ communication (GJC) is involved in embryonic morphogenesis, in synchronization of heart contractions, regulation of cell proliferation. GJs found in almost all tissues with the notable exception of adult skeletal muscle. The aim of this study was to investigate the possible involvement of GJC in muscle development. Primary myoblast cultures originated from newborn rats were applied as our *in vitro* model. The Cx43 expression had a peak around 2–3 days just before the myoblast fusion. Active coupling between the neighboring cells was also detectable by dye transfer studies at 2–3 days which declined at day 4 (time of fusion).Genetic modification of gap junctions can help to evidence the involvement of GJC in myoblast fusion. Two-day-old cultures were transfected with wild type (wt) and dominant negative (dn) Cx43 DNA +eGFP construct and we followed their influence for the cell proliferation and differentiation. Cells, transfected with wt showed bit smaller proliferation than the control and were more involved in myotube formation. Dn expression retard the GJ communication of the cell and it caused a significant increase in proliferation of green cells and we found less green myotubes in these cultures than in the control. In summary, we observed the upregulation of Cx43 GJ expression at an early stage of skeletal muscle differentiation preceding myoblast fusion and genetic modification of GJ resulted modified myoblast fusion proposing that GJC is involved in early muscle differentiation.

C1-023P
Modulation of enzymatic activities by molting cycle in hepatopancreatic R cells of *Marsupenaeus japonicus*

L. Zilli[2], A. M. Giudetti[1], M. Leo[1], R. Schiavone[2], S. Vilella[2] and **G. V. Gnoni**[1]
[1]*Biochemistry Lab., Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, University of Lecce, Lecce, Italy,* [2]*Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Comparative Physiology Lab., University of Lecce, Lecce, Italy. E-mail: gabriele.gnoni@unile.it*

Four cell types compose the crustacean hepatopancreas: E (embryonic), R (resorptive), F (fibrillar) and B (blister-like)

cells. The physiological activity played by these cells seems to be affected by different physiological/environmental conditions such as the molting cycle, nutritional state, osmoregulation, etc. The aim of the present study was to evaluate whether the activities of different enzymes, i.e. acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), plasma membrane calcium ATPase (PMCA), sarco-endoplasmic calcium ATPase (SERCA) were expressed in the R cells and then affected by the molting cycle. ACC and FAS are involved in the de novo fatty acid synthesis being ACC generally considered the rate limiting step of this metabolic pathway. The characteristics of ACC and FAS have been extensively studied in both mammalian and fish liver. In this respect, however, no information is so far available in marine invertebrates. The calcium pump activities were investigated since the epithelial cells of the crustacean hepatopancreas play an important role in Ca^{2+} balance. ACC and FAS activities were assayed at different temperatures in fresh cells coming from hepatopancreas of shrimps at the pre-moult stage. The highest activities of both ACC and FAS were found at 25 °C. When these enzymatic activities were measured as dependence of moult stage, intermoult was the stage in which both ACC and FAS activities showed their maximum value. SERCA and PMCA activities were detected only in early premoult stage. The results obtained demonstrated that the molting cycle affects important metabolic pathways and are in agreement with the role carried out by the R cells mainly represented by lipid and calcium storage.

C1-024P
Modification of functional state of excitatory amino acid receptors changes the conditions of field excitatory post-synaptic potentials formation in the CA1 hippocampal region

Y. S. Garkun and V. A. Kulchitsky
Brainstem Physiology Lab., Institute of Physiology, Minsk, Belarus. E-mail: garkun@fizio.bas-net.by

Functional state of the excitatory amino acid receptors depends on a conformational state of the peptides forming a structure of these receptors in the membranes of the cells. If the conformational state of these peptides is changed, the functional state of the glutamatergic receptors is changed also. Experimental series were performed on the 400 μm-thick hippocampal slices from the 3- to 5-week-old rats. Population spikes and field excitatory post-synaptic potentials (fEPSPs) recording in the CA1 regions were carried out under electrical stimulation of the Schaffer collaterals. Functional state of an interneuronal communications, which excitate firing of the pyramidal neurons, is modulated by an addition of agonists and antagonists of the excitatory amino acid receptors (kainic acid, ibotenic acid, kynurenic acid). Plasticity of the nervous system permits to sustain a natural activity and functional mobility of neural networks under the action of the acids in a 10^{-5}–10^{-8} M concentration. Under the higher concentration of the acids (10^{-4}–10^{-3} M) populations of neurons loose their ability to function as a whole. In particular, an epileptoformic activity in the rat hippocampal CA1 area was detected when slices were perfused with the kynurenic acid – the antagonist of the excitatory amino acid receptors. Kynurenic acid concentration was known to rise in the cerebrospinal fluid under the convulsions. Regular formation of the epileptoformic activity allows to assume a disinhibition phenomena of unknown receptors in the CA1 hippocampal slice area under the perfusion of the kynurenic acid (10^{-3}–10^{-5} M). Disinhibition should be accompanied by the activation of neurons up to their pathologic activity.

C1-025P
Early effects of ionizing radiation on rat brain NTPDase activity

A. Horvat, S. Petrovic, M. Milosevic, I. Stanojevic and M. Demajo
Laboratory for Molecular Biology and Endocrinology, "Vinca" Institute of Nuclear Sciences, Belgrade, Serbia, Serbia and Montenegro. E-mail: ahorvat@vin.bg.ac.yu

The ecto-adenosine triphospho diphosphohydrolase (NTPDase) is the integral membrane protein that, in the presence of divalent cations (Ca^{2+} or Mg^{2+}), hydrolyses the extra cellular nucleoside tri- and di-phosphate, since their nucleotide-hydrolyzing site is outwardly orientated. By hydrolyzing ATP and ADP it is the major inactivating agent in purine tri- and di-phosphate signalling. It has been reported that ionizing radiation induces tissue damage through different simultaneous pathways. The disturbance of some ion-transporting ATPases by irradiation in different tissues has been reported. The aim of this work is to study the modulation of ecto-NTPDase activity from rat brain nerve terminals after whole body irradiation with γ-rays from a ^{60}Co source one hour post-irradiation. Female rats were divided into three groups: the control group (C) were under physiological conditions, animals whole body irradiated (9.6 Gy, 10.7 cGy/min) were termed as the irradiated (R) group. During irradiation, the animals were confined in plywood boxes. Because of the immobilization stress as a control in respect to the R group, the third group of animals were treated as the irradiated group but not subjected to irradiation (I). One hour after irradiation, membranes of nerve endings were isolated from whole brains and the hydrolysis of ATP or ADP were determined under *in vitro* conditions. The hydrolysis of ATP was not affected by immobilization or irradiation. On the contrary, single whole body irradiation increased ADP hydrolysis by 30% when compared to I animals (0.051 and 0.038 μmol Pi/mg/min, respectively; $P < 0.01$). These findings suggest that irritation may affect brain cell functions, in part, by modulating NTPDase activity.

C1-026P
Molecular mechanism of Na,K-ATPase inhibition by ouabain: ouabain dephosphorylates cofilin through the Ras/MEK/ERK pathway

J. Jung[1,2], S. Choi[1], E. C. Choi[2] and K. Lee[1]
[1]Center for Cell Signaling Research and Division of Molecular Life Sciences, College of Pharmacy, Ewha Womanīs University, Seoul, South Korea, [2]College of Pharmacy, Seoul National University, Seoul, South Korea. E-mail: zec00@snu.ac.kr

We reported previously that phosphorylated cofilin-TPI complex interacts with Na,K-ATPase and enhances the pump activity through the phosphorylation of cofilin via Rho mediated signaling pathway [1, 2]. Therefore, we hypothesized that dephosphorylation of cofilin may be involved in the molecular mechanism of Na,K-ATPase inhibition by ouabain. Dephosphorylation of cofilin by ouabain was confirmed in a time- and dose-dependent manner using an antibody that specifically detects the phosphorylated form of cofilin. Dephosphorylation of cofilin by ouabain in HeLa cell was blocked by Ras dominant negative form of Ras N17 as well as MAPK/ERK kinase inhibitor PD98059, suggesting that ouabain dephosphorylates cofilin through the activation of Ras/MEK/ERK pathways. Immunoprecipitation assay indicates that ouabain caused the phosphorylated cofilin to dissociate from Na,K-ATPase by inducing dephosphorylation of cofilin and it was blocked by the pre-treatment with PD98059. Ouabain-sensitive $86Rb^{+}$-uptake

indicates that Na,K-ATPase activity was increased by the pretreatment of PD98059 in a dose-dependent manner even in the presence of ouabain. In conclusion, our data suggest that ouabain inhibits the Na,K-ATPase activity through the dephosphorylation of cofilin that is regulated by Ras/MEK/ERK pathway.

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C1-027P
Nucleotide binding to Na^+/K^+-ATPase

M. Kubala[1,2], Z. Lansky[1,2], R. Ettrich[3], J. Plasek[1], J. Teisinger[2] and E. Amler[2]
[1]*Institute of Physics, Charles University, Czech Republic,* [2]*Institute of Physiology, Czech Academy of Sciences, Czech Republic,* [3]*Institute of Physical Biology, University of Southern Bohemia, Czech Republic. E-mail: mkubala@centrum.cz*

Na^+/K^+-ATPase is one of the most important enzymes in the metabolism of all animal cells. This enzyme exports sodium and imports potassium ions across the plasma membrane against the concentration gradient. Such a transport requires energy, which is gained by ATP hydrolysis. Despite of tremendous effort of many research groups, it is still not understood, how this molecular pump works, particularly because we lack relevant structural information. The single ATP-binding site was identified on the major cytoplasmic loop connecting transmembrane helices 4 and 5 (H4-H5-loop). In our previous work we tested influence of point mutations on the ATP-binding affinity using the fluorescence analog TNP-ATP and isolated H4-H5-loop of Na^+/K^+-ATPase. We found that besides the previously reported amino acid residues Lys480, Lys501, Gly502 and Arg544, further four amino acid residues, Asp443, Glu446, Phe475 and Gln482, contribute to the enzyme-ATP interaction. This set of amino acids forming the ATP-binding pocket of Na^+/K^+-ATPase is complete, as deduced from our computer model. Interestingly, the largest effect was observed after mutations of Arg423 and Glu472, which are rather distant from the nucleotide-binding site. We showed that these two residues form a hydrogen bond, which is essential for the connection of two opposite halves of the binding pocket. Up to now, it is not clear how the ATP reaches the phosphorylation site, which is more than 2 nm distant. We show that ATP influences dynamics of the isolated loop and that also such a simplified system can yield interesting information about the mechanism of phosphorylation.

C1-028P
Changes in the expression of NTPDases in the human cardiovascular diseases

Á. Kittel[1], B. Sperlagh[1], I. Matkó[2], N. Müllner[3] and A. L. Kiss[4]
[1]*Department of Pharmacology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary,* [2]*Cardiac Surgery Clinic, Semmelweis University, Medical School, Budapest, Hungary,* [3]*Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Medical School, Budapest, Hungary,* [4]*Department of Human Morphology and Developmental Biology, Semmelweis University, Medical School, Budapest, Hungary. E-mail: kittel@koki.hu*

Extracellular nucleoside effects were observed in the cardiovascular system long ago and showed the role of adenosine as an extracellular signaling molecule [1]. This antithrombotic and anti-inflammatory mediator compound is generated by the successive actions of NTPDase1/CD39 (ectonucleoside triphosphate diphosphohydrolase1) and 5(-nucleotidase/CD73. Expression of this protein is abundant especially in caveolar microdomains of endothelial and smooth muscle cells [2,3]. NTPDase1 is targeted to caveolae but so far there is no data available what changes occur in the expression and localization pattern of NTPDase1 during pathological processes. Our investigations were performed on control/healthy and pathological human cardiac tissue blocks obtained from aortic valve replacement surgery or aorto-coronary bypass operations. Immunohistochemistry on ultracryo sections, Western blot analysis, HPLC analysis of the adenine nucleotides and nucleosides and enzyme histochemistry for demonstration of ecto-ATPase activity were applied. We concluded that aging and pathophysiological states evoke changes in ATP-metabolism and in the expression of NTPDase1. We suppose that unlike endothelial cells, NTPDase2 is the enzyme responsible for the high ecto-ATPase activity of cardiac muscle cells. The higher ectonucleotidase activity and enhanced production of inosine by the pathological samples may be a marker for human cardiovascular disease.

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C1-029P
Specific inhibition of ATP-dependent K^+ transport in intact mitochondria by polyclonal antibodies against mitoKIR (55 kDa protein)

E. V. Kachaeva[1,2], A. E. Negoda[1] and G. D. Mironova[1,2]
[1]*Laboratory of Mitochondrial Transport, Institute of Theoretical and Experimental Biophysics, RAS, Pushchino, Moscow region, Russian Federation,* [2]*Department of Biophysics and Biomedicine, Pushchino State University, Pushchino, Moscow region, Russian Federation. E-mail: zhenechka@rambler.ru*

The important physiological role of the ATP-dependent K^+ channel of the inner mitochondrial membrane [mitoK(ATP)] and key role of this channel in cardioprotection makes many scientists to investigate it more thoroughly. However the molecular structure of this channel has not been determined yet. The aim of this study was to obtain the specific polyclonal antibodies (ABs) against channel subunit of rat liver mitoK(ATP) (molecular weight 55 kDa), purified to electrophoretically homogenous state. The specificity of the ABs obtained was verified by Western blot analysis. The inhibitory analysis of these ABs was carried out by usage of two models allowing to reveal ATP-dependent K^+ transport: energy-dependent K^+ influx into mitochondria and DNP-induced K^+ efflux. Both models used in our experiments demonstrated that ABs against the protein 55 kDa in dose-response way inhibited ATP-dependent K^+ transport in rat liver mitochondria. It should be noted that these ABs inactivated by boiling during 5 min lost their inhibitory activity. ABs against this protein did not affect the other functions of mitochondria, such as respiration and oxidative phosphorilation. Therefore the observed inhibitory effect was not connected with the changes in respiration rate and membrane potential dissipation. These ABs are tissue-specific, because they did not influence ATP-dependent K^+ transport in intact rat heart mitochondria. Hence we can say that the protein with the m.w. 55 kDa belongs to the mitochondrial ATP-dependent potassium channel. The results make us closer to determination of the molecular structure of the channel subunit of the mitoK(ATP) and promote further study of its physiological role.

C1-030P
The 5 Å Structure of Heterologously Expressed Plant Aquaporin PM28A

W. Kukulski[1], A. Schenk[1], T. Braun[1], M. Karlsson[2], P. Kjellbom[2], D. Fotiadis[1] and A. Engel[1]
[1]*M.E. Müller Institute, Biozentrum, University of Basel, Basel, Switzerland,* [2]*Department of Plant Biochemistry, Lund University, Lund, Sweden. E-mail: w.kukulski@unibas.ch*

One of the major integral proteins in the spinach leaf plasma membrane is PM28A. Its water channel activity was shown to be regulated by phosphorylation at the C-terminus and in the first cytosolic loop. To assess its structure, PM28A was heterologously overexpressed in the methylotrophic yeast Pichia pastoris, purified and reconstituted into two-dimensional crystals in the presence of lipids. The crystals were analyzed by electron (EM) and atomic force microscopy (AFM). Electron diffraction revealed the crystals to be ordered to high resolution; diffraction spots were observed corresponding to a resolution of 2.96 Å. A three-dimensional structure at 5 Å resolution was determined by cryo electron crystallography. Comparison with known aquaporin structures demonstrates the well conserved overall structure of water channels from all organisms. However, the specific regulation mechanism of PM28A remains to be elucidated. Due to the favorable crystal packing, the phosphorylation sites at the C-terminus as well as in the B-loop are experimentally accessible, allowing studies concerning the gating of the water channel.

C1-031P
Blocking anthrax at the PA channel

V. A. Karginov[1], E. M. Nestorovich[2], M. Moayeri[3], S. H. Leppla[3], N. E. Fahmi[4], I. I. Vaisman[5], S. M. Hecht[4] and S. M. Bezrukov[2]
[1]*Innovative Biologics, Inc., Manassas, Virginia, United States of America,* [2]*Laboratory of Physical and Structural Biology, NICHD, National Institutes of Health, Bethesda, Maryland, United States of America,* [3]*Bacterial Toxins and Therapeutics Section, NIAID, National Institutes of Health, Bethesda, Maryland, United States of America,* [4]*Pinnacle Pharmaceuticals, Inc., Charlottesville, Virginia, United States of America,* [5]*School of Computational Sciences, George Mason University, Manassas, Virginia, United States of America. E-mail: vak@innovbio.com*

The two toxins playing a key role in anthrax pathogenesis are formed by three polypeptides secreted by Bacillus anthracis: protective antigen (PA) which either combines with lethal factor (LF) to form lethal toxin (LeTx), or with edema factor (EF) to form edema toxin (EdTx). LF and EF are enzymes that target substrates within the cytosol; PA provides a heptameric trans-membrane pore to facilitate LF and EF transport into cytosol. Here we demonstrate a novel approach to disable the toxin: high-affinity blockage of the PA pore by unique low-molecular weight compounds that prevent LF and EF entry into the cells. Guided by the sevenfold symmetry and predominantly negative charge of the PA pore, we designed cyclic molecules of sevenfold symmetry using β-cyclodextrin chemically modified to add seven positive charges. Several derivatives of β-cyclodextrin were evaluated for their ability to protect RAW 264.7 macrophages from anthrax lethal toxin cytotoxicity. Per-6-aminoalkyl-β-cyclodextrins displayed inhibitory activity, and they were protective against anthrax lethal toxin action at low micromolar concentrations. By channel reconstitution into planar lipid bilayers and high-resolution conductance recording, we show that they interact strongly with the PA pore lumen, blocking PA-induced transport at nanomolar concentrations. One of the aminoalkyl derivatives completely protected the highly susceptible Fischer F344 rats from anthrax lethal toxin. We anticipate that this approach will serve as the basis for a structure-directed drug discovery program to find new and effective treatments for anthrax.

C1-032P
Topology of the N-terminal part of equinatoxin II, an eukaryotic pore-forming toxin, in the final pore

K. Kristan[1], G. Viero[2], M. Dalla Serra[2] and G. Anderluh[1]
[1]*Department of Biology, University of Ljubljana, Biotechnical Faculty, Ljubljana, Slovenia,* [2]*ITC-CNR Istituto di Biofisica, Sezione di Trento, Trento, Italy. E-mail: katarina.kristan@bf.uni-lj.si*

Equinatoxin II (EqtII) is an eukaryotic pore-forming toxin produced by the sea anemone *Actinia equina*. It is a 20 kDa, basic, cysteinless protein, with sphingomyelin dependent activity. It forms 2-nm, tetrameric, cation selective pores in natural and model lipid membranes. The combination of N-terminal amphipatic α-helix and β-sandwich core with an aromatic cluster is the basis of toxin's efficiency in pore-formation. Previous results have shown, that parts of EqtII crucial for its cytolytic mechanism are an exposed aromatic cluster and the N-terminal α-helix. Recent data showed that the N-terminal region (1–30 AA) of the molecule requires flexibility and is the only part undergoing large conformational changes during the pore-formation. The region between D10-N28 is in an α-helical arrangement in the membrane and participates in the formation of the pore walls. The aim of our present study was to obtain more information about the topology of the N-terminus (1–10 AA) in the final pore. Therefore, a number of EqtII mutants were produced and the technique of planar lipid bilayers (PLM) was used. The results show that Asp3 is positioned within the pore lumen, where it is partly responsible for the toxin's cation selectivity. The results also propose that the N-terminus of the toxin extends through the pore to the other (*trans*) side of the membrane.

C1-033P
Localization of subunits of the V-ATPase complex

S. Kronenberg, D. Venzke, M. Diepholz and B. Böttcher
Department of Structural and Computational Biology, European Molecular Biology Laboratory, Heidelberg, Germany. E-mail: kronenberg@embl.de

Vacuolar H^+-ATPases (V-ATPases) are located in the endomembrane system of eukaryotic cells and in the plasma membranes of specialized cells in higher eukaryotes. They are large (about 900 kDa) membrane bound complexes composed of two functional domains, V_0 and V_1, connected by a stalk region. The membrane embedded part V_0 is built by the subunits a, c, ć, ć, and d, while the hydrophilic part V_1 is made of the subunits (AB)3, C, D, E, F, G, and H. V-ATPases function as ATP hydrolysis driven proton pumps which action is involved in a variety of intra- and intercellular processes. Several of these reactions are due to the complexation of V-ATPase subunits with other proteins. To learn more about the underlying mechanisms for these interactions knowledge of the exact structural arrangement of the different subunits is necessary. Therefore, single subunits of the yeast V-ATPase complex were localized by means of specific labelling. One approach was to introduce a 30 kDa green fluorescent protein (GFP) at the N- or C-terminus stably into the sequence of the subunits a, C, and E by means of homologous recombination. This allows the homogenous integration of labelled proteins in the complex. The GFP forms a globular structure, which can be seen as additional density in the electron microscope. In addition to that we raised a monoclonal antibody against a subcomplex of subunits E and G. Binding of the antibody can also be detected by electronmicroscopy. The localization of specific subunits will be discussed.

C1-034P
Effect of cystein substitution in S4 segments on $Ca_v3.1$ channel activation and inactivation

M. Kurejova[1], L. Lacinova[1] and N. Klugbauer[2]
[1]*Laboratory of Biophysics, Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovakia,* [2]*Institute of Clinical and Experimental Pharmacology and Toxicology, Alberts-Ludvig University, Freiburg, Germany.*
E-mail: martina.kurejova@savba.sk

We have investigated the contribution of individual S4 segments of $Ca_V3.1$ calcium channels to the channel gating by substitution of arginines in each segment to cysteines (R180C, R834C, R1379C and R1717C). Ion currents through the wild type (WT) and the four mutant $Ca_V3.1$ channels transiently expressed in HEK 293 cells were investigated using the whole cell configuration of patch-clamp technique. 2 mM calcium was used as charge carrier. Current–voltage (I–V) relations, maximal current densities, voltage dependences of activation and inactivation were compared. The most prominent effect on voltage dependence of channel activation and on maximal current density was found in the domain I mutant. The current density decreased from 83.6 ± 1.1 pA/pF (WT) to 27.7 ± 0.4 pA/pF (R180C) ($P < 0.001$). This mutation shifted half-maximal activation voltage from –45.0 ± 1.2 mV to –53.4 ± 0.9 mV ($P < 0.001$) without affecting significantly the slope factor. The most prominent effect on channel inactivation was observed in the domain III mutant R1379C. Both, the half maximal inactivation voltage and the slope factor were significantly changed from values –68.2 ± 1.3 mV and 5.3 ± 0.4 mV to –84.2 ± 0.9 mV and 8.3 ± 0.2 mV for WT and R1379C channel, respectively ($P < 0.001$ each). Values for the half maximal inactivation and the slope factor for other channels were: –74.0 ± 1.7 mV* and 7.6 ± 0,4 mV*** for (R180C) –76.7 ± 1.5*** mV and 6.6 ± 0.2** mV (R834C) and –75.0 ± 1.5 mV** and 6.4 ± 0.3 mV* (R1717C) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). We conclude that the cysteine substitutions uncover the different effects of the four domains for the channel gating.

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C1-035P
Dual face of ceramide: non-apoptotic Cer-stimuli modulate antigen-specific T-cell activation through blocking plasma membrane ion channels

C. Detre[1], **E. Kiss**[1], Z. Varga[3], K. Ludányi[2], K. Pászty[4], Á. Enyedi[4], G. Panyi[3], É. Rajnavölgyi[2] and J. Matkó[1]
[1]*Department of Immunology, Eötvös Lorand University, Budapest, Hungary,* [2]*Department of Immunology, University of Debrecen, Debrecen, Hungary,* [3]*Department of Biophysics & Cell Biology, University of Debrecen, Debrecen, Hungary,* [4]*National Medical Center, Budapest, Hungary. E-mail: endre.kiss@cerberus.elte.hu*

Sphingomyelinase-mediated ceramide release in the plasma membrane of T-lymphocytes induced by different stimuli (e.g. ligation of Fas or TNF death receptors, irradiation, stress, inflammation, anticancer drugs) plays a pivotal role in apoptosis signaling, but recently non-apoptotic or even costimulatory Fas signaling have also been reported under specific conditions. The exact mechanisms behind these effects are, however, still poorly understood. Therefore, we investigated the effects of membrane ceramide release on the activation and fate of T-cells, in a murine, virus-antigen specific T-helper cell line and in immunological synapse (IS) models, where T-cells were triggered by antigen presenting cells (APC). C2-ceramide induced massive apoptosis in the majority of TH-cells, but only above a certain threshold stimulus (> 25 μM in strength or > 30 min in duration). Below this threshold C2-Cer was non-apoptotic for the T-cells, as confirmed by several early and late apoptotic markers (PS translocation, mitochondrial depolarization, caspase-3 activation, DNA-fragmentation). The non-apoptotic ceramide stimuli strongly inhibited the calcium response and several downstream signal events (e.g. Erk1/2-, JNK-phosphorylation, CD69 expression or IL-2 production) during antigen-specific T-cell activation in the IS, similarly to T-cell activation through cross-linking of T-cell receptor by anti-CD3 antibody. The release phase of the calcium signal (from ER) was moderately affected by ceramide, while the influx phase was remarkably reduced in both amplitude and rate. Inhibition of Kv1.3 potassium channels, formation of Cer-channels, the consequent plasma membrane depolarization and possibly the newly recognized voltage-gated Ca^{2+}-channels may control this suppressed Ca^{2+}-signaling, in a concerted way. These results suggest that non-apoptotic Fas stimuli, received from other, encountered activated lymphocytes in the lymph nodes, may negatively regulate the antigen-specific T-cell activation/response and thus may set even the T-cell repertoire.

C1-036P
Comparative analysis of membrane channels and receptors on mammalian brain using diverse two-dimensional gel electrophoresis

S. Kang[1], K. Fuchs[2], W. Sieghart[2] and G. Lubec[1]
[1]*PROTOMICS, Department of Pediatrics, Vienna, Vienna, Austria,* [2]*Division of Biochemistry and Molecular Biology, Brain Research Institute, Vienna, Vienna, Austria. E-mail: pcmt@dreamwiz.com*

Over the past decade, our understanding of the structural and function of membrane proteins has advanced significantly as well as how their detailed characterization can be approached experimentally. But although membrane proteins represent 20–30% of currently sequenced genomes, only 0.2% of solved structures are membrane proteins. As a result, comparatively little is known about how membrane how their structure and proteins function is defined by an amino acid sequence. The great disparity between understanding of soluble proteins and membrane proteins has occurred largely because of the many practical problems of working with membrane proteins, which are poor solubilization and stability. Thus, it is essential that we develop methods to overcome this technical barrier if we hope to make more rapid progress in understanding membrane protein structure and function. In this report, three kinds of two dimensional electrophoresis [IEF, cationic detergent and BN-PAGE combined with SDS-PAGE] were employed to GABAAR complex, because It is not only the important major inhibitory neurotransmitter in mammalian brain but also a good example of isolation and stabilization of membrane protein complex.

C1-037P
Systemic hypertension in transgenic mice overexpressing translationally controlled tumor protein

M. J. Kim[1,2], J. S. Kwon[3], S. N. Lee[1,2], Y. H. Kim[3], J. K. Suh[1,2], M. C. Cho[3], G. T. Oh[2] and K. Lee[1,2]
[1]*College of Pharmacy, Ewha Womans University, Seoul, South Korea,* [2]*Center for Cell Signaling Research and Division of Molecular Life Sciences, Ewha Womans University, Seoul, South Korea,* [3]*Department of Internal Medicine, College of Medicine, Chungbuk National University, Cheonju, South Korea.*
E-mail: cofilin2@hanmail.net

Translationally Controlled Tumor Protein (TCTP) is a growth-related protein under both the transcriptional and the transla-

tional control. It is ubiquitously found in most cell types and highly conserved among the various species, suggesting its essential cellular function. TCTP has been reported to have correlation with cell cycle progression and malignant transformation, and to exert anti-apoptotic action in the cell, whereas to trigger histamine release in basophil at the extracellular level. Although TCTP is regarded as a multi-functional protein, it remains unknown about its wide-ranging primary physiological function. We first identified TCTP as a Na,K-ATPase binding protein by yeast two-hybrid screening and as a cytoplasmic repressor of Na,K-ATPase in HeLa cells [Jung J, Kim M, Kim MJ, Moon J, Lim JS, Kim M and Lee K. (2004) J. Biol. Chem. 279:49868–49875]. Since Na,K-ATPase inhibition by cardiac glycosides and potassium-deprivation causes hypertension, we hypothesized that the overexpession of TCTP *in vivo* might be associated with the pathogenesis of hypertension. Thus, we generated transgenic mice constitutively overexpressing TCTP. The follow-up study using a non-invasive computerized tail-cuff system revealed that systolic blood pressure in both male and female transgenic mice was elevated compared to non-transgenic mice. Carotid arterial systolic and diastolic blood pressure as well as heart left ventricular systolic and end-diastolic blood pressure was increased in transgenic mice compared to those of non-transgemic mice using catheter-based micromanometry. Our new finding suggests that as a negative regulator of Na,K-ATPase activity, TCTP seems to play a key role in maintaining the cells' ion homeostasis and dysregulation of its gene expression may lead to disease progression, such as hypertension.

C1-038P
The relationship between plasma membrane Ca^{2+}-ATPase isoforms composition and non-genomic regulation of calcium transport by neuroactive steroids in PC12 cells

I. Kawecka, J. Szemraj, J. Bartkowiak and L. Zylinska
Neurochemical Laboratory, Department of Medical Biochemistry, Medical University of Lodz, Lodz, Poland.
E-mail: iwokaw@csk.am.lodz.pl

Plasma membrane calcium ATPase (PMCA), responsible for Ca^{2+} extrusion, consists of four main isoforms. PMCA1 and 4 are present in all tissues, PMCA2 and 3 preferable in excitable ones. The least known mechanism of PMCA regulation is non-genomic steroids action. It was shown that some steroids regulate membrane enzymes activity participating in neuronal calcium homeostasis. Because almost all of cellular processes are Ca^{2+} dependent, we tested if a non-genomic steroid action may be related to PMCA and calcium homeostasis. We obtained the stably transfected PC12 cells with suppressed expression of isoforms 2 and 3. Inhibition of gene isoforms expression on the mRNA level by RT-PCR method, and on the protein level (using specific antibodies) was confirmed. Next, we analyzed the effect of both PMCA isoforms elimination on overall basic Ca^{2+} transport, and its modulation by 17-αE, 17-βE, PREG, PREGS, DHEA, DHEAS. Our results indicate that the diminished enzyme amount in modified membranes correlated with reduced PMCA basal activity, probably due to the lack of more active PMCA2 and 3 isoforms. Neuroactive steroids affected both, the Ca^{2+} transport activity and calmodulin stimulation. These effects depended on PMCA isoforms composition as well as the steroids structure. These data suggest that steroids in isoform-dependent manner may regulate Ca^{2+} transport via non-genomic way. Further studies are needed to elucidate the molecular mechanisms of specific interaction of PMCA isoforms with the steroids.

C1-039P
Application of laser cytomonitoring method for investigation of working of cell membrane ion-transport system

A. F. Lobkov and E. V. Tereshkin
Faculty of Biology, Department of Biophysics, Moscow State University, Moscow, Russian Federation. E-mail: sasha@moldyn.org

Cellular size and volume are regulated by various interrelated protein systems of a membrane. Some pathological states involve abnormalities in regulation of membrane proteins transport function. The size and the volume of erythrocyte are important indexes of functional and structural state of a cell and ion-transport peptides [1]. Elaboration of methods that can characterize functioning of transport membrane proteins is actual. Original multi-purpose analytical system (laser cytomonitoring – LC) was used to determine size distribution function of erythrocytes [2]. System working bases on well-known method of small-angle light scattering on small-size objects. In this work concentration of intracellular calcium in erythrocytes was increased *in vitro*. Calcium ionophore-peptide A23187 was used. Density and volume distribution of cells were detected depending on calcium concentration. Problem of effectiveness of LC at erythrocytes investigation was examined. Resuspended in various environments erythrocytes were studied. Size distribution functions of erythrocytes were received according to ionophore A23187 concentration. Volume distribution of erythrocytes that was obtained by means of laser cytomonitoring method was compared to natural cell volume. Gage functions were plotted. Cellular size change kinetic was observed during 90 min after ionophore addition. Results were obtained to show sensitivity of laser cytomonitoring to erythrocytic volume changes. This method makes it possible to detect kinetic of cellular sizes changes.

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C1-040P
$Ca_v1.2$ calcium channels modify threshold and firing frequency of action potential bursts in hippocampal CA1 pyramidal cells.

L. Lacinová[1], S. Moosmang[2], N. Haider[2], F. Hofmann[2] and T. Kleppisch[2]
[1]Institute for Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovakia, [2]Institute for Pharmacology and Toxicology, Technische Universität, München, Germany.
E-mail: lubica.lacinova@savba.sk

Hippocampal pyramidal cells express two forms of L-type calcium channels, $Ca_v1.2$ and $Ca_v1.3$. We examined the role of the prevailing $Ca_v1.2$ channel for the excitability of these neurons in a mouse line with an inactivation of the $Ca_v1.2$ gene in the cortex and the hippocampus (N-S Arch. Pharmacol., 2004, 369, Suppl. 1, R83). Voltage- and current-clamp recordings in the whole-cell configuration were performed. Confirming the knockout on a functional level, the density of dihydropyridine-sensitive calcium currents was largely reduced in CA1 pyramidal cells from the $Ca_v1.2$ mutants. This did not alter the resting membrane potential (–70.8 ± 0.9 mV in control vs. –69.9 ± 1.0 mV in mutant mice) and slightly, but not significantly, increased the input resist-

ance of CA1 pyramidal neurons measured at a membrane potential of –70 mV (110.7 ± 12.1 MÙ in control vs. 89.9 ± 5.8 MÙ in mutant mice). There was also no difference between the genotypes in the maximal slope of the ascending and the descending phase of single action potentials (AP) induced by brief 5 ms current pulse. The threshold potential for generation of a single AP was shifted from –44.6 ± 2.2 mV in control to –32.7 ± 6.0 mV in mutant mice ($P \leq 0.05$). Likewise, the threshold for generation of an AP burst from the resting membrane potential of –70 mV was drastically altered in CA1 neurons from mutant mice (–35.6 ± 4.2 mV) compared with the control (–46.7 ± 1.4 mV; $P < 0.01$). Moreover, the AP frequency in bursts activated after 500 ms long hyperpolarizations to 80 mV to speed up recovery of Na^+ channels from voltage dependent inactivation was significantly enhanced in CA1 pyramidal cells from $Ca_v1.2$ mutants. Our data suggest that the $Ca_v1.2$ channel activity may modulate the excitability of CA1 hippocampal neurons.

C1-041P
The distribution of the secretory pathway Ca^{2+}-ATPase (SPCA1) protein in neuronal and glial cells

R. Murin[1,2], **J. Lehotsky**[2], A. Urikova[2], P. Kaplan[2], D. Dobrota[2], S. Verleysdonk[1] and L. Raeymaekers[3]
[1]Department of Physiological Chemistry, University of Tuebingen, Tübingen, Germany, [2]Department of Medical Biochemistry, Comenius University, Martin, Slovakia, [3]Laboratory of Physiology, Leuven University, Leuven, Belgium.
E-mail: lehotsky@jfmed.uniba.sk

The neural cells of the brain have important secretory functions. They secrete many neurotransmitters and secretory proteins. The Golgi apparatus as a calcium store may regulate this secretion by secretory Ca^{2+}-ATPase (SPCA1). While the presence of SPCA1 in the brain has already been shown the cell-type specific expression pattern has not been established. We investigated the presence and distribution of the SPCA1 pump protein in homogenates prepared from both the rat brain and the cell cultures of neurons and glial cells. Western blot analysis showed that SPCA1 is clearly present in homogenates from whole brain as well as in primary cell cultures of: (i) neurons, (ii) astrocytes, (iii) oligodendrocytes and (iv) ependymal cells. Surprisingly, the signal quantity in all types of analyzed cells was not remarkable different. However, a very weak signal could be detected in cultures of microglial cells. In addition, as shown by immunocytochemistry, the SPCA1 pump within the cells was mostly localized to tubular structures in the perinuclear region of the Golgi apparatus. These results suggest that in spite of morphological and functional differences between neural cell types, most of them contain SPCA1 pump protein localized to structures distinct from endoplasmic reticulum. The pump may play a major role in the refilling of Ca^{2+} stores and it may contribute to intracellular Ca^{2+} signaling in neural cells.

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C1-042P
Transforming growth factor-beta blocks alveolar fluid reabsorption by inhibiting the epithelial sodium channel ENaC

R. E. Morty, I. Vadasz, A. Olschewski, W. Seeger and O. Eickelberg
Department of Internal Medicine, University of Giessen Lung Centre, Giessen, Germany. E-mail: rory.morty@innere.med.uni-giessen.de

Transforming growth factor-beta (TGF-beta) is a key mediator of physiological and pathophysiological processes in the lung, including pulmonary hypertension, fibrosis, and acute respiratory distress syndrome. We report here that TGF-beta, applied to the alveolar space, blocked transepithelial active sodium in an isolated, ventilated and perfused rabbit lung model by up to 90%. Transport was determined by ^{22}Na transit from the alveolar to the vascular space of the lung. This transepithelial active sodium transport block was accompanied by a 100% increase in epithelial lining fluid volume, although there was no change in the paracellular epithelial or endothelial permeability. TGF-beta thus potently provoked alveolar oedema. These effects were abrogated by prior application of the cell-permeable TGF-beta kinase inhibitor SB431542, and also by the cell-permeable endocytosis inhibitor phalloidin oleate, suggesting that both TGF-beta signalling and endocytosis mediated these effects. In cell culture studies, TGF-beta was without effect on Na^+,K^+-ATPase activity, since it did not alter the ouabain-sensitive $^{86}Rb^+$-uptake by A549 human lung epithelial cells. However, a 30 min exposure of A549 cells to TGF-beta significantly impaired amiloride-sensitive sodium currents, as evaluated by patch-clamp. This effect was also blocked by SB431542 and phalloidin oleate. These effects were immediate, and therefore not dependent on transcriptional regulation of the ENaC gene. Our findings therefore indicate a novel signalling pathway by which TGF-beta can rapidly block active sodium transport in epithelial cells by promoting the endocytosis of sodium channels.

C1-043P
The role of membrane ATPase in aluminium uptake by red blood cells

A. A. Moshtaghie
Department of Biochemistry, Laboratory of Clinical Biochemistry, Isfahan University of Medical Sciences, Isfahan, Iran.
E-mail: moshtaghie@pharm.mui.ac.ir

It is now well documented that aluminium interferes with iron metabolism and causes hypochromic anemia in chronic renal failure undergoing dialysis. Investigation of probable mechanism of aluminium uptake by human erythrocytes and the role of ATPase activity in this process was the major aim of this study.

Methods: Fresh packed red blood cells were prepared and further washed with saline, cells were incubated in Earl's medium (pH 7.4) containing varying concentrations of aluminium (0–160 µg/L) as aluminium potassium sulfate at 37 °C. Aluminium content of cells were determined using flame less atomic absorption spectrophotometry with standard methods.

Results: There was significant increase in aluminium content of the cells in comparison to control. Addition of 5 mM glucose caused an elevation of red cell aluminium, whereas depletion of red cells from ATP caused a marked reduction in aluminium uptake. Both oubain and vanadate when added to the medium, caused a significant reduction in aluminium uptake in line with decrease in ATPase activity.

Conclusion: Aluminium uptake by red cells might be achieved either through transferrin receptor membrane during heme synthesis in reticulocytes or probably by an active transport process in erythrocytes during aluminium elevation in the blood. The exact mechnism and how ATPases involve in this process should however be elucidated by further investigation.

C1-044P
Electrophysiological and immunohistochemical studies of a organophosphate pesticide on neuron K^+ channels modulated by muscarinic receptor

A. R. Murgia[1], I. Zanardi[1], M. Basso[2], S. Deplano[2], C. Falugi[2] and G. Prestipino[1]
[1]*Institute of Biophisics of Genoa, National Research Council, Genoa, Italy,* [2]*Department of Biology, University of Genoa, Genoa, Italy. E-mail: murgia@ge.ibf.cnr.it*

Organophosphates (OPs) are pesticides, largely employed in European countries for several purposes: agricultural, garden and domestic pest control. These insecticides are neurotoxic compounds that have their target in the cholinergic neuromuscular system, by inhibiting the activity of acetylcholynesterase with consequent alteration of all functions of the cholinergic neurotransmission system. The interference with this system affects human health because of impairment of neuronal development, as well as of memory and psychomotor speed and affective symptoms. At long term, nervous system disorders may occur: for instance, increasing the incidence of Parkinsonism. Our work has been focused on Cidial, the commercial neurotoxic pesticide used in agriculture. It has been tested on K^+ currents of cerebellum granular cells in primary culture obtained from 7-day-old Wistar rat, using the patch-clamp technique in the whole-cell configuration. We have described the biophysical and pharmacological properties of the interaction of the pesticide with the fast activating and inactivating currents as well as the non-inactivating current present on neuron cells. The experiments with typical molecular effectors of muscarinic receptors have shown that voltage-dependent K^+ channels are modulated by these compounds. These data have been confirmed by immunohistochemical tests using muscarinic ACh receptor antibodies. We have also investigate the subtype of K^+ channels involved in the modulation by muscarinic receptor with antibody against voltage-dependent potassium channels.

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C1-045P
Determination of structure and functional properties of cytoplasmic terminus of vanilloid receptor TRPV1

V. Mrazikova[1], E. Jindrova[1], J. Teisinger[2], V. Vlachová[2] and R. Ettrich[1]
[1]*Laboratory of High Performance Computing, Institute of Physical Biology of USB and Institute of Landscape Ecology of AS CR, Nove Hrady, Czech Republic,* [2]*Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic. E-mail: mrazikova@greentech.cz*

The vanilloid receptor TRPV1, a member of TRP channel family, has function as a multimodal signal transducter of noxious stimuli in the mammalian somatosensory system [1]. The TRPV1 is consisted of six transmembarane-spanning domains with a pore forming region between fifth and sixth domains, and cytoplasmically located C- and N-terminal regions. Although structural and functional studies have been done [2,3], the possible contributions of terminal regions to vanilloid receptor function remain elusive. To determine structure and functional properties of the cytoplasmically located tails, the DNA fragments encoding for the N- and C-terminus were cloned to the expression vectors and transformed to *Escherichia coli* strain. Overexpressed proteins were purified by affinity chromatography and used for structural analysis by a wide range of low resolution methods. Experimental results were combined with homology and energetic modeling techniques and we propose a three-dimensional structure of the C-terminus.

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C1-046P
ER distribution and calcium signalling in glioma C6 cells

P. Pomorski, B. Targos and J. Barańska
Laboratory of Signal Transduction, Cellular and Molecular Neurobiology, Nencki Institute of Experimental Biology, Warsaw, Poland. E-mail: pp@nencki.gov.pl

Distribution of ER cisternae in glioma C6 cells treated with cytochalasin D and jasplakinoide was studied by laser confocal microscopy. Its significant rearrangement was found following actin cytoskeleton manipulations. Changes of ER distribution were accompanied by cell shape changes and lead to local ER concentration alterations. Obtained results were used to construct computer model of calcium signal modulation by ER cisternae translocation. Calculation results suggest that rearrangement of the endoplasmic reticulum elements may be responsible for modulation of calcium signal strength. We have also noticed that even if the endoplasmic reticulum concentration levels are local, resulting changes in free calcium concentration are global and evenly distributed throughout the cell. Used mathematical method proved to be powerful tool which made us able to understand the chemical dynamics of non-equilibrium processes of calcium transient formation. Presented data show how Ca^{2+} signal resulting from IP_3 provoked release of calcium from the endoplasmic reticulum may depend on the cytoskeleton structure.

C1-047P
Using yeast to study transport and structure–function relationship in aquaglyceroporins.

N. Pettersson, S. Karlgren, B. Nordlander and S. Hohmann
Department of Cell and Molecular Biology, Lundberg Laboratory, Göteborg University, Göteborg, Sweden. E-mail: nina.pettersson@gmm.gu.se

Aquaporins are small membrane proteins that transport water while the closely related aquaglyceroporins also can be permeable to polyols, urea and even arsenic. These substances can pass the pore in both directions by facilitated diffusion. Aquaporins are represented in organisms ranging from archaea to human, and their discovery was awarded the Nobel Prize in chemistry in 2003. Eleven different aquaporins (0–10) have been identified in mammals. Of these, AQP3, 7, 9 and 10 are aquaglyceroporins. They are expressed in a tissue-specific manner and play key roles

in the regulation of water balance. Examples for relevant applications are transpiration, water retention in the kidneys and glycerol transport following fat metabolism. Aquaglyceroporins are probably also an entry point for arsenic in the liver. To study the function of aquaglyceroporins, we have developed a test system in *Saccharomyces cerevisiae*. When exposed to a hyperosmotic stress, yeast cells uses glycerol as a compatible solute to regain the turgor pressure decreased by water loss. When aquaglyceroporins are expressed in such cells, they cause sensitivity to hyperosmotic stress, due to glycerol loss through the aquaglyceroporins. When expressed in a strain deficient in glycerol production, the sensitivity of that strain to high levels of certain polyols is suppressed because the polyol can be taken up by the cell through the aquaglyceroporin. We have employed this system of conditional osmotic shock to design a genetic screen which has made it possible to identify residues responsible for the regulation of the yeast aquaglyceroporin Fps1. The genetic screen is being further developed to unravel key residues in channel specificity as well as the mode of action of potential inhibitors. We are also using this system to study mammalian aquaglyceroporins.

C1-048P
The caspase-3 cleavage product of PMCA4b is activated and is targeted to the basolateral membrane of polarized MDCKII cells

K. Pászty[1], G. Antalffy[2], A. R. Penheiter[3], L. Homolya[1], R. Padányi[2], A. Iliás[4], A. G. Filoteo[3], J. T. Penniston[5] and Á. Enyedi[2]

[1]*Membrane Research Group of the Hungarian Academy of Sciences, Budapest, Hungary,* [2]*National Medical Center, Budapest, Hungary,* [3]*Department of Biochemistry and Molecular Biology, Mayo Foundation, Rochester, MN, United States of America,* [4]*Institute of Enzimology, Hungarian Academy of Sciences, Budapest, Hungary,* [5]*Neuroscience Center, Massachusetts General Hospital, Boston, MA, United States of America.*
E-mail: paszty@biomembrane.hu

The calmodulin-activated plasma membrane Ca^{2+}ATPases (PMCAs) are responsible for ejecting Ca^{2+} from the cytosol and thus maintaining intracellular Ca^{2+} homeostasis. During Ca^{2+} signaling, when intracellular Ca^{2+} concentration rises, Ca^{2+}-calmodulin binds to the C-terminal regulatory domain of PMCAs and increases their activity. At resting Ca^{2+} concentrations, on the other hand, when calmodulin is dissociated, the regulatory domain binds to the catalytic core and inhibits the pump's activity. Recently, we demonstrated that hPMCA4b is a target for caspase-3 cleavage during apoptosis and that this cleavage removes the entire C-terminal regulatory domain, leaving behind a 120 kDa catalytic fragment of the protein. To analyze the characteristics of the 120 kDa fragment we overexpressed the corresponding truncated mutant in COS-7 and MDCKII cells. This technique made it possible to clearly define the properties of the caspase-3 fragment and show that it is fully and constitutively active; it forms a phosphoenzyme intermediate and has high Ca^{2+} transport activity in the absence of calmodulin. When this fragment of hPMCA4b was stably expressed in MDCKII cell clones it was targeted without degradation to the basolateral plasma membrane. In summary, our studies emphasize that the caspase-3 cleavage product of hPMCA4b is fully and constitutively active and the carboxyl terminus is not required for proper targeting of hPMCA4b to the plasma membrane. Our studies are aimed to provide a firm ground for future experiments to elucidate the physiological role of hPMCA4b in apoptosis.

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C1-049P
Does the mere binding of nucleotide to sarcoplasmic reticulum Ca^{2+}-ATPase result in Ca^{2+} occlusion? As a rule, no, but in the presence of high [Ca^{2+}] and solubilizing detergent, binding of AMPPCP almost does

M. Picard[1], C. Toyoshima[2] and P. Champeil[1]

[1]*Département de Biologie Joliot-Curie, Service de Biophysique des Fonctions Membranaires, CEA Saclay & CNRS URA 2096, Gif-sur-Yvette, France,* [2]*Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan.*
E-mail: picard@dsvidf.cea.fr

Crystalline forms of detergent-solubilized sarcoplasmic reticulum Ca^{2+}-ATPase, obtained in the presence of either a substrate analog, AMPPCP, or a transition state complex, ADP.fluoroaluminate, were recently described to share the same general architecture despite the fact that, when studied in a test tube, these forms show different functional properties. Here, we show that the differences in the properties of the E1.AMPPCP and the E1.ADP.AlFx membraneous (or solubilized) forms are much less pronounced when these properties are examined in the presence of 10 mM Ca^{2+} (the concentration prevailing in the crystallization media) than when they are examined in the presence of the few μM Ca^{2+} known to be sufficient to saturate the transport sites. This concerns various properties including ATPase susceptibility to proteolytic cleavage by Proteinase K, ATPase reactivity towards SH-directed Ellman's reagent, ATPase intrinsic fluorescence properties (here described for the E1.ADP.AlFx complex for the first time), and also the rates of $^{45}Ca^{2+}$–$^{40}Ca^{2+}$ exchange at site "II". These results solve the above paradox at least partially, and suggest that the presence or absence of a previously unrecognized Ca^{2+} ion in the Ca^{2+}-ATPase.AMPPCP crystals should be re-investigated. A contrario, they emphasize the fact that the average conformation or dynamics of the E1.AMPPCP complex under usual conditions in the test tube differs from that found in the crystalline form. The extended conformation of nucleotide revealed by the E1.AMPPCP crystalline form might be only indicative of the requirements for further processing of the complex, towards the transition state leading to phosphorylation and Ca^{2+} occlusion.

C1-050P
Identification of ATP-dependent K^+ channel subunits in human heart mitochondria with 2D electrophoresis

E. Pankotai[1,3], E. Kovács[2], M. Kollai[1], C. Szabó[1] and Z. Lacza[1,3]

[1]*Institute of Human Physiology and Clinical Experimental Research, Semmelweis University, Budapest, Hungary,* [2]*Department of Heart and Vascular Surgery, Semmelweis University, Budapest, Hungary,* [3]*Biotech Hungary KFT, Budapest, Hungary.*
E-mail: pankotaie@yahoo.com

Background: Mitochondrial ATP-dependent K-channel (mitoK_{ATP}) openers are key mediators of ischemic pre-conditioning and represent a novel target for cardioprotective therapies. The present study aimed to isolate mitochondria from human heart tissue and identify the putative mitoK_{ATP} subunits with immunoblotting approaches.

Methods: Atrial and papillary muscle samples were obtained during heart surgery procedures. Mitochondria were isolated

with Percoll gradient purification. The mitochondrial proteins were subjected to one- and two-dimensional SDS-PAGE electrophoresis followed by immunoblotting against the sulphonylurea-receptor (SUR2) and the inwardly rectifying K^+ channel (Kir6.2).

Results: We successfully isolated mitochondria from human heart samples. Conventional 1D Western blotting showed that both Kir6.2 and a small molecular weight SUR2-like protein are similarly present in human and rat tissues. Two dimensional Western blot was applied to separate the proteins based on their respective pI value. The pI and MW of the Kir6.2 subunit corresponds well with the calculated value (MW = 45 kDa, pI = 9), while the SUR2 immunoreactive protein is significantly smaller than the previously known form (MW = 28 kDa, pI = 8), indicating that mitochondria contain a novel SUR2 variant.

Conclusions: We successfully isolated mitochondria from human heart and identified possible subunits of the mitoK_{ATP} channel. Separating mitochondrial proteins with 2D electrophoresis makes it possible to use proteomics tools to further characterize the proteins in question.

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C1-051P
Alterations of Na^+, K^+-ATPase and Ca^{2+}-ATPase in colon carcinomas

M. Panayiotou[1], S. V. Hizhnyak[2], A. A. Kaplia[3], D. Kyriakopoulou[4], M. Stavropoulos[4] and N. Papageorgakopoulou[1]
[1]Laboratory of Biochemistry, Department of Chemistry, University of Patras, Patras, Greece, [2]Laboratory of Biochemistry, Department of Biochemistry, Taras Shevchenko National University of Kyiv, Kiev, Ukraine, [3]Palladin Biochemistry Institute, National Academy of Sciences of Ukraine, Kiev, Ukraine, [4]Department of Surgery, University Hospital of Patras, Patras, Greece. E-mail: mpanayot@upatras.gr

Abnormal ion homeostasis accompanies intensive cellular proliferation at tumor growth. The objective of this study was to compare the qualitative and quantitative changes of the ion-transporting ATPases in human colon carcinoma tissues at different stages, benign normal tissues, including: determination of Ca^{2+}-ATPase and Na^+, K^+-ATPase activities and detection of the enzyme levels by Western blot analysis. Our results demonstrate that both the Na^+, K^+- ATPase and Ca^{2+}-ATPase activities are decreased in carcinomas, as also the levels of the Na^+, K^+-ATPase catalytic α-subunit, as compared to the normal mucosa. The detected levels of the Na^+, K^+-ATPase α-subunit varied in different carcinomas. Together with the loss of the enzymatic activity the results indicate that the expression of the functional Na^+,K^+-ATPase is down-regulated in human colon carcinoma, by immunoblotting. On the other hand, Ca^{2+}-ATPase expression appeared to be increased in colon carcinomas (sigmoid and descendind) as was shown by Western blot analysis using monoclonal antibodies against SERCA3 isozyme. The characterization of the details of transport ATPases expression at cancer development will be relevant for understanding of the regulatory mechanisms of ion homeostasis in malignancy.

C1-052P
The loss of SERCA3 expression in colon tumours correlates with their degree of malignancy

J.-P. Brouland[1], P. Gélébart[2], T. Kovács[3], J. Grossmann[4], C. Chomienne[5] and **B. Papp**[5]
[1]Service d'Anatomie Pathologique, Hopital Lariboisiere, Paris, France, [2]Department of Biochemistry, University of Alberta, Canada, [3]Institute of Haematology and Immunology, National Medical Center, Budapest, Hungary, [4]Ev. Krankenhaus Bethesda GmBH, Mönchengladbach, Germany, [5]Inserm EMI-00-03 U-718, Institut Universitaire d'Hématologie, Hopital Saint-Louis, Paris, France. E-mail: bela.papp@chu-stlouis.fr

Sarco/endoplasmic reticulum calcium transport ATPases (SERCA) accumulate calcium from the cytosol into the endoplasmic reticulum. Three SERCA genes are known in humans, that code for several isoforms that arise by alternative splicing. We have shown that normal colonic epithelium expresses SERCA3 abundantly, and that SERCA3 expression is induced during differentiation of colon cancer cells *in vitro*. Here we investigated SERCA3 expression by immunohistochemistry in foetal and normal adult colon epithelium, in hyperplastic polyps, in adenomas and in well, moderately and poorly differentiated colon adenocarcinomas. Strong SERCA3 staining could be observed in normal foetal or adult epithelium, as well as in hyperplastic polyps. In adenomas SERCA3 staining was heterogeneous, and inversely correlated with the degree of displasia of the tumour. SERCA3 staining was barely detectable in moderately differentiated adenocarcinomas and absent in poorly differentiated cancers. Defects of SERCA3 expression thus occur already early during the multistep process of colon tumorigenesis (i.e. in adenomas). The extent of the deficiency of SERCA3 expression correlates with the degree of loss of differentiation that takes place during the low grade to high grade adenoma and moderately to poorly differentiated adenocarcinoma sequence. In conclusion, intracellular calcium sequestration of colon tumours is different when compared to normal tissue. This defect is correlated to the degree of the malignancy of the lesions and may contribute to the establishment of a neoplastic phenotype. SERCA3 therefore constitutes a useful new marker for the investigation of colon tumour biology and phenotype.

C1-053P
Correlation of structure with kinetics in activation of PMCA by calmodulin

A. R. Penheiter[1], A. G. Filoteo[1], **J. T. Penniston**[2] and A. J. Caride[1]
[1]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota, United States of America, [2]Laboratory of Membrane Biology, Neurosurgery Service, Mass. General Hospital,, Harvard Medical School, Boston, Massachusetts, United States of America. E-mail: john@penniston.com

The resting state of the plasma membrane Ca pump (PMCA) is a closed one in which the regulatory C-terminus interacts with the catalytic core and reduces pumping of Ca. Activation can occur by binding of calmodulin followed by opening of PMCA. Our analysis of activation by a fluorescent calmodulin derivative (TA-Cam) shows a three-step activation pattern in which the 1st step increases the fluorescence and each of the last two steps decrease the fluorescence [Penheiter, A.R., et al. (2003) Biochemistry 42:12115]. When peptide C20, (LRRGQILWFRGLNRIQTQIK), representing the N-terminal part of the Cam-binding domain, binds to TA-Cam, only an increase in fluorescence is seen, comparable to the increase seen in the 1st step of PMCA

activation. When a longer peptide (C28) replaces C20, the increase in fluorescence is followed by a decrease, just as is the case for PMCA. C28 also binds to calmodulin 20 times more tightly than C20. A study of numerous peptides shows that all of the determinants for peptide binding are present in a 20 residue peptide ILWFRGLNRIQTQIKVVKAF, shifted five residues downstream from C20 [1]. This is in accordance with the NMR structure of C20-Cam [2] that shows Cam in the extended state. All of these data are consistent with a scheme in which the observed three steps of activation of PMCA correspond to three different conformations of the Cam-PMCA complex. Step 1 is the binding of the extended form of calmodulin, steps 2 and 3 are the opening of PMCA, rearrangement and collapse of calmodulin.

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C1-054P
14-3-3 epsilon: A protein partner for plasma-membrane Ca^{2+} pump isoform 4 and Na^+/Ca^{2+} exchanger isoform 2

L. Coletto[1], **M. Pulina**[1,3], A. Rimessi[2], M. Brini[1,3], R. Rizzuto[2] and E. Carafoli[1]
[1]*Venetian Institute of Molecular Medicine, Padova, Italy,* [2]*Department of Experimental and Diagnostic Medicine, University of Ferrara, Ferrara, Italy,* [3]*Department of Biological Chemistry, University of Padova, Padova, Italy.*
E-mail: maria.pulina@unipd.it

The PMCA pump is the product of four genes. PMCA1 and PMCA4 are ubiquitously distributed, PMCA2 and PMCA3 are essentially restricted to some brain domains. This could depend on isoform specific interactions with protein partners. We have used the N-terminal portion of PMCA4 and PMCA2 as bait in an yeast two hybrid approach to identify partners. This domain was chosen because of its low homology in the PMCA isoforms. The screening was performed with a human brain cDNA library. Protein 14-3-3 epsilon protein was identified as an interacting partner. Coimmunoprecipitation experiments using PMCA2 and PMCA4 and protein 14-3-3 epsilon showed interaction with PMCA4 but not with PMCA2. To investigate the effect of 14-3-3 protein on pump activity, PMCA4 was co-expressed with 14-3-3 epsilon in HeLa cells and the effects on cellular Ca^{2+} homeostasis were monitored using recombinant aequorins targeted to the cytoplasm, to the endoplasmic reticulum and to the sub-plasma membrane domain. The experiments showed inhibition of pump activity in cell co-expressing 14-3-3 epsilon. Silencing of the 14-3-3 epsilon gene with siRNA cells cotransfected with PMCA4 confirmed the inhibition. Similar work was performed on the Na^+/Ca^{2+} exchanger (NCX). As a bait the large cytoplasmic loop of NCX isoform 2, typical of neurons, was chosen. The screening yielded one interaction partner, the 14-3-3 epsilon protein. The interaction was confirmed in immunoprecipitation and immunofluorescence experiments. To investigate the functional effects of interaction NCX2 and 14-3-3 epsilon were co-expressed in CHO cells together with aequorin targeted to the cytoplasm. The experiments showed an increase of Ca^{2+} in the cytoplasm, suggesting a reduction of NCX2 activity in the presence of 14-3-3 epsilon.

C1-055P
Expression and distribution of the Kv1 (Shaker) family in kidney epithelium

C. G. Rolando, S. H. Carolina and E. P. Laura
Department of Physiology, Laboratory of Ionic Channels, Universidad Nacional Autónoma de México, Mexico City, Distrito Federal Mexico. E-mail: rolandocarrisoza@yahoo.com.mx

Ion channels are membrane proteins that mediate the movement of ions across the cellular membranes. The voltage gated potassium channels (Kv) integrate one of the most widely studied families in excitable cells. However, less is know about the function and distribution of Kvs in epithelia. The urinary regulation of sodium and potassium ions occurs in the kidney inner medullary collecting ducts (IMCD). The goal of this work is the identification and distribution of Kvs in epithelial cells from the rat kidney. Applying RT-PCR, Western blot and immunohistochemistry techniques, we demonstrated the presence of members of the Shaker subfamily (Kv1.x): the Kv1.1, Kv1.2, Kv1.3, Kv1.5 and Kv1.6 channels in the collecting duct and proximal tubules through different kidney sections. The polarity of these channels was also determined by immunofluorescence. These Kv channels may help to the sodium reabsorption and to maintain the resting membrane potential in the IMCD.

C1-056P
Sigma receptors regulate the apoptosis/proliferation balance through K^+ and Cl^- ion channel modulation in tumor cells

A. Renaudo, H. Guizouarn and O. Soriani
CNRS UMR FRE2127, Physiology, University of Nice Sophia-Antipolis, Nice, France. E-mail: renaudo@obs-vlfr.fr

In the last decade, a lot of progress has been made in the understanding of cell mechanisms which are presiding over cell destiny. In that domain, both Cl^- and K^+ channels have been shown to play a crucial role in cell proliferation and apoptosis by controlling cell volume regulation. The sigma-1 receptor is a small protein of 28 kDa, overexpressed in tumor cells and which has yet been shown to inhibit K^+ channels. However, until now, the function of this receptor remains enigmatic. We have studied in two cancer cell model, NCI-H209 (a Small Cell Lung Cancer line) and Jurkat (a Leucemic T cell line), the influence of sigma-1 receptors on those events through the regulation of volume-regulated Cl^- (ORCC) and K^+ channels (Kv). We have demonstrated that: (i) the pharmacological sigma-1 receptor activation induces a strong inhibition of both ORCC and Kv in the two cell lines; (ii), activation of sigma-1 receptors leads to a dramatic inhibition of cell proliferation characterized by an arrest in the G1 phase of cell cycle through p27Kip1 accumulation; (iii), stimulation of sigma-1 receptors protects cells against Staurosporine or Fas-L-induced apoptosis through KCl efflux (AVD) inhibition. These results lead to the apparently paradoxical conclusion that activation of sigma-1 receptors inhibits both proliferation and apoptosis in tumor cells. However, because the level of activation of sigma-1 receptor is rather low *in vivo*, we can speculate that it is strong enough to protect tumor cells against apoptosis but not sufficient to slow down proliferation. Altogether our results suggest that sigma-1 receptors may be used as a target to modulate the balance between apoptosis and cell proliferation and open a new track in the field of cancer treatment.

C1-057P
The Second Sodium pump: Isolation and characterization

F. J. Romero, M. A. Rocafull, L. E. Thomas and J. R. del Castillo
Fisiología Gastrointestinal, Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas (IVIC), Caracas, Venezuela. E-mail: fromero@ivic.ve

Small intestinal epithelial cells transport Na^+ by two different active mechanisms: The Na/K pump (ouabain-sensitive Na/K ATPase) and the Second Sodium pump, associated with the ouabain-insensitive Na ATPase (BBA, 812:402, 1985; BBA, 812:413, 1985; ABB, 419:190, 2003). These pumps and their ATPases have multiple functional differences, but it has not been so far possible to isolate the biochemical entity related with the Second Sodium pump. To identify the protein related to the Second Sodium pump, basolateral plasma membranes of small intestinal epithelial cells from guinea pig were solubilized with C12E9. Proteins were separated by a combination of gel filtration and concanavalin-A-sepharose affinity chromatography. The solubilized fraction, which contained the Na ATPase (181 ± 6.5 nmol Pi/mg/min) and the Na/K ATPase (381 ± 8.4 nmol Pi/mg/min), was gel filtered in Sepharose 6B. The Na/K and Na ATPases co-purified. These ATPases were separated by concanavalin-A-sepharose affinity chromatography. The purified Na ATPase had a specific activity of 1650 ± 104 nmol Pi/mg/min. SDS-PAGE of the enzyme showed a/b subunits of 100 and 45 kDa, respectively. Polyclonal antibodies against Na/K ATPase did not recognize the purified Na ATPase (Western blot analysis). Kinetic properties of the purified enzyme were similar to those of the native membrane-bound enzyme, indicating that it had not been substantially altered during the purification procedure. The purified Na ATPase was Mg-dependent, stimulated by Na^+, inhibited by furosemide and vanadate and insensitive to ouabain. Thus, the basolateral plasma membrane Na ATPase is structurally and functionally dissociable from the Na/K ATPase. It appears as a new member of the P-type ATPases.

C1-058P
Short chain fatty acid-induced differentiation results in modulated plasma membrane Ca^{2+}ATPase expression in gastric and colon cancer cells

P. Ribiczey[1], B. Papp[2], A. Tordai[1], Á. Enyedi[1] and T. Kovács[1]
[1]*Institute of Haematology and Immunology, National Medical Center, Budapest, Hungary,* [2]*INSERM EMI-00-03 Laboratoire de Biologie Cellulaire Hématopoiétique, Institut Universitaire d'Hématologie, Hôpital Saint-Louis, Paris, France. E-mail: ribiczey@biomembrane.hu*

Ca^{2+}ATPases play a key role in the maintenance and restoration of asymmetrical calcium distribution between the cytosol, intracellular organelles and the extracellular medium. This asymmetry is essential for normal calcium signalling that controls cell proliferation, differentiation or apoptosis. Recently, defects in the sarco/endoplasmic reticulum Ca^{2+}ATPase 3 (SERCA3) expression were shown in various gastric/colon cancer cells and tissues. We demonstrated that SERCA3 expression is markedly enhanced during cell differentiation. Here, we show differentiation-induced changes in the expression of the plasma membrane Ca^{2+}ATPases (PMCAs) in several gastric/colon cancer cell types. PMCA1b is the major isoform in the untreated cancer cell lines, whereas the expression level of PMCA4b is significantly lower. Differentiation of these cells initiated with short chain fatty acids (SCFAs) resulted in a marked induction of PMCA4b expression, while the level of PMCA1b did not change or was only slightly increased. The upregulation of PMCA4b expression during differentiation was demonstrated both at protein and mRNA levels, and it correlated well with the induction of other differentiation markers. At the same time, the expression level of the Na^+/K^+ATPase or that of the housekeeping SERCA2 did not change significantly or was reduced. A marked increase in PMCA-dependent calcium transport activity of microsomal membranes obtained from SCFA-treated gastric/colon cancer cells supported further the induction of PMCA4b expression and function. Our data indicate a suppressed PMCA4b expression in several gastric/colon cancer cell types and provide evidence for differentiation-induced, isoform-specific regulation of PMCA gene expression.

C1-059P
Photoaffinity labeling of ATP synthase by mono- and bifunctional 3′-biotinylated 8-azidoadenine nucleotides

R. Genswein[1], O. Eger[1], M. Stolz[1], Y. Kagawa[2] and **H.-J. Schaefer**[1]
[1]*Institute for Biochemistry, Department of Chemistry and Pharmacy, Johannes-Gutenberg-University, Mainz, Germany,* [2]*Department of Biochemistry, Jichi Medical School, Tochigi-ken, Japan. E-mail: jschaef@uni-mainz.de*

In order to characterize nucleotide binding sites of ATP synthases, we have synthesized various mono- and bifunctional photoactivatable ATP analogs [1]. The six nucleotide binding sites – three catalytic and three non-catalytic – of ATP synthases are located on the F1 complex of the enzyme alternately at the interfaces between the major subunits alpha and beta as demonstrated by photoaffinity labeling and photoaffinity cross-linking using mono- and bifunctional photolabels [2,3]. In 1994, this interfacial location of all the nucleotide binding sites was confirmed impressively by X-ray analysis of the F1ATPase from beef heart mitochondria [4]. The introduction of an additional biotin residue, yielding 3′-biotinyl-8-azido-ATP [5], is advantageous for an easy detection of labeled proteins. Irradiation of the F1ATPase from the thermophilic bacterium PS3 (TF1) in the presence of 3-biotinyl-8-azido-ATP resulted in the nucleotide-specific inactivation of the enzyme as well as in the nucleotide-dependent labeling of alpha and/or beta subunits. In addition, 3′-biotinyl-8-azido-ATP could be used successfully to label V1ATPase from *Manduca sexta* [5]. Dimerization of 3′-biotinyl-8-azido-ADP resulted in the formation of the bifunctional diadenine dinucleotide 3′-dibiotinyl-8-diazido-AP4A. Irradiation of TF1 in the presence of this photolabel yielded the nucleotide-specific inactivation of TF1 and the nucleotide-dependent formation of alpha-beta cross-links. All these results demonstrate the suitability of the biotinylated azidonucleotides for photoaffinity labeling and photoaffinity cross-linking of ATP binding proteins.

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C1-060P
Application of FLIPR platform to the generation of K^+ channel cell based assays

A. Di Silvio, G. M. Stucchi, M. Micheletti, E. Redaelli, A. Taddei and **L. Scarabottolo**
Cell Biology, Axxam, San Raffaele Biomedical Science Park, Milano, Italy. E-mail: lia.scarabottolo.ls@axxam.com

K^+ channels form a large and diverse group of distinct ion channel families which play critical roles in a wide variety of physiological processes, including heart rate, muscle contraction, neurotransmitter release, neuronal excitability, insulin secretion, epithelial electrolyte transporter, cell volume regulation, and cell proliferation. Over the last decade, the human genome project, together with an intense cloning effort, has identified more than 80 K^+ channel-related genes. This, coupled with the progress toward understanding the distribution, the molecular composition and the contribution of K^+ channels to native currents, has made K^+ channels increasingly attractive as therapeutic drug targets. In this work we illustrate the application of Fluorimetric Imaging Plate Reader (FLIPR) platform to the generation of cell based assays for different K^+ channel classes, suitable for high throughput screening (HTS) of compounds. In particular we have focused our attention on the: Kv2.1 channel, belonging to the voltage-gated family, EAG2, belonging to the voltage-gated ether a go-go family, and Kir6.2-SUR1 and Kir6.2-SUR2A, belonging to the KATP sensitive family. We have stably transfected CHO Dukx or CHO K1 with the human cDNAs and we have used FLIPR technology to develop functional assays, suitable for HTS. Kv2.1 and EAG2 are voltage gated, outward delayed rectifier, non-inactivating potassium channels; to detect their functionality we have used KCl injection, able to provoke a strong and sustained channel dependent cell membrane depolarization, recorder as RFU (relative fluorescent units) increase after loading the cells with a membrane potential sensitive dye. Kir6.2 is a weak potassium inward rectifier ATP sensitive K^+ (KATP) channel which assembles as heteromultimers with the SUR (sulfonylurea) receptors. Functionality of both Kir6.2-SUR1 and Kir6.2-SUR2A could be assessed as a strong cell membrane hyperpolarization obtained upon injection of the respective specific channel openers: Diazoxide and Pinacidil; while second injection of a channel blocker, such as Glybenclamide, showed an inhibition of the previously evoked current. All the FLIPR data were validated by electrophysiological experiments. We have demonstrated that FLIPR technology may represent a very sensitive and reliable tool for the study of K^+ channel functionality. Furthermore FLIPR based assays result to be particularly robust and suitable for high throughput screening of molecules, for the identification of specific channel modulators.

C1-061P
Analysis of SERCA and PMCA proteins in developing chick cerebellum

M. R. Sepúlveda, J. Palacios and A. M. Mata
Dept. Bioquímica y Biología Molecular y Genética, Fac. Ciencias, Universidad de Extremadura, Badajoz, Spain.
E-mail: rosarios@unex.es

The Ca^{2+}-ATPases from the sarco(endo)plasmic reticulum (SERCA) and from the plasma membrane (PMCA) are key components in the regulation of the intracellular Ca^{2+} in neuronal cells. However, the physiological role of these Ca^{2+} pumps in specific processes during development is not yet understood. In this work, we have analyzed the functional expression of both SERCA and PMCA proteins and their distribution in developing chick cerebellum. An increase in ATPase activity and Ca^{2+} transport with the stages of development was observed in membrane vesicles prepared from embryos to hatching. Western blot assays using specific antibodies showed that the content of the SERCA protein increased with development while that of PMCA protein remained constant at all stages analyzed. Inmunohistochemical assays in sagittal sections revealed that the developmental expression patterns of these proteins are linked to the organization of the cerebellar cortex and maturation of cell types.

C1-062P
Nucleotide mediated plasma membrane H(+)-ATPase fluorescence quenching

J. G. Sampedro and Y. G. Ruiz-Granados
Laboratory of Nutrición Molecular, Área Académica de Nutrición, Instituto de Ciencias de la Salud-ICSA, Universidad Autónoma del Estado de Hidalgo-UAEH, Pachuca, Hidalgo, Mexico.
E-mail: sampedro@lycos.com

The plasma membrane H(+)-ATPase from the yeast *Kluyveromyces lactis* was isolated to 90–95% purity. Sigmoid dependence of activity on ATP concentration was observed with Hill number = 1.5, $S_{0.5}$ = 0.8 mM ATP and turnover number $36s^{-1}$. The addition of ADP or AMP-PNP (5 mM) resulted in 60% quenching of the intrinsic fluorescence of the H(+)-ATPase. Trp505 is the only Trp residue located in the nucleotide binding domain (N) and therefore, this Trp seems to be responsible of fluorescence changes. Fluorescence titration with AMP-PNP or ADP revealed the presence of two nucleotide binding sites showing high and low affinity for ATP (K_{d1} = 0.49 and K_{d2} = 1.26 mM ATP) but the same affinity for ADP (K_d = 1.08 mM ADP). It is proposed that in a putative dimeric structure the H(+)-ATPase subunits alternate between two conformational states during catalysis which could be the origin of its cooperative kinetics. Both Trp NBS-modification and fluorescence quenching by acrylamide of the native and denatured H(+)-ATPase indicated that Trp505 is located near the protein surface and becomes exposed when nucleotide is bound; further supporting that this amino acid is the responsible for fluorescence changes. Enzyme kinetics and fluorescence titration with nucleotides suggested that in the H(+)-ATPase the N-domain (N) equilibrates between three different states (ADP-N $\leftrightarrow$ ADP + N + ATP $\leftrightarrow$ ATP-N) before the P-domain becomes phosphorylated. In the catalytic cycle the high affinity for ATP will forward the cycle toward ATP hydrolysis, where Brownian motion would lead the complex ATP-N close to the P-domain for phosphate transfer.

C1-063P
CLIC proteins form redox-sensitive ion channels immunologically related to native brain microsomal anion channels

H. Singh and R. H. Ashley
Department of Biomedical Sciences, The University of Edinburgh, Edinburgh, United Kingdom. E-mail: harpreet.singh@ed.ac.uk

Chloride Intracellular Channel (CLIC) proteins are soluble ~30 kDa proteins that autoinsert into membranes to form molecular components of intracellular anion channels. We expressed mammalian CLIC1 and CLIC4 as cleavable His-tagged fusion proteins and incorporated them into voltage-clamped planar lipid bilayers to examine their channel behaviour. CLIC1 channels showed a saturating conductance of ~40 pS in KCl under reducing conditions, with sublevels of ~20 and ~10 pS, suggesting the presence of independently conducting "protomers". Protomers may contain several monomers, because CLIC1 only appears to have one transmembrane domain. The channels were inhibited by an affinity-purified anti-CLIC1 antibody, but

not by control Ig. The antibody also inhibited similar channels reconstituted from rat brain microsomal membranes, suggesting that CLIC proteins may be widely expressed components of intracellular anion channels. CLIC1 contains six cysteine residues, and redox titration using a GSSG/2GSH coupled buffer system led to a sequential reduction in conductance as oxidation progressed, resulting in near-complete channel closure. This is consistent with the idea that channel gating may be regulated by reversible disulphide bond formation. Cysteine-reactive chemicals resulted in a side-specific inhibition (or blockade) of CLIC1 activity, implying that at least one cysteine residue is located near the pore-forming region, possibly on the luminal side of the channel. CLIC1 also inserted into planar bilayers under strongly oxidizing conditions to form ~20 pS ion channels. Future experiments may help define the stoichiometry of the channels, and determine how their activity may be controlled in cells.

C1-064P
Potassium channels in brain mitochondria

A. Szewczyk, J. Skalska, B. Kulawiak and M. Glab
Laboratory of Intracellular Ion Channels, Nencki Institute of Experimental Biology, Warsaw, Poland.
E-mail: adam@nencki.gov.pl

In the inner mitochondrial membrane a potassium selective channels are present. They play probably involved in cytoprotective action in various cell types. In this study we have begun to characterize the potassium channels present in brain mitochondria. Previously, the presence of ATP-regulated potassium channel was observed in hippocampal mitochondria. The channel activity was measured after reconstitution of purified inner mitochondrial membrane into planar lipid bilayer. The activity of potassium channel was recorded. The mean conductance of the channel was 250 pS in 50/450 mM KCl gradient. Single-channel activity of this reconstituted protein showed properties of the big-conductance potassium (BK) channel: it was activated by Ca^{2+} and blocked by charybdotoxin. Additionally, stimulation of the channel activity was observed upon application of BK channel openers, benzimidazolone derivatives, NS1619 and NS004. Additionally, the effect of BK channel openers on neuronal cells survival was studied.

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C1-065P
Role of SH-groups in *Escherichia coli* and *Enterococcus hirae* FOF1-ATPase functioning

A. Poladian, N. Mnatsakanyan, K. Bagramyan and
A. Trchounian
Department of Biophysics, Yerevan State University, Yerevan, Armenia. E-mail: trchounian@ysu.am

The oxidation–reduction states of thiol-groups in the form of cysteine residues can modulate the enzymatic and transport activity of membranes. In *Escherichia coli*, the number of accessible SH-groups in membrane vesicles was shown [1] to be increased by ATP or by formate, suggesting an interaction between the FOF1-ATPase and hydrogenase 4 or hydrogenase 3, components of formate hydrogenlyases 2 (FHL-2) or 1 (FHL-1), under fermentation conditions. This would lead to formation of a protein–protein complex [2] within which the energy could be transferred via a dithiol–disulfide interchange. It is suggested that the effects with SH-groups is due to interaction of FoF1 with FHL-2 when external formate was absent, and with FHL-1 upon adding formate. The SH-groups from FoF1 are proposed to be involved in the oxidation of formate through FHL-2 or FHL-1. E. hirae FOF1 might have direct involvement with K^+ uptake Trk-like or Ktr1 system. The findings about a fixed stoichiometry of K^+ influx via Ktr1 with H^+ efflux through FOF1 and the FOF1-ATPase activity strongly stimulated by K^+ [3] seem to be arguments in a close relationship of FOF1 with Ktr1. The energy of ATP might be transferred from FOF1 to Ktr1 through a dithiol–disulfide interchange so that ATP may cause a change in SH-groups. Addition of ATP or NAD^{++}NADH to the membrane vesicles from *E. hirae* grown under anaerobic conditions at pH 8.0 was shown to cause a ~1.4-fold increase in the number of SH-groups. This was inhibited with *N*-ethylmaleimide. The increase was higher when ATP and NAD^{++}NADH both were added. The change was absent in the presence of *N*,*N*′-dicyclohexylcarbodiimide or sodium azide. This was also absent in atp mutant with defect in the FOF1-ATPase and, in addition, it was less in potassium ion-free medium. Results are discussed indicating that Ktr1 may be regulated by NAD or NADH mediated conformational changes.

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C1-066P
Ionic migration through glycine channel

K. B. Tereshkina and K. V. Shaitan
Department of Biology, Chair of Bioengineering, M.V. Lomonosov Moscow State University, Moscow, Russian Federation.
E-mail: ksenia@moldyn.org

The glycine receptor is a member of the ligand gated ion channel superfamily of neurotransmitter receptors. This receptor transmits a fast neurotransmission in the CNS. It is a transmembrane protein with oligomeric structure. The glycine receptor is composed of five subunits. Each subunit consists of four α-helices and has four transmembrane domains TM1-TM4. These domains form a central ion channel. It is believed that the TM2 domains of each receptor subunit line the pore of the channel and thus are critical transmembrane component for the functional activity of the receptor [1]. There are various hypotheses about 3D structure of the pore. In these work the structure with two positively charged rings of Arg and one negatively charged ring of Asp was considered. Mutation studies have proved these charged rings to be important determinants of channel conductance [2]. In the present work the following extended TM2 chain of the human neuronal glycine receptor α1 subunit was considered: MDAAPARVGLGITTVLTMTTQSSGSRA. The homomeric receptor was studied by means of molecular dynamics method with standard protocol [3]. The carboxyl terminus of peptides was bound with *N*-methylamine, the amino terminus of one was bound with acetyl for reduction of end effects. Hydrocarbon hull was used to simulate membrane environment. Migration of F^-, Cl^-, Br^-, I^-, Na^+, K^+, Ca^{2+} through the channel was investigated. Hydrated ions were considered as well as unhydrated. Three type of ionic behaviour was found to take place during migration, namely free migration, stop due narrow channel and attraction to charged atoms of the channel. Dependence of migration rate on time was plotted. Friction and diffusion coefficients were found for each ion and aquated complex. Only negatively charged ions were revealed to pass through the channel. Diffusion coefficients change depending on a part of the channel. Diffusion of ion is non-linear and involves several factors.

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C1-067P
Change dynamic of erythrocytes size under modification of protein ion-transport system of membrane

E. V. Tereshkin and A. F. Lobkov
Faculty of Biology, Chair of Biophysics, Moscow State University, Moscow, Russian Federation. E-mail: ram@moldyn.org

Size and volume of cell are related with ion-transport membrane processes. Some transport processes in membrane affect cellular size [1]. Currently the methods allow observing cellular size changes dynamics at high resolution are slightly developed. Here multi-purpose analytical system (laser cytomonitoring – LC) was used [2,3]. Technique of small-angle light scattering on suspended particles in various liquid environments of small-size objects is a basis of the system. LC makes it possible to obtain size distribution functions of particles and to observe their time evolution. The experiments detected density and volume distribution function kinetics. Normal human erythrocytes, Tris–buffer with 230 mosM were used. Ca concentration in cell was raised by sodium vanadate. The buffer solution contained 1 mM Na_3VO_4 and 2 mM Ca^{2+}. It is well-known that Na vanadate serves as erythrocyte calcium pomp inhibitor at concentrations high as 0.5 mM. Incubation of erythrocytes with vanadate resulted in Ca^{2+} accumulation and cell degradation. Kinetics of erythrocytes size distribution functions was detected during 90 min at 5–30 s. time resolution. LC was found allow detecting cellular size change kinetic as an integral index, which characterizes work of ion-transport protein system of membrane.

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C1-068P
Determination of the membrane topology of a haloacid transporter of *Burkholderia cepacia* MBA4

J. Tsang, M. Yu and V. Tam
Department of Botany, Molecular Microbiology Laboratory, University of Hong Kong, Hong Kong, Hong Kong SAR, PR China. E-mail: jshtsang@hkucc.hku.hk

Burkholderia cepacia MBA4 is a natural soil isolate. In batch culture it produces a single dehalogenase (Deh4a) that metabolizes the degradation of 2-haloacids such as monochloroacetate. The enzyme has been purified and characterized and the structural gene has been cloned and sequenced. Three hundred and 53 bases downstream of *deh4a* is an ORF encoding a putative haloacid transporter (Deh4p) of 552 amino acids. Pfam analysis of the putative amino acid sequence of Deh4p revealed that it is probably a member of the major facilitator superfamily (MFS) transporters. It has the signature of family 1: sugar transporter family proteins. The signature [LIVMF]–xG–[LIVMFA]–x(2)–Gx(8)–[LIFY]–x(2)–[EQ]–x(6)–[RK] is found between residues 130–155. Comparative analysis of Deh4p predicted that it contains twelve transmembrane domains, typical property of the sugar transporters. In order to confirm the structural property of the protein we decided to investigate the topology of this membrane protein. PhoA is alkaline phosphatase which only works in the periplasmic space while beta-galactosidase (LacZ) is an enzyme that is only functional in the cytoplasm. DNA fragments encoding various lengths of *deh4p* were amplified and fused in-frame with a *pho-lac* cassette. These recombinant plasmids were transformed and the fusion proteins expressed in *E. coli*. The activities of PhoA and LacZ were examined by X-phos and/or Red-Gal plates. The colour of the colonies indicates the location of the corresponding enzymes and thus determines the topology of the membrane protein. The making of the constructs and the colour evaluation results will be presented.

C1-069P
Mitochondrial biogenesis during differentiation of myoblasts

M. Comelli[1,2,3], **L. Tomasetig**[1,2,3], A. Francesconi[4] and I. Mavelli[1,2,3]
[1]Department of Biomedical Sciences and Technologies, University of Udine, Udine, Italy, [2]Department of Biomedical Sciences and Technologies, M.A.T.I. Center of Excellence, Udine, Italy, [3]Centro Interdipartimentale di Medicina Rigenerativa, University of Udine, Udine, Italy, [4]Institute of Clinical Pharmacology and Toxicology, University of Udine, Udine, Italy.
E-mail: ltomasetig@mail.dstb.uniud.it

Differentiation is accompanied in different cell models by mitochondrial biogenesis, as indicated by increases in mtDNA, marker enzyme activities and mRNA levels. Nevertheless, the connections between mitochondrial biogenesis and bioenergetics during myogenesis have not been completely established yet. In the present study, we used a rat myoblast cell line (H9c2) to investigate the energy metabolism and mitochondrial biogenesis during myogenesis. Cardiac differentiation was induced by culturing H9c2 in the presence of retinoic acid (1). Differentiation was monitored by reduction of cell proliferation over 7 days and expression of specific markers, i.e. troponin I and myosin heavy chain. Concomitantly, the activity of citrate synthase, a matrix mitochondrial enzyme which closely correlates with mitochondrial content during mitochondrial biogenesis (2), increased significantly. Upon cardiac differentiation the oxidative phosphorylation enzyme complexes (OXPHOS) were analyzed by a differential/functional proteomic approach by 2D electrophoresis (1D BN-PAGE, 2D SDS PAGE) and immunoblotting (3). The results indicate a different assembly of the OXPHOS complex V (F1-F0) in the differentiated cells and specifically a marked reduction of the amount of non-assembled F1 with respect to the whole complex. The results, though preliminary, suggest that myogenesis should be accompanied by a better coordination among the pathways controlling mitochondrial biogenesis and OXPHOS complexes assembly with respect of fast-proliferating undifferentiated myoblasts.

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C1-070P
Regulation of poly-(R)-3-hydroxybutyrate biosynthesis in *Escherichia coli*

M. C. Theodorou[1], C. A. Panagiotidis[2], A. A. Pantazaki[1] and D. A. Kyriakidis[1]
[1]*Department of Chemistry, Laboratory of Biochemistry, Aristotle University of Thessaloniki, Thessaloniki, Greece,* [2]*Department of Pharmaceutical Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece. E-mail: marina_theodor@hotmail.com*

Short-chain poly-(*R*)-3-hydroxybutyrate (PHB), a member of the PHAs family, is a ubiquitous constituent of prokaryotic and eukaryotic cells where it forms complexes with other macromolecules and is referred to as cPHB. Antizyme (Az) is a polyamine-inducible, non-competitive inhibitor of ornithine decarboxylase, the key enzyme of polyamine biosynthesis and it is a member of the NtrC-NifA family of sigma^{54}-RNA polymerase transcriptional activators. Az is identical to the AtoC protein, which is a transcriptional regulator of the *atoDAEB* operon. This operon, which is inducible by acetoacetate encodes the structural enzymes involved in short-chain fatty acid metabolism. AtoC/Az together with AtoS, the sensor kinase whose gene is located upstream of the *atoC* gene, constitute a two-component system, responsible for *atoDAEB* acetoacetate induction and positive regulation of cPHB biosynthesis in *Escherichia coli*. Here we report that polyamines can positively modulate the levels of poly-(*R*)-3-hydroxybutyrate (cPHB) biosynthesis in *E. coli*. Increased amounts of cPHB are synthesized in *E. coli* upon spermidine but not putrescine addition in the growth medium. This enhancement is up to the level of the positive regulation that AtoS-AtoC two-component system exerts on cPHB upon acetoacetate induction. A slight enhancement by spermidine was observed in DE*atoSC* cells, with cPHB amounts to remain in lower levels than their isogenic *atoSC*$^{+}$ cells. Simultaneous addition of acetoacetate and spermidine declines the amounts of cPHB to the basal levels. *N*-acetyl-spermidine, the first spermidine derivative upon entry in the cells, results in higher amounts of cPHB but at lower levels than spermidine.

C1-071P
The Ca^{2+} affinity of cardiac sarco(endo)plasmic reticulum Ca^{2+} ATPase is an important determinant of normal cardiac function and is tightly regulated by phospholamban

P. Vangheluwe[1], M. Tjwa[4], W. E. Louch[2], A. Van Den Bergh[3], P. Herijgers[3], E. G. Kranias[5], K. Sipido[2], L. Raeymaekers[1] and F. Wuytack[1]
[1]*Laboratory of Physiology, Leuven, Belgium,* [2]*Laboratory of Cardiology, Leuven, Belgium,* [3]*CEHA, Leuven, Belgium,* [4]*CTR, K.U.Leuven, Leuven, Belgium,* [5]*Department of Pharmacology, University of Cincinnati, Cincinnati, Ohio, United States of America. E-mail: peter.vangheluwe@med.kuleuven.ac.be*

The sarco(endo)plasmic reticulum (SR) Ca^{2+}-ATPase SERCA2a is a major determinant of cardiac relaxation and contraction. *SERCA2*$^{b/b}$ mice, in which SERCA2a was replaced by the higher Ca^{2+}-affinity isoform SERCA2b, suffered from impaired cardiac contraction/relaxation and developed left ventricular hypertrophy [Ver Heyen et al. (2001) Circ. Res. 89:838]. Cardiac SERCA2 expression was reduced by 50% which was shown to compensate for the high Ca^{2+} affinity of SERCA2b in the heart [Antoons et al. (2003) Circ. Res. 92:881]. In this study, we now demonstrate that the observed 2-fold upregulation of the SERCA2 inhibitor phospholamban (PLB) is protective in *SERCA2*$^{b/b}$ hearts through the reduction of the apparent Ca^{2+} affinity of SERCA2b. Indeed, the fraction of Ser16-phosphorylated PLB was reduced making PLB a stronger inhibitor. Also, ablation of PLB by crossing *SERCA2*$^{b/b}$ with *PLB*$^{-/-}$ mice worsened the overall phenotype and exacerbated the hypertrophic response. This is the first report of compensatory elevated cardiac PLB expression which benefits overall *in vivo* function and cardiac remodelling. Moreover, this study further illustrates the importance of the apparent Ca^{2+} affinity of SERCA2 to maintain normal cardiac excitation–contraction coupling. Reduced SERCA2 expression, increased PLB levels and a lowered fraction of phosphorylated PLB adequately serve the same goal in *SERCA2*$^{b/b}$, i.e. counteract the increased Ca^{2+} pump rate of SERCA2b in the submicromolar Ca^{2+} concentration range. In conclusion, Ca^{2+}-uptake activity in the low Ca^{2+} concentration range is a more important parameter than the maximal pumping rate and must be tightly regulated in the cardiomyocyte to avoid excessive cytosolic Ca^{2+} removal and the development of hypertrophy.

C1-072P
ATP2C2 encodes a novel isoform of secretory pathway Ca^{2+}-transport ATPase

J. Vanoevelen[1], L. Dode[1], K. Van Baelen[1], R. Fairclough[2], L. Missiaen[1], F. Wuytack[1] and L. Raeymaekers[1]
[1]*Physiology, Molecular Cell Biology, K.U.Leuven, Leuven, Belgium,* [2]*Endocrine Unit, Centre for Diabetes, Oxford, United Kingdom. E-mail: jo.vanoevelen@med.kuleuven.ac.be*

The family of P-type Ca^{2+}-transport ATPases consists of three subfamilies: plasma-membrane Ca^{2+} ATPases (PMCA), sarco(endo)plasmic-reticulum Ca^{2+} ATPases (SERCA) and secretory-pathway Ca^{2+} ATPases (SPCA). The present study focuses on the SPCA-branch of the family. The human SPCA1 protein (encoded by the *ATP2C1* gene) is a Ca^{2+}/Mn^{2+}-transport ATPase that localizes to the Golgi apparatus of eukaryotic cells. Together with SERCA2b, SPCA1 maintains the proper ionic milieu in the Golgi lumen for post-translational protein processing. Mutations in *ATP2C1* result in the human skin disorder Hailey-Hailey disease. Here we report the identification of the gene encoding a second SPCA isoform and functionally characterized the protein. The encoding gene is designated *ATP2C2* and its protein product is referred to as hSPCA2. The human *ATP2C2* gene consists of 27 exons that span a region of 95 kb on chromosome 16. While hSPCA1 is ubiquitously expressed, hSPCA2 mRNA is found mainly throughout the gastro-intestinal tract, in lung and in a number of secretory glands. Immunocytochemistry demonstrated the protein in the Golgi of colon epithelial cells. Upon overexpression in COS-1 cells, the hSPCA2 protein also showed a Golgi-like distribution. The overexpressed protein is a functional Ca^{2+}/Mn^{2+}-transporting enzyme because it forms a phosphorylated reaction intermediate and because hSPCA2-overexpressing COS-1 cells accumulate more Ca^{2+} and Mn^{2+} than control cells. This additional uptake was insensitive to the SERCA-specific inhibitor thapsigargin. Half-maximal activation of the enzyme was observed at 0.27 μM-free Ca^{2+}, which is similar to hSPCA1. This study shows for the first time that two distinct pumps can mediate thapsigargin-insensitive Ca^{2+} uptake into the Golgi apparatus.

C1-073P
Structural features of the vacuolar ATPase from electron microscopy, NMR spectroscopy and H/D exchange mass spectrometry

S. Wilkens, Z. Zhang, Y. Zheng, N. Kitagawa and E. Kish-Trier
Department of Biochemistry, University of California, Riverside, California, United States of America.
E-mail: stephan.wilkens@ucr.edu

Vacuolar ATPases (V-ATPases; V1V0-ATPases) are large, membrane bound, multi subunit protein complexes which function as ATP hydrolysis driven proton pumps. The vacuolar ATPase is made of two domains: a water soluble V_1, and a membrane bound V_0. ATP hydrolysis taking place on the V_1 is coupled to proton transport through the V_0. In the cell, the activity of the vacuolar ATPase is regulated by a substrate dependent dissociation. The dissociation is reversible and results in V_1 and V_0 domains which are incapable of MgATP hydrolysis and proton translocation, respectively. We have used electron microscopy (EM) and image reconstruction to generate three dimensional (3-D) structural models of the V-ATPases from bovine brain and yeast. Antibody labeling and difference imaging was used to determine the binding sites of individual subunits and subunit domains in the V-ATPase. The binding positions of subunits A,H,G,C,a,d, and AC45 have been studied and will be discussed. A comparison of the structural models of intact V-ATPase and isolated V_1 and V_0 domains reveals that the vacuolar ATPase undergoes significant structural changes during substrate dependent dissociation. We speculate that the observed structural changes in the isolated V-ATPase domains are responsible for the silencing of the MgATPase and proton translocation activities of the individual V_1 and V_0 domains. Protein NMR spectroscopy and hydrogen/deuterium mass spectrometry (HXMS) are used to obtain high resolution structural information for individual subunits and to study subunit-subunit interaction, respectively.

C1-074P
Algogenic effects of RFa peptides at the periphery are independent on their modulatory action on the acid sensing ionic channels (ASICs)

Y. Yudin[1], R. Waldmann[2], A. Baron[2], Z. Tamarova[1], O. Krishtal[1] and M. Lazdunski[2]
[1]*Department of Cellular Membranology, Bogomoletz Institute of Physiology, Kiev, Ukraine,* [2]*Institut de Pharmacologie Moleculaire et Cellulaire, Sophia Antipolis, Valbonne, France.*
E-mail: yudin_e@mail.ru

Acid sensitive ion channels are widely expressed in mammalian sensory neurons. Knock-out experiments indicate at their participation in several modalities of perception comprising mechanosensitivity, nociception and, more specifically, acid sensation. RFa peptides affect the activity of ASICs by slowing down their desensitization. This implies that RFa peptides could be algogenic at the periphery. Using the skin-n.saphenous preparation *in vitro* we have found that RFa peptides have a strong excitatory effect predominantly on the C-fibers (76% tested fibers). However, there is no correlation in the sensitivity of C-fibres to RFa peptides, protons and amiloride (the channel blocker of ASICs): 74% of tested RFa-sensitive C-fibres were insensitive to protons and in 67% of cases the response to peptides was insensitive to amiloride. This negative result, however, is not decisive. The subtype of ASICs most abundantly and specifically expressed in the sensory neurons is ASIC3. It has been shown that the sustained component of the current through the ASIC3 channel is not inhibited by amiloride. We tested the peripheral action of RFa peptides in the *in vivo* experiments on ASIC3 knock-out (ASIC3 –/–) mice. We have found that subcutaneous injection of mammalian RFa peptide NPSF (2 mM) in the area of the n.saphenous innervation results in a clearly nociceptive behavior both in ASIC3 –/– and wild-type (C57BL/6J) mice. There was no significant difference in the total time of licking of injected paw in the groups of ASIC3 –/– (195 ± 22 s) and C57BL/6J (227 ± 21 s) animals. Thus the loss of ASIC3 gene did not alter the nociceptive behavior induced by administration of RFa peptides. Our data indicate that RFa peptides act at the peripheral nociceptive pathways and powerfully excite cutaneous C-fibres. Their excitatory/algogenic action cannot be interpreted only in terms of their interaction with ASICs channels. Other still unknown mechanisms of nociception are most probably involved.

C1-075P
Translationally controlled tumor protein interacts with sorting nexin 6

T. Yoon and K. Lee
College of Pharmacy, Center for Cell Signaling Research and Division of Molecular Life Sciences, Ewha Woman's University, Seoul, South Korea. E-mail: yts65@hanmail.net

Translationally controlled tumor protein (TCTP), also known as IgE-dependent histamine-releasing factor (HRF), p23 and fortilin, has both extra- and intracellular functions. To better understand the intracellular function of TCTP, we performed yeast two-hybrid assay using TCTP as a bait and identified the sorting nexin 6 (SNX6). Since TCTP has been reported to interact with Na,K-ATPase and inhibit its activity, the interaction of TCTP with SNX6 as well as Na,K-ATPase was confirmed by immunoprecipitation and confocal microscopy. The deletion analysis showed that the N-terminal 1-166 amino acid region of SNX6 containing the Phox domain is essential for the association with TCTP. The $^{86}Rb^+$ uptake assay showed that Na,K-ATPase activity was increased by the overexpression of SNX6, but not by the overexpression of its deletion mutant which is unbound to TCTP. We also found that insulin stimulates the translocation of SNX6 to the plasma membrane. These results suggest that SNX6 may increase Na,K-ATPase activity by acting as a negative regulator of TCTP and that the Na,K-ATPase activation by insulin may be caused by the interaction of TCTP with SNX6.

C1-076P
Non-genomic effect of DHEA and its sulfate on plasma membrane Ca^{2+}-ATPase activity *in vitro*

L. Zylinska, I. Kawecka and J. Szemraj
Department of Molecular Neurochemistry, Medical University, Lodz, Poland. E-mail: luska@csk.umed,lodz.pl

The aim of our study was to compare the effect of dehydroepiandrosterone (DHEA) and its sulfate derivative on hydrolytic activity of plasma membrane calcium pump (PMCA) purified from excitable (rat cortical synaptosomes) and non-excitable (human erythrocytes) cells. Both types of cell membranes contained different composition of the PMCA isoforms. To elucidate if the hormone action could depend on structure of PMCA protein, we assayed the hormone effect on Ca^{2+}-ATPases pre-treated for 15 and 40 min with a trypsin. The full length and trypsin-treated Ca^{2+}-ATPases were next incubated with 10^{-9} and 10^{-7} M concentration of steroids. The ATPase activity was also examined in the presence of naturally existing activator-calmodulin. In examined Ca^{2+}-ATPases both steroids tested differently altered their activity. DHEA significantly decreased the activity of synaptosomal enzyme, whereas the increase of erythrocyte enzyme activity was

observed, particularly in the trypsin-treated samples. In contrary, a substantial enhancement of the activity was detected for both enzymes in the presence of DHEAS, and this effect was more pronounced for erythrocyte calcium pump treated with protease. The steroids altered the potency for stimulation of calcium pump activity by calmodulin, showing different mechanisms of action in dependence on isoform compositions and structural feature of the steroids. Our results suggest that plasma membrane Ca^{2+}-ATPase could be a target for non-genomic action of DHEA and DHEAS at biologically and pharmacologically relevant concentrations.

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C2 – ABC Transporter Proteins

C2-001
Multidrug resistance proteins: versatile transporters of anionic drugs and metabolites

P. Borst[1], N. Ono[1], K. van de Wetering[1], P. Wielinga[1], J. Wijnholds[1], H. Yamaguchi[2] and N. Zelcer[1]
[1]*Division of Molecular Biology and Center of Biomedical Genetics, The Netherlands Cancer Institute, Amsterdam, The Netherlands,* [2]*Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto, Japan.*
E-mail: p.borst@nki.nl

Three types of active drug transporters belonging to the ABC transporter family can give rise to Multidrug Resistance (MDR) of cancer cells, the MDR1 P-glycoprotein (ABCB1), the Breast Cancer Resistance Protein (ABCG2), and the Multidrug Resistance Proteins (MRPs) of the ABCC sub-family (1). The human genome contains nine MRP genes and eight of these have been shown to be able to transport organic anions, such as drugs conjugated to glutathione, sulfate or glucuronate. In addition, selected MRPs may transport a variety of endogenous compounds, such as leukotriene C4 (MRP1), bilirubin glucuronides (MRP2, MRP3), prostaglandins E1 and E2 (MRP4, Ref. 2), cGMP (MRP4, MRP5, MRP8, Ref. 3), and several glucuronosyl-, or sulfatidyl steroids. In my lecture I shall review the current evidence for the physiological function of the MRPs, their role in drug resistance to anti-cancer agents, and in the disposition of drugs modified by conjugation to acidic moieties. Special attention will be given to allosteric modulation of MRPs (4) and to morphine disposition.

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C2-002
New ABC transporters associated with multidrug resistance in cancer

M. M. Gottesman, J. K. Paterson, K. G. Chen, J. P. Annereau and G. Szakács
Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD, United States of America.
E-mail: mgottesman@nih.gov

ATP-dependent (ABC) transporters, such as ABCB1 (MDR1, P-glycoprotein), ABCC1 (MRP1), and ABCG2 can confer multidrug-resistance (MDR) on cancer cells by energy-dependent efflux of anti-cancer drugs. To explore the possible role of all 48 known human ABC transporters in drug-resistance in cancer, we have used real-time (RT)-PCR [Szakács, G., Annereau, J. P., Lababidi, S., Shankavaram, U., Arciello, A., Bussey, K. J., Reinhold, W., Guo, Y., Kruh, G. D., Reimers, M., Weinstein, J. N., and Gottesman, M. M.: Predicting drug sensitivity and resistance: profiling ABC transporter genes in cancer cells. *Cancer Cell* **6**: 129–137, 2004] and micro-array analysis [Annereau, J. P., Szakács, G., Tucker, C. J., Arciello, A., Cardarelli, C., Collins, J., Grissom, S., Zeeberg, B., Reinhold, W., Weinstein, J., Pommier, Y., Paules, R. S., and Gottesman, M. M.: Analysis of ABC transporter expression in drug-selected cell lines by a micro-array dedicated to multidrug resistance. *Mol Pharmacol* **66**: 1397–1405, 2004] to measure mRNA levels for these transporters in the NCI panel of 60 cancer cells, for which resistance to almost 100 000 different drugs is known, and in various *in vitro*-selected, drug-resistant cancer cell lines. The quantitative RT-PCR analysis shows that expression of at least one-half of human ABC transporters can be correlated with specific drug-resistance of cancer cells in the NCI-60 panel, and the use of micro-array analysis also shows expression of specific ABC transporters other than ABCB1, ABCC1, and ABCG2 in drug-selected cancer cells. We have focused on ABCB5 (expressed in melanomas), ABCC2 (expressed in melanomas and drug-selected cells), and ABCB6 (expressed in arsenite and cisplatin-resistant cells) to evaluate the specific role of these transporters in drug-resistance in cancer. In addition, the RT-PCR analysis has revealed some drugs that specifically kill ABC-transporter-expressing cells. These agents show promise for targeting MDR cancer cells expressing known drug-resistance genes such as ABCB1.

C2-003
Structure and mechanism of bacterial ABC transporters

K. P. Locher[1] and E. L. Borths[2]
[1]*Institute of Molecular Biology and Biophysics, Swiss Federal Institute of Technology, Zürich, Switzerland,* [2]*Department of Chemistry, California Institute of Technology, Pasadena, CA, United States of America. E-mail: locher@mol.biol.ethz.ch*

ATP-binding cassette (ABC) transporters couple ATP hydrolysis to the translocation of diverse substrates across cell membranes. Human ABC transporters have been associated with various diseases and with multi-drug resistance of cancer cells, whereas bacterial homologs mediate nutrient uptake and drug extrusion. Recently, crystal structures have been solved of two bacterial, full-length ABC transporters, the lipid A flippase MsbA and the vitamin B12 transporter BtuCDF. We have studied the functional properties of *E. coli* BtuCDF in detergent solution and liposomes. In addition, we have labeled BtuCD with fluorescent probes to probe the dynamics of the transporter. The results and proposed mechanism of transport will be discussed.

C2-004
Function of the transport machinery TAP in the cellular immune system

R. Abele, M. Herget, S. Schrodt, M. Chen, J. Koch and **R. Tampé**
Institute of Biochemistry, J. W. Goethe-University, Biocenter, Frankfurt, Germany. E-mail: tampe@em.uni-frankfurt.de

The adaptive immune system has evolved to protect vertebrates against numerous pathogens. The transporter associated with antigen processing (TAP) plays a key role in the cellular immune response. The ABC-transporter TAP translocates peptides mainly derived from proteasomal degradation into the endoplasmic reticulum, where these peptides are loaded onto MHC class I molecules. At the cell surface, MHC complexes display their antigenic cargo to cytotoxic T-lymphocytes, which eventually eliminate infected or transformed cells. Due to its key function in the compartmentalization of antigens (connecting the inside with the outside), the antigen transport machinery is the target of sophisticated strategies, by which viruses or tumors evade immune surveillance. Structural and functional aspects of the TAP complex as well as viral inhibition strategies are discussed.

C2-005
Energetics of CFTR channel gating studied through temperature dependence of transition rates

L. Csanády[1], A. C. Nairn[2] and D. C. Gadsby[3]
[1]*Department of Medical Biochemistry, Semmelweis University, Budapest, Hungary,* [2]*Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY, United States of America,* [3]*Laboratory of Cardiac/Membrane Physiology, The Rockefeller University, New York, NY, United States of America. E-mail: csanady@puskin.sote.hu*

CFTR channel gating was recorded at temperatures from 15 to 35 °C. Opening and closing rates were extracted, and the enthalpies of the transition states (DH#) for these gating steps determined from Eyring plots. For partially phosphorylated wild-type channels, both opening and closing rates were highly temperature sensitive (DH# = 103 ± 6 kJ/mol for opening and 60 ± 4 kJ/mol for closing). DH# values obtained in the presence of protein kinase A were similar (86 ± 10 and 78 ± 8 kJ/mol respectively). DH# for non-hydrolytic reversal of channel opening, obtained from the temperature dependence of the slow closure of ATP-hydrolysis deficient NBD2 mutant K1250R, was 43 ± 2 kJ/mol. Activation free energies (DG#) and entropies (DS#) were calculated from transition state theory. DG# was 76, 70, and 77 kJ/mol for opening, normal closing, and non-hydrolytic closure. The entropy of the open state is higher than that of the closed state (TDSopen-TDSclosed = 61 kJ/mol). Part of the entropy increase upon opening is already seen in the transition state (TDS# = 27 kJ/mol). In contrast, no entropy increase accompanies formation of the transition state for closure (TDS# = –10 kJ/mol). We conclude: gating of partially and fully phosphorylated channels is qualitatively similar; the gating cycle is asymmetric, with high DS# for opening but not for closure. Recent work suggests that channel opening is driven by formation of an NBD1/NBD2 dimer, and closure by disruption of this dimer upon ATP hydrolysis at NBD2. The high DS# for opening suggests that the dimer interface is partially desolvated in the transition state. The small DS# for closure is consistent with ATP hydrolysis being rate limiting; this transition state precedes rehydration (and hence disruption) of the dimer interface. [NIH DK51767, TW5761].

C2-006
The role of the conserved glycines of signature regions of MRP1 multidrug transporter in the catalytic mechanism

Z. Szentpétery[1], A. Kern[1], K. Liliom[1], B. Sarkadi[2], A. Váradi[1] and É. Bakos[1]
[1]*Laboratory of Active Transport Proteins, Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary,* [2]*National Medical Center, Institute of Haematology and Immunology, Hungarian Academy of Sciences, Budapest, Hungary. E-mail: szentpetery@enzim.hu*

A key element of the structural model of ABC-ATPases is the interaction of the two ABC-domains. They complement each other's active sites on a way that the ABC-signature motif (LSGGQ) of one subunit interacts with the gamma-phosphate of the ATP bound at the Walker motifs of the opposite subunit. In order to investigate the role of the signature motifs in the ATP hydrolysis of MRP1, the conserved glycines of the LSGGQ were substituted for aspartic acids (G771D, G1433D). The mutants were expressed in Sf9 insect cells and the catalytic activity was assayed by ATPase- and vesicular transport experiments. ATP binding and the transition-state formation were studied by using a labeled photoreactive ATP analog. We found that the signature mutants were transport - and ATPase-incompetent, they could not present the transition-state formation, although showed normal ATP binding. In the ortho-vanadate-cleavage reaction of the mutant variants, the nucleotide- as well as the transported substrate – protein interactions were further studied and we found that the glycines are not essential in the nucleotide-induced interaction between the two-nucleotide binding sites. However, the effect of substrates on the cleavage reaction was significantly different in the mutant variants than in the wild type. While the transported substrates stimulated the formation of the post-hydrolytic complex in the wild type, this reaction was inhibited in the signature mutants. Our results suggest that the conserved glycine residues in both LSGGQ segments are part of the intramolecular conformational network, which is responsible for the accelerated hydrolytic activity upon interaction of the protein with its transported substrates.

C2-007P
Functional aspects of multidrug efflux pumps – lessons learnt from P-glycoprotein and its bacterial homologue LmrA

K. Pleban[1,4], S. Kopp[1], E. Csaszar[2], W. N. Konings[3], G. F. Ecker[4] and **P. Chiba**[1]
[1]*Functional Biology Lab, Center for Physiology and Pathophysiology, Institute of Medical Chemistry, Medical University of Vienna, Vienna, Austria,* [2]*Max. F. Perutz Laboratories, University Departments. at the Vienna Biocenter, Mass Spectrometry Facility, University of Vienna, Vienna, Austria,* [3]*Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, the Netherlands,* [4]*Pharmacoinformatics Lab, Department of Pharmaceutical/Medicinal Chemistry, University of Vienna, Vienna, Austria. E-mail: peter.chiba@meduniwien.ac.at*

P-glycoprotein, a human energy dependent multidrug efflux pump, plays an important role for resistance to cancer-chemotherapy and early ADMET profiling in drug development. LmrA, a structural and functional homologue of P-glycoprotein confers resistance to 17 of 20 clinically most frequently administered antibiotics. Propafenone-type substrates were used for photo-affinity labeling of the proteins. Proteolytic degradation

and subsequent identification of labeled peptide fragments by MALDI-TOF mass spectrometry led to identification of transmembrane segments that are involved in the formation of the binding sites. MsbA is the only full length structurally resolved ATP-binding cassette transporter conforming to the predicted structures of P-gp and LmrA and was thus used as a template for the generation of protein homology models. For both transporters, affinity labeled peptides mapped to the transmembrane domain – transmembrane domain interfaces. Though the models represent a static structure, labeling patterns associated with different steps of the catalytic cycle provided evidence that the interface undergoes considerable rearrangement during the transport cycle. Available data suggest that substrate binding at domain interfaces may be a general feature of multispecific drug efflux pumps.

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C2-008P
Mining ABC Transporters of *Mycobacterium tuberculosis*

P. Chakrabarti
Bose Institute, Kolkata, India.
E-mail: parulchakrabarti@hotmail.com

The genes encoding ABC transporters occupy 2.5% of the genome of *Mycobacterium tuberculosis*. However, none of these putative ABC transporters has been characterized so far. We have undertaken for the first time molecular and functional characterization of two such ABC transporters of *M. tuberculosis*. The *drr operon* encoding polypeptides similar to ABC transporters is present in a 50 kb virulence fragment of the genome of *M. tuberculosis* which contains seven genes involved in the biosynthesis of an surface-exposed antigenic lipid, phthiocerol dimycocerosate(DIM). Signature-tagged mutagenesis in *drr operon* led to the strong growth inhibition of *M. tuberculosis* in the lungs of intravenously infected mice and in export of DIM to the cell surface. Thus *drr operon* assumes a particular significance and presents an attractive drug target. We developed expression systems in *E. coli* and *M. smegmatis* for tandem expression of DrrA and DrrB and characterized them as an ATP-binding protein and an integral membrane protein respectively. In both the expression systems DrrA and DrrB behave as a functional doxorubicin efflux pump. When expressed in *M. smegmatis*, DrrAB conferred resistance towards a broad range of clinically relevant, structurally unrelated antibiotics in mycobacteria, much like LmrA of *Lactococcus lactis*, counterpart of the human P-glycoprotein. The resistant phenotype could be reversed by verapamil and reserpine, two potent inhibitors of ABC transporters. Site-directed mutagenesis furnished important information on the amino acid residues involved in the ATP-binding and doxorubicin accumulation in DrrAB-mediated transport process. Identification of its inhibitors would be of particular use considering that the *drr operon* is essential for the survival of *M. tuberculosis* in macrophages. Oligopeptides play important roles in bacterial nutrition and signaling. Oligopeptides are probably a valuable form of nutrient during long-term survival of *M. tuberculosis* in the macrophage when they remain protected from specific and nonspecific immune system and many antibacterial drugs. Oligopeptides are taken up by the oligopeptide (Opp) transport system, a member of the ABC transporter family. In the putative Opp ABCD transport system of *M. tuberculosis*, OppB and C are two membrane spanning proteins, OppD where two nucleotide binding domains are fused in one protein, is a nucleotide binding protein and OppA is a substrate binding lipoprotein. Since substrate specificity for ABC transporter in bacteria is determined by their substrate binding protein, the *oppA* gene, encoded by the open reading frame Rv1280c in the genome of *M. tuberculosis* H37Rv, was amplified from the cosmid clone, MTCY50 by PCR, cloned, sequenced and expressed in *E.coli* BL21(DE3). The purified protein showed preference for glutathione as substrate. Mutational studies of the expressed protein enabled us to identify residues essential for substrate binding. These will be discussed.

C2-009P
Over expression and functional reconstitution of Pdr5p

R. Ernst[1], K. Kuchler[2] and L. Schmitt[1]
[1]Institute of Biochemistry, University of Frankfurt, Frankfurt, Germany, [2]Medical Biochemistry, Medical University Vienna, Vienna, Austria. E-mail: ernst@em.uni-frankfurt.de

The ABC-transporter Pdr5p from *S. cerevisiae* is a key element of the pleiotrophic drug-resistance (PDR). PDR is phenomenological and functional similar to the human multi drug-resistance. However, it was recently proposed that Pdr5p among other membrane protein is involved in lipid homeostasis. As a prerequisite for a detailed study of this suggestion, we were able to over-express and purify Pdr5p in sufficient quantities. Furthermore, we could show that Pdr5p remained functional in detergent solution as well as in the reconstituted state. After reconstitution in liposomes we were able to show, that Pdr5p is a broad specific translocase for fluorescently labeled phospholipids. In conclusion, these results serve as a starting point to decipher the molecular role and properties of this ABC-transporter.

C2-010P
New children in the ABC super-family: the *Tetrahymena thermophila* ABC set

G. Friso[1], G. Iannone[2] and G. Cercignani[1]
[1]Dipartimento di Fisiologia e Biochimica, Università di Pisa, Pisa, Italy, [2]Dipartimento di Patologia Animale, Profilassi e Igiene degli alimenti, Università di Pisa, Pisa, Italy.
E-mail: giorgio.friso@pi.ibf.cnr.it

ABC (ATP-Binding Cassette) proteins are integral membrane proteins that can bind and hydrolyze ATP by means of specific amino acid sequences. One of the most extensively studied ABC proteins in man is PgP (also known as P170) which is responsible of multidrug resistance in cancer cells, resulting in their ability to withstand anticancer drugs most commonly used in clinical oncology trials. A single report in the ABC protein literature suggests the presence of a protein similar to human PgP in the ciliate genus Tetrahymena. This organism is an outstanding model for molecular biology studies, since it is easily grown in axenic culture, is readily amenable to manipulations and, like all ciliates, is the single-cell life form which is most near to Metazoa in the evolutionary tree. Here we present a study on ABC proteins in the species *Tetrahymena thermophila*. Using simultaneously four different approaches (genomics, bioinformatics, proteomics and immunobiochemistry) we sorted out six ABC-like proteins in the recently sequenced *T. thermophila* genome, resulting in a first characterization of a set of ABC proteins in this organism. We therefore propose the *T. thermophila* model system as a new tool to investigate ABC protein functions and inhibitors.

C2-011P
Analysis of missense PXE-mutants of *ABCC6/MRP6*: the first steps toward the allele-specific therapy

K. Fülöp[1], A. Iliás[1], E. Sinkó[1], L. Homolya[2] and A. Váradi[1]
[1]*Laboratory of active transport proteins, Institute of Enzymology BRC HAS, Budapest, Hungary,* [2]*National Medical Center, Institute of Haematology and Immunology HAS, Budapest, Hungary.*
E-mail: fulop@enzim.hu

While a spectrum of mutations within the *ABCC6* gene is clearly responsible for PXE, the functional relationship between altered *ABCC6* gene products and the PXE phenotype is still unknown. Recently, we have described the first studies of the transport activity of human *ABCC6*. We have established that this protein actively transports at least two anionic glutathione conjugates, and this transport is abolished by three missense mutations in *ABCC6* that are known to cause PXE. These new findings establish that aberrant transport is one of the primary determinants in the PXE phenotype. Furthermore, our published data indicate that *ABCC6* is localized to the plasma membrane in polarized kidney-derived (MDCKII) cells, and it is targeted to the basolateral membrane compartment. We have embarked upon a project to study 10 missense mutant variants of *ABCC6* (identified in PXE patients) by determining their transport activity utilizing insect cell expression and vesicular transport assays, as well as their subcellular localization in polarized kidney cells. We have found that a set of mutants possess no ATP-dependent transport activity, but are targeted correctly to the basolateral membrane. Another set of mutations is characterized with full transport activity but impaired targeting. In these mutants incomplete N-glycosylation was also observed. The data we obtain can contribute in better understanding the highly heterogeneous forms of PXE by providing the molecular basis of the aberrant function of the ABCC6 protein. Furthermore, the classification of missense mutations may provide the basis of allele-specific therapy of PXE.

C2-012P
ΔF508 CFTR with mutations in two arginine-framed tripeptide sequences escapes from ER quality control but remains thermally unstable

T. Hegedűs, A. Aleksandrov, L. Cui, M. Gentzsch, X.-B. Chang and J. R. Riordan
Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, S.C. Johnson Medical Research Center, Scottsdale, AZ, United States of America.
E-mail: hegedus@mayo.edu

Most cystic fibrosis (CF) patients carry the ΔF508 mutation in the CFTR chloride channel protein resulting in its misfolding, retention in the endoplasmic reticulum (ER), and proteasomal degradation. Therefore characterization of the retention and attempts to rescue the mutant CFTR are a major focus of CF research. Earlier we had shown that four arginine-framed tripeptide (AFT) signals in CFTR participate in the quality control. Now we have mutated these four AFTs in all possible combinations and found that simultaneous inactivation of two of them (R29K and R555K) is necessary and sufficient to overcome ΔF508 CFTR retention. Immunofluorescence staining of BHK cells expressing this variant indicates that it matures and is routed to the plasma membrane. Acquisition of at least some wild-type structure was detected in the pattern of proteolytic digestion fragments. Functional activity at the cell surface was evident in chloride efflux assays. However, single channel activity of the rescued mutant measured in planar lipid bilayers diminished as temperature was increased from 30 to 37 °C. These findings indicate that absence of Phe 508 causes not only a kinetic folding defect but also steady-state structural instability. Therefore effective molecular therapies developed to alleviate disease caused by ΔF508 and probably other misfolding mutants will require overcoming both their kinetic and steady state impacts.
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C2-013P
Subcellular localization of the ABCG1 and ABCG4 transporters in mammalian cells.

L. Seres[1], J. Cserepes[2], N. B. Elkind[1], B. Sarkadi[1,2] and **L. Homolya**[1]
[1]*Cell Biology Laboratory, Research Group for Membrane biology and Immunopathology, Hungarian Academy of Sciences, Budapest, Hungary,* [2]*Department of Molecular Cell Biology, National Medical Center, Budapest, Hungary.*
E-mail: homolya@biomembrane.hu

Most ATP binding cassette (ABC) transporters localize to the plasma membrane, however, several ABC half transporters such as TAP1, TAP2 and ABCD proteins reside in intracellular compartments. Subcellular localization of members of the ABCG subfamily is only partially clarified. ABCG2 and ABCG5/G8 are expressed in the plasma membrane, whereas the localization of ABCG1 and ABCG4 has not been clearly demonstrated. Previously, we have expressed ABCG1 and ABCG4 in Sf9 insect cells, and shown their substrate-stimulated ATPase activity. Our results also supported the hypothesis of homo- and heterodimerization of these proteins. The main focus of the present study was to investigate the cellular localization of the ABCG1 and ABCG4 proteins in mammalian cells. To visualize these proteins we have tagged them with various fluorescent proteins (eGFP, CFP and YFP) positioned either C- or N-terminally to the transporter. In addition, we have generated an anti-ABCG1 monoclonal antibody that allows us to detect the native, untagged protein. We have expressed ABCG1 and ABCG4 in different cell lines (HEK 293, COS-7, N2a and HepG2) by using a transient expression system and studied their subcellular localization by confocal laser scanning microscopy. We found that the tagged versions of ABCG1 and ABCG4 were localized to intracellular compartments, mostly to the endoplasmic reticulum. However, immunostaning of the untagged ABCG1 revealed that this protein was targeted almost exclusively to the plasma membrane. In conclusion, our results suggest that tagging of ABCG1 and ABCG4 with fluorescent proteins can greatly influence their subcellular localization.

C2-014P
H662 is the ''linchpin'' of ATP-hydrolysis in the nucleotide-binding domain of the ABC-transporter HlyB

S. Jenewein[1], J. Zaitseva[1], B. Holland[2] and L. Schmitt[1]
[1]*Institute of Biochemistry, University of Frankfurt, Frankfurt, Germany,* [2]*Institut de Génétique et Microbiologie, Université de Paris XI, Paris, France. E-mail: jenewein@stud.uni-frankfurt.de*

The ABC-transporter Haemolysin B (HlyB) is a central element of the *E.coli* Haemolysin A secretion machinery, a paradigm of Type I secretion. It energizes the transport of the cellular toxin Haemolysin A that targets to host cell membranes to form an aqueous transmembrane pore that results in cell lysis. Here we describe the crystal structure of the soluble HlyB-NBD (nucleotide binding domain) with H662 replaced by Ala in complex with

ATP/Mg^{2+}. The dimer shows a composite architecture, in which two intact ATP molecules are bound at the interface of the Walker A motif and the C-loop, provided by the two monomers. ATPase measurements confirm that H662 is essential for activity and that ATP-hydrolysis is the rate-limiting step during the catalytic cycle. Based on these data, we propose a model supported by the crystal structure, in which H662, highly conserved among ABC-transporters, acts as a "linchpin", holding together all required parts of a complicated network of interactions between ATP, waters, Mg^{2+}, and amino acids both in cis and trans, necessary for inter-monomer communication and ATP-hydrolysis through a novel mechanism not previously apparent for ABC-ATPases.

C2-015P
Feature based drug/protein interaction in multispecific proteins – Lessons learnt from multidrug efflux pumps ABCB1 and ABCG2

S. Kopp[1], K. Pleban[1,2], S. E. Bates[3], G. F. Ecker[2] and P. Chiba[1]
[1]*Functional Biology Lab, Center for Physiology and Pathophysiology, Institute of Medical Chemistry, Medical University of Vienna, Vienna, Austria,* [2]*Pharmacoinformatics Lab, Department of Pharmaceutical/Medicinal Chemistry, University of Vienna, Vienna, Austria,* [3]*Cancer Therapeutics Branch, National Cancer Institute, Bethesda, United States of America.*
E-mail: stephan.kopp@meduniwien.ac.at

The multidrug resistance transporters ABCB1 (P-glycoprotein) and ABCG2 (BCRP, MXR, ABCP) represent druggable targets in cancer therapy. On the other hand these plasma membrane proteins also play an important role as antitargets for a number of therapeutically administered drugs. Both transporters demonstrate remarkably broad and partly overlapping substrate specificity. Propafenone analogs, which are inhibitors of ABCB1 and ABCG2, have been used in a selectivity profiling approach to tune the activity of the compounds for selectivity towards one or the other drug efflux pump. Data demonstrate that a more than 100-fold difference in activity can easily be accomplished by triggering charge of the compounds. While neutral molecules inhibit ABCG2 stronger than ABCB1, the opposite is observed with analogs containing a tertiary nitrogen atom. Presence of the wt arginine residue in position 482 led to a decrease in activity of compounds containing chargeable tertiary amine functions. This indicates that amino acid residue R482 is located in proximity of the propafenone-binding site.

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C2-016P
Intestinal transfer of the pesticide diazinon: P-glycoprotein induction and mediated-efflux

S. Lecoeur[1], S. Cavret[2] and B. Videmann[1]
[1]*Laboratory of Xenobiotic Metabolism and Toxicology, Animal Health Department, National Institute for Agronomic Research, Marcy l'Etoile, France,* [2]*Laboratory of Food Safety, Animal Production Department, University of ISARA-Lyon, Lyon, France.*
E-mail: s.lecoeur@vet-lyon.fr

As one of the main sources of contamination by pesticides is oral exposure, the study of mechanisms governing their bioavailability is of primary interest. The purpose of this work was to investigate, *in vivo* and *in vitro*, the interaction of diazinon, a widely used organophosphorus pesticide, with intestinal P-glycoprotein. Oral administration of diazinon (2–20 mg/kg, 5 days, or 10 mg/kg, 2–12 days) increased intestinal mdr1a mRNA and P-glycoprotein in rats, in a dose- and time-dependent manner. The intestinal cell-line Caco-2 was used for *in vitro* transfer studies. Cell exposure to 25 M diazinon showed a secretory-directed transport. The efflux rate was significantly decreased in the presence of metabolic inhibitors (sodium azide and 2deoxy-Dglucose), the P-gp inhibitor valspodar, and MRP inhibitors (probenecid, and 1-chloro-2,4-dinitrobenzene) in a lesser extent. The efflux rate of 10 Mμ vinblastine, a P-glycoprotein substrate, was decreased in the presence of 100 M diazinon. Long-term pre-exposure of cells to low doses of diazinon increased P-glycoprotein expression and enhanced the efflux of pesticide by the intestinal cell line monolayer. This efflux was significantly decreased in the presence of valspodar and 1-chloro- 2,4-dinitrobenzene. These results suggested the involvement of efflux proteins in the mechanisms governing the transfer of diazinon, and showed that repeated exposure to low doses of pesticide may lead to up-regulated P-gp functions in the intestine of mammals.

C2-017P
Preparation of an ABC transport system for solid-state NMR analysis

V. Lange, L. Krabben and O. Hartmut
NMR-supported structural biology, Forschungsinstitut für Molekulare Pharmakologie, Berlin, Germany.
E-mail: vlange@fmp-berlin.de

The aim of our project is to analyse the structure of an ABC transport system via solid-state magic angle spinning (MAS) NMR techniques. The ATP-binding cassette (ABC) transporters represent a large family of proteins responsible for translocation of small biochemical compounds across cell membranes. The ABC transport complex YqiXYZ from *Geobacillus stearothermophilus* is a member of this superfamily. The proteins are likely to be quite stable in the thermophile *Geobacillus stearothermophilus*. These properties of the system make it well suited for solid-state NMR investigations. The ABC importer was expressed in *E. coli* both in rich and in minimal media. Via solubilization not only the membrane proteins were purified but also the whole complex including the ABC domains stayed together under our experimental conditions. The ATP-binding proteins carry a His-tag that facilitates purification. The function of the ABC transporter is analysed by studying the ATPase activity. For the ATPase activity test it was necessary to purify the substrate-binding protein YqiX, with the assumed specificity for arginine, in addition to the complex components. In order to show the purified ABC transport system is functional, its ATPase activity was measured. For the analyses via solid-state NMR an incorporation of the YqiYZ complex into liposomes is a prerequisite. Another requirement for the practice of the NMR technique are isotopically labelled membrane domains of the import complex.

C2-018P
Evidence for a molecular dialogue between the P-glycoprotein and volume-sensitive outward rectifying chloride channels in resistant MCF7 cells

M. Marin and F. Le Foll
Laboratory of Ecotoxicology UPRESEA 3222, Department of Biology, University of Le Havre, Le Havre, France.
E-mail: matthieu.marin@univ-lehavre.fr

The P-glycoprotein (P-gp), encoded by the mdr1 gene and responsible for the multi-drug resistance (MDR) phenotype, is thought to be involved in volume-sensitive chloride currents. In

the present study, the possible coupling between P-gp and swelling-activated chloride channels has been re-examined in MCF7 cells with 1) sensitive (MDR^-), 2) resistant (MDR^+) and 3) reversed resistant (MDRREV) phenotypes. The experimental approach is mainly based on measurements of P-gp activity and electrophysiological recordings. Western blot analysis shows that incubation of cells with doxorubicin induces P-gp expression in a reversible manner. Verapamil and cyclosporine A abolished both survival of MDR^+ cells exposed to doxorubicin and expulsion of the fluorescent probe calcein. One hour exposure of MDR^+ cells to hypotonicity resulted in an inhibition of P-gp activity while DIDS provoked a complete abolition of the hypotonic-induced calcein accumulation. Hypotonic challenges induced swelling-activated chloride currents (ICl-swell) in MDR^-, MDR^+ and MDR-REV MCF7 cells. For the first time, we demonstrate by electrophysiological recordings that ICl-swell are faster activated and of a larger density in MDR^+ than in MDR^- cells. Doxorubicin and vincristine rapidly and reversibly inhibit ICl swell uniquely in MDR^+. Intracellular dialysis of MDR^+ cells with C219 anti P-gp antibody abolished the sensitivity of ICl-swell to doxorubicin and led to a response pattern very close to that of MDR^- cells. Taken together, these results strongly suggest that the P-glycoprotein is functionally coupled to ICl-swell in resistant MCF7 cells.

C2-019P
Human ABCG5 and ABCG8 proteins: in quest of function

M. Müller[1], I. Klein[2], S. Kopacsi[3], A. Varadi[2] and B. Sarkadi[1]
[1]*Department of Molecular Cell Biology, National Medical Center, Budapest, Hungary,* [2]*Institute of Enzymology, Budapest, Hungary,* [3]*Corvinus University, Budapest, Hungary.*
E-mail: mullerm@biomembrane.hu

The ATP binding cassette (ABC) super family of membrane transporters is one of the largest protein classes known, members of the super family are involved in trafficking of biological molecules across membranes, host-defense mechanism to xenobiotics. ABCG5 and ABCG8 are members of the G subfamily of ABC transporters expressed in liver, intestine and colon. They are proposed to function as heterodimers, regulating dietary sterol absorption and excretion. Mutations in either of them cause sitosterolemia, a rare condition with increased intestinal absorption and decreased biliary excretion of dietary sterols into bile. Here we report the funcional expression of ABCG5 and ABCG8 proteins in a baculovirus expression system, identified by immunoblotting. The function of the proteins was followed by ATPase assays. We found a low but distinct vanadate sensitive activity, using an inactive mutant (K96M) of ABCG5 as a negative control. We could stimulate the basal activity to twofold by adding an androgen analog 3DAndrostene. Our results are promising to work out a functional assay to test the possible substrate candidates for these proteins.

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C2-020P
Structural and functional studies of MDR-related ABC-transporters by site-directed mutagenesis, photoaffinity labelling and protein homology modelling

S. Pferschy[1], M. Peer[1], S. Kopp[1], K. Pleban[1,2], G. F. Ecker[2], P. Mazurkiewicz[3], K. Holzmann[4] and P. Chiba[1]
[1]*Functional Biology Lab., Center for Physiology and Pathophysiology, Institute of Medical Chemistry, Medical University of Vienna, Vienna, Austria,* [2]*Pharmacoinformatics Lab., Department of Pharmaceutical/Medicinal Chemistry, University of Vienna, Vienna, Austria,* [3]*Centre for Molecular Microbiology and Infection, Department of Infectious Diseases, Imperial College, London, United Kingdom,* [4]*Institute of Cancer Research, Medical University of Vienna, Vienna, Austria.*
E-mail: sandra.pferschy@meduniwien.ac.at

One major obstacle in chemotherapeutic treatment of many human cancers is the occurrence of cross-resistance to a panel of drugs, when exposed to a single drug. This type of resistance has been termed multidrug-resistance (MDR). One major mechanism for MDR is related to the expression of the bacterial LmrA (*Lactococcus lactis*) and the human P-glycoprotein (P-gp) which are members of the ABC-transporter family of proteins. At present high-resolution crystal-structures are not available and the molecular mechanism of transport is still incompletely understood. We are using a combined approach of 3D-homology modelling and site-directed mutagenesis of residues being potentially involved in substrate binding (i.e. TM3, TM5 and TM6 for LmrA and TM3, TM5, TM8 and TM11 for P-gp). These mutants are used for creating a GATEWAYTM (Invitrogen, Carlsbad, CA, USA) library enabling homologous and heterologous expression of wild type and mutated LmrA and P-gp in any compatible expression system. Recombinant proteins are characterized by photoaffinity-labeling, cytotoxicity assays and flow cytometric uptake assays of fluorochromes. This will allow elucidation of structure and function of these and other ABC-transporters involved in MDR.

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C2-021P
ABC transport proteins as mediators of the multixenobiotic resistance (MXR) defence mechanism in aquatic organisms

R. Sauerborn Klobucar, R. Zaja and T. Smital
Laboratory for Molecular Ecotoxicology, Department for Marine and Environmental Research, Rudjer Boskovic Institute, Zagreb, Croatia. E-mail: rsauer@irb.hr

One of the most intriguing cellular defence strategies evolutionary developed in aquatic organisms is the activity of the multixenobiotic resistance (MXR) mechanism first described in early 1990s by Kurelec and co-workers. Analogous to the well-known multidrug resistant (MDR) mechanism, MXR in aquatic organisms is mediated by expression of the same transmembrane ATP-dependent proteins. MDR/MXR results from the rapid efflux of a wide variety of potentially toxic xenobiotics out of the cell. The best-

studied ABC protein in aquatic organisms is the P-glycoprotein, while our recent studies demonstrate the presence of the MRP-related genes in fish and some non-vertebrate species. Numerous studies performed during the last decade support the proposed MXR role as a general, broadly distributed biological system in aquatic organisms used as a "first line of defense" against endogenous and exogenous toxins. However, recently demonstrated environmental presence of the so-called chemosensitizers or inhibitors of the MXR defense in aquatic organisms could cause increase in intracellular accumulation and toxic effects of other xenobiotics normally effluxed by MXR transport proteins. As a consequence, within the field of ecotoxicology there is an increasing interest for better characterization and identification of all proteins possibly involved in MXR phenomenon and our intention is to stimulate a more efficient and fruitful collaboration between ecotoxicologists and experts for ABC transport proteins.

C2-022P
Structural genomics of bacterial transporters and their human homologues

P. C. Stolt, M. Klein, S. Surade, C. Muenke and H. Michel
Laboratory of Hartmut Michel, Department of Molecular Membrane Biology, Max Planck Institute for Biophysics, Frankfurt, Germany. E-mail: peggy.stolt@mpibp-frankfurt.mpg.de

While crystallization of soluble cytoplasmic proteins for structural analysis by X-ray diffraction has been very successful, the purification and crystallization of membrane proteins still proves quite challenging, with structures of less than 100 membrane proteins available at this time. We have begun a structural genomics project to analyze over 200 transporters and other membrane protein targets from two bacterial and one archaeal species – *A. aquifex*, *S. typhimurium*, and *P. furiousis*. In addition, purification and crystallization of some of the human homologues of these transporters will also be attempted as part of the European Membrane Protein Consortium (E-MeP) project. This analysis will not only lead to new crystal structures of membrane proteins, but also increase knowledge of overexpression systems as well as solubilization, purification, and crystallization conditions useful for these types of proteins, which will help eliminate the bottlenecks encountered when attempting structural analysis of membrane proteins in general. Here, we endeavor to overexpress and purify members of two families of secondary transporters from *S. typhimurium*, the Amino acid/Polyamine/Organocation (APC) family as well as the Proton-coupled Oligopeptide Transporter (POT) family. Expression and purification will be attempted using different *E. coli* strains, vector systems, and affinity tags, as well as various detergents for solubilization, to achieve sufficient amounts of soluble protein for crystallization trials. The expression and purification of the human homologues of these receptors will be attempted using both bacterial expression systems and the Pichia pastoris eukaryotic expression system, with which our laboratory has had previous success.

C2-023P
ABCA1 membrane protein in cholesterol removal and in Ca^{2+}-activated exofacial translocation of phosphatidylserine

I. Kasza[1], A. Váradi[2], B. Sarkadi[1] and **K. Szabó**[2]
[1]*Research Group of the Hungarian Academy of Sciences, Institute of Haematology and Immunology, National Medical Center, Budapest, Hungary,* [2]*Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary. E-mail: k.szabo@biomembrane.hu*

The ATP-binding cassette protein A1 (ABCA1) plays a key role in cellular apolipoprotein-mediated cholesterol and phospholipid removal pathway and it has been also implicated in the exofacial translocation of phosphatidylserine (PS). However, it is not yet clear whether ABCA1 translocates cholesterol and phospholipids directly or acts as a regulator of other proteins responsible for the transmembrane movement of lipids. Our functional studies, performed using the baculovirus-Sf9 insect cell system indicated that ABCA1 might not be a primary active transporter, but rather, a regulatory protein. In order to study the function of ABCA1 protein and its potential interaction with intracellular proteins in various cell environments, we established stable mammalian cell lines expressing wild-type and mutant ABCA1 proteins by using retroviral transduction systems. The mRNA and protein expression levels were followed by RT-PCR and Western blotting, while subcellular localization of ABCA1 protein were studied by using immunofluorescent staining of HA-tagged ABCA1 variants, analysed by flow cytometry and confocal microscopy. The elevated level of ApoA1-dependent ^{3}H-cholesterol efflux from the cells expressing the wild type and the HA-tagged ABCA1 protein indicated that the expressed proteins were functional. However, expression of the wild type ABCA1 influenced the Ca^{2+}-stimulated PS translocation in a cell type dependent manner. Further studies are in progress to determine the effects of expression of mutant forms of ABCA1 either with substitution known to inhibit other ABC transporters or with mutations known to cause serious dysfunctions in lipid metabolism.

C2-024P
The *Drosophila MRP/CG6214* gene encodes a high capacity organic anion transporter

F. Szeri[1], A. Iliás[1], J. N. Tarnay[2], S. Robinow[3] and A. Váradi[1]
[1]*Laboratory of Active Transport Proteins, Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary,* [2]*Cell and Molecular Biology, University of Hawaii, Honolulu, HI, United States of America,* [3]*Department of Zoology, University of Hawaii, Honolulu, HI, United States of America. E-mail: szeri@enzim.hu*

ATP-binding cassette transporters are involved in the transport of substrates across biological membranes and are essential for many cellular processes. Phylogenetic analyses identify the *Drosophila MRP/CG6214* gene as the *Drosophila* orthologue to four human genes encoding multidrug resistance-associated proteins: MRP1, MRP2, MRP3, and MRP6. To reveal the function of this recently identified protein we have initiated its biochemical characterization using the Sf9/baculovirus heterologous expression system. Functional studies, such as vesicular transport assays, ATPase activity measurements, and vanadate trapping experiments, indicate that *Drosophila* MRP is a high capacity ATP-dependent vanadate-sensitive organic anion transporter of leukotriene C4 and estrogen-metabolite estradiol-17-β-d-glucuronide.

C2-025P
Application of a human multidrug transporter (ABCG2) as selectable marker in gene therapy

O. Ujhelly[1], J. Cervenak[1], G. Várady[1], N. Kucsma[1], L. Chen[2], S. Stein[2], C. Özvegy[3], A. Váradi[4], M. Grez[2], B. Sarkadi[3] and K. Német[1]
[1]*Department of Experimental Gene Therapy, National medical Center, Budapest, Hungary,* [2]*Department of Molecular Virology, Georg-Speyer-Haus, Frankfurt, Germany,* [3]*Membrane Research Group, National medical Center, Budapest, Hungary,* [4]*Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary. E-mail: oujhelly@hotmail.com*

Stem cell-based gene therapy is often unsuccessful because of the relatively low number of genetically modified cells with repopulating capabilities. To provide a selective advantage to the

modified cells we applied the human ABCG2 protein, a resident xenobiotic transporter in stem cells, as a selectable marker. This protein is active as a homodimer, and its relatively small cDNA is an advantage in gene therapy applications. In the present study the gene therapy application of a mutant form of ABCG2 (R482G) was investigated. ABCG2 variants were expressed in haematopoietic stem cells alone or co-expressed with the therapeutic gene ($gp91^{phox}$) of X-linked chronic granulomatous disease (X-CGD) by an efficient retroviral transduction system. Transgene expression was determined by Western blotting, immunohistochemistry and flow cytometry analysis. To estimate the multidrug resistance phenotype, functional assays of ABCG2 were performed. The differentiation of the transduced cells was followed by *in vitro* clonogen and *in vivo* mouse transplantation experiments. High proportion of transgene positive cells could be detected in the ABCG2 transduced cells, where the mutant ABCG2 protein selectively protected the cells against clinically applicable cytostatic drugs as mitoxantrone (MX) and doxorubicin. Expression of the $gp91^{phox}$ protein in human $gp91^{phox}$ knock out hematopoietic progenitor cells corrected the mutation responsible for X-CGD after MX selection. Overexpression of ABCG2 did not affect hematopoietic cell maturation both *in vitro* and *in vivo*. We suggest that the mutant ABCG2 protein is an ideal candidate for human stem cell protection and for use as a selectable marker in gene therapy.

C2-026P
Biogenic amine production in *Lactobacillus brevis*

W. A. Wolken and J. S. Lolkema
Molecular Microbiology, Groningen University, Haren, the Netherlands. E-mail: w.a.m.wolken@rug.nl

Lactic acid bacteria are a group of bacteria used extensively in food and beverage fermentations, for instance, in the dairy and wine making industries. Decarboxylation of acids can improve the hygienic quality and the taste and texture of cheese and wine and, as such, give added value to the final product. In contrast, the production of biogenic amines by decarboxylation of amino acids has a deleterious effect on consumers due to toxicity or intolerance. In *Lactobacillus brevis* a putative tyrosine decarboxylation pathway was identified consisting of a decarboxylase and a precursor/product exchanger organized in one operon structure. External tyrosine is taken up by the tyrosine/tyramine exchanger and decarboxylated to form tyramine. Subsequently, this tyramine is transported out of the cell by the same transporter in antiport to the tyrosine that is internalized. The consumption of an internal proton and the net translocation of one positive charge out of the cell lead to the formation of a proton motive force, which the cell, for instance, can use for ATP production. The gene coding for the tyrosine/tyramine exchanger (*tyrP*) has been cloned and expressed in *Lactococcus lactis* under the control of a nisin promoter. Upon expression of *tyrP* initial uptake rates of radioactively labeled tyrosine increases 12-fold and a strong exchange activity (tyrosine for tyramine), not present in control cells is detected. Further characterization of the transporter as well as the cloning of *tyrDC*, coding for tyrosine decarboxylase, is currently in progress.

C2-027P
Molecular cloning and functional characterization of hemolysin gene from *Vibrio furnissii*

Y.-K. Wang and **T.-K. Wu**
Bioorganic Chemistry and Molecular Evolution, Department of Biological Science and Technology, National Chiao Tung University, Hsin-Chu, Taiwan PR China. E-mail: tkwmll@mail.nctu.edu.tw

The halophilic bacterium *Vibrio furnissii* is an enteric pathogen that causes acute diarrheal illness after consumption of contaminated seafood. Hemolysin is a major virulence factor in pathogenic gram-negative bacteria. A hemolysin from *Vibrio furnissii* has been purified to homogeneity by ammonium sulfate precipitation, Phenyl-Sepharose 6 Fast Flow, and antibody-conjugated Sepharose 4B chromatographies. It had a molecular mass of ~63 kDa and exhibited cytotoxicity against Chinese Hamster Ovary cell in cell culture. Results from Edman degradation of hemolysin showed (A-V-V-P-A-G-T-R-L-A-D-V-Q-E-F-V-R-G-N-C) sequence, which is homologous to ABC transporter superfamily. Genetic analysis and DNA sequence of the hemolysin gene showed high sequence similarity to bacterial extracellular solute-binding proteins, family 5.

C3–Receptor Proteins and Membrane Organization

C3-001
Pathways regulating the internalization of activated immune receptors

F. M. Brodsky[1], V. L. Crotzer[2], A. P. Jackson[3] and A. Stoddart[4]
[1]G.W. Hooper Foundation, Departments of Biopharmaceutical Sciences and Microbiology and Immunology, University of California, San Francisco, CA, USA, [2]Department of Microbiology and Immunology, Indiana University, Indianapolis, IN, USA, [3]Department of Biochemistry, Cambridge University, Cambridge, UK, [4]Department of Hematology/Oncology, University of Chicago, Chicago, IL, USA. E-mail: fmarbro@itsa.ucsf.edu

Receptors in the plasma membrane can associate with lipid raft domains and thereby initiate signaling. Numerous pathways have been invoked in subsequent receptor internalization and these can lead to signal attenuation or signal amplification. In studying the behavior of activated T cell receptors and B cell receptors we have characterized a pathway by which these receptors can signal via kinases in lipid raft domains and subsequently regulate their uptake by clathrin through clathrin heavy chain phosphorylation. Previously localization to raft and clathrin domains was considered mutually exclusive but our data indicate cooperative interactions. Such cross-talk between raft domains and internalization pathways is highly relevant for receptor dynamics at both the T cell and B cell immunological synapse. In recent studies of B cell receptor uptake in cells that can be conditionally depleted of clathrin, we observed plasticity of receptor internalization routes and a hierarchy of preference for these routes in wild-type cells. The cooperation between raft signaling and clathrin-mediated uptake was further confirmed in these cells and actin was implicated all routes of receptor internalization. Finally, we have demonstrated that when all pathways of internalization for the B cell receptor are blocked, receptor signaling is amplified, indicating that for this particular receptor, uptake leads to attenuation of signaling. These observations, combined with our earlier studies of epidermal growth factor receptor and T cell receptor suggest

that shared mechanisms for regulating the internalization of signaling receptors can lead to different functional consequences for receptor signaling.

C3-002
Retrograde filopodial transport of activated EGF receptors

T. M. Jovin, D. S. Lidke, B. Rieger and D. J. Arndt-Jovin
Molecular Biology, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany. E-mail: tjovin@gwdg.de

The growth factor EGF bound to quantum dots (QDs) activates the cognate receptor tyrosine kinase (erbB1, EGFR) on cell surfaces. The QDs are readily internalized and traffic in the endosomal compartments [1]. Thanks to the great photostability of the QDs, these processes are readily visualized over extended periods of time by confocal laser scanning and wide-field microscopy. The QD ligands can be visualized down to the single nanoparticle level, leading to the unexpected finding of a mechanism for the systematic retrograde transport of QD-EGF-EGFR complexes along filopodial cellular extensions. This mechanism requires activation and interaction of at least two receptor molecules. The transport rates determined by particle tracking and MSD analysis are compatible with or exceed those characteristic of the treadmilling activity of the actin bundles constituting the core of the filopodia [2]. Cytochalasin D, that disrupts polymerized actin cytoskeleton, and specific inhibitors of the EGFR kinase activity prevent transport but not diffusion of the receptor. Retrograde transport occurs prior to internalization of the ligand-receptor complex at the base of the filopodia and is presumed to be mediated by an actin-associated adapter and/or motor protein. These results imply that filopodia serve as sensory organelles for the cell, probing for the presence and concentration of effector molecules far from the cell body, and thereby coupling remote sensing to cellular response via directed transport of activated receptors. QDs are excellent ligands for biophysical studies of cellular activities, particular in combination with the numerous expression probes of cell surfaces receptors, such as EGFR, available and under development.

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C3-003
Digital-like signal transduction? Investigations by single-molecule observations

A. Kusumi
Kusumi Membrane Mechanism Project, ICORP, JST, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan. E-mail: akusumi@biol1.bio.nagoya-u.ac.jp

Using single molecule techniques, the movement, localization, and activation reactions of single signaling molecules have been investigated in living cells. One of the major findings by using single molecule techniques is that the activation period for each individual signaling molecule is often shorter than a second, namely, activation of single signaling molecules occurs like a short pulse although signaling molecules collectively exhibit activation lasting over a minute, a time course which is the same as that detected biochemically. Therefore, many cellular signaling processes may have adopted digital or frequency-modulated signal transduction mechanism in the sense that each signaling event or the elementary step for the signal transduction process may take place like a transient pulse-like on-signal. Such a pulse-like activation is likely based on transient cooperative formation and disassembly of the signaling complex. As examples of such digital signal transduction, first, I will talk how raft molecules in the cell membrane form transient (0.7 s) signaling platforms upon stimulation. The second topic is visualization of temporary (shorter than 0.6 s) activation of individual H-Ras molecules, and signal transfer from Ras to Raf in the transient activated-Ras signaling complex. These observations were made possible by simultaneous observation of dual color images of single molecules (two kinds of single molecules) and single molecule fluorescence resonance energy transfer, carried out in living cells. In both cases, the transient signaling may be supported by transient formation of signaling molecular complexes. In the former case, the stabilized raft provides the key platform, whereas in the latter case, a scaffolding protein may play critical roles.

C3-004
Cross-talk among membrane receptors: regulation of mast cells' secretory response.

J. Abramson, A. E. Barbu and **I. Pecht**
Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel. E-mail: israel.pecht@weizmann.ac.il

Current understanding of the stimulus-response coupling networks triggered by the multi-chain immuno-recognition receptors (MIRRs) has markedly advanced while knowledge of its regulation is only emerging. Control of the secretory response of mast cells to the type I Fcε receptor (FcεRI) stimulus is a major topic of our interest. Several mast cell membranal receptors capable of inhibiting both immediate and late responses have so far been identified. However, their mode(s) of operation are only partly resolved . Moreover, control of mast cells response to the FcεRI by desensitization, a wide spread process of control of many receptors, is still hardly understood. We have previously shown that the FcεRI-mediated degranulation is efficiently suppressed upon clustering the inhibitory receptor-Mast cell function-associated antigen (MAFA), previously discovered and characterized in our laboratory. MAFA clustering is also suppressing the FcεRI - induced secretion of *de novo* synthesized cytokines and leukotriens and we have now shown that it interferes with activation of Erk-1/2 and p38 MAP kinase resulting in a selective suppression of cytokine gene transcription and leukotriene synthesis. Dok-1 and Dok-2 were found to undergo tyrosine phosphorylation upon MAFA clustering with the concomitant increase in Dok-1 binding to RasGAP. This is apparently essential for MAFA-mediated down-regulation of RasGTP levels , suppression of Erk activity and the subsequent reduced cytokine and leukotriene *de novo* synthesis. Both Dok molecules also undergo tyrosine phosphorylation upon FcεRI clustering. Further, RBL-2H3 cells over-expressing Dok-1 exhibit significantly lower Ras and Erk-1/2 activation, and a concomitantly reduced level *de novo* synthesis and secretion of TNF-a and LTC4. These findings suggest that Dok-1 functions as a built-in autoregulatory element, keeping in check the FcεRI induced de-novo synthesis of pro inflammatory mediators. Most recently, we found that MAFA, so far considered only as an inhibitory receptor, may also produce activating signals as its clustering alone (without an activating stimulus of the FcεRI) induces rapid activation of MAP kinases (Erk-1/2, p38, JNK), which subsequently enhances transcription of several genes (e.g. MCP-1).

C3-005
Role of rafts and rabs in Alzheimer's disease

L. Rajendran, M. Honsho, T. Zahn, P. Verkade and K. Simons
Max-Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany. E-mail: rajendra@mpi-cbg.de

Lipid–lipid immiscibility gives rise to lateral heterogeneities in the membrane plane, a subset of which are termed lipid rafts. Lipid rafts in cell membranes are sub-microscopic and sphingolipids and cholesterol in the outer exoplasmic leaflet play a crucial role in the assembly of these domains. Cholesterol and cholesterol enriched domains have been implicated in the pathogenesis of Alzheimer's disease. Formation of senile plaques harboring the amyloid β-peptide is an invariant feature of Alzheimer's disease. This insoluble 40-42 amino acid peptide (Aβ40 or 42) is generated by the sequential cleavage by β-and γ-secretases. Neither the exact cellular sites of the cleavages nor the mechanism by which these peptides are secreted into the extracellular milieu are currently well established. We have studied the involvement of raft clustering proteins such as flotillins and several rab-GTPases and their corresponding mutants in the amyloidogenic processing of the amyloid precursor protein (APP). Knock-down of flotillin-2 decreased the β-cleavage to more than 50% implying that raft clustering process plays a major role in the amyloidogenic processing. Subcellular localization studies with rab proteins as markers for the different endosomal compartments have helped us identify a subset of early endosomes to be involved in the β-cleavage. The functional implications of rab proteins in the pathogenesis will also be discussed.

C3-006
Effect of lipid environment on signaling of ErbB2 receptor tyrosine kinase in Herceptin resistant and sensitive cell lines

J. Szöllösi
Department of Biophysics and Cell Biology, University of Debrecen, Debrecen, Hungary. E-mail: szollol@jaguar.dote.hu

The ErbB2 (HER2) protein is a member of the EGF receptor (ErbB) family of transmembrane receptor tyrosine kinases. Its medical importance stems form its frequent overexpression in breast and other cancers. Humanized antibodies against ErbB2 (i.e. Herceptin) have been introduced into clinical practice and were found to have cytostatic effect in ~40% of ErbB2 positive breast tumors. Our working hypothesis is expression levels of ErbB kinases, their interactions and activity within multimolecular complexes and their lipid environment will determine the outcome of ErbB2 directed therapy. Our comparison of Herceptin resistant (JIMT-1, MKN-7) and sensitive (SKBR-3, N-87) cell lines demonstrates the importance of ErbB2 association patterns involving integrins and lipid rafts. Flow cytometric FRET and confocal microscopic measurements revealed colocalization and molecular proximity between β1-integrins and ErbB2, as well as their association with lipid rafts. A weak functional interaction between ErbB2 and β1-integrin and the fact that ErbB2 did not co-patch with β1-integrins upon crosslinking imply that ErbB2 and β1-integrin define two distinct molecular association clusters from a functional point of view. Although Herceptin-sensitive cell lines expressed more ErbB2 and fewer β1-integrin molecules on their surface than their resistant counterparts, this finding probably does not explain the Herceptin resistant phenotype due to the weak interaction between β1-integrins and ErbB2. It is proposed that in the resistant cell line active ErbB2 homodimers that bind Herceptin with high affinity are scarce, and signaling that drives proliferation may originate from other ErbB kinase dimers such as the ErbB2-ErbB3 heterodimer. Our results imply that the true significance of the expression profile of proteins involved in oncogenesis can only be understood after characterizing their molecular interactions.

C3-007P
Study of interactions between typical and atypical antipsychotic drugs and their membrane receptors using QSAR methods – a way for new neuroleptics drug design

S. Avram[1], A. L. Milac[2], A. N. Dabu[3], A. J. Petrescu[2] and M.-L. Flonta[1]
[1]*Department of Physiology and Biophysics, University of Bucharest, Bucharest, Romania,* [2]*Structural Biochemistry Group, Institute of Biochemistry, Bucharest, Romania,* [3]*Department of Neurosurgery, University Hospital, Bucharest, Romania.*
E-mail: speranta@bio.bio.unibuc.ro ; a_speranta2001@yahoo.com

A series of typical and atypical antipsychotic drugs: aripiprazole, chlorpromazine, clozapine, flufenazine, flupentixol, haloperidol, loxapine, mesoridazide, molindone, olanzapine, perphenazine, pimozide, promazine, quetiapine, risperidone, sertindole, thiothixene, tioridazide, trifluoperazine, compazine, ziprazidone have been studied using quantitative structure activity relationship analysis. Using the computational softwares (e.g. Tinker and Schrodinger) the antipsychotic affinity (–log *Ki*) for 12 receptors: dopaminergic D2, serotoninergic(5-HT7, 5-HT6, 5HT2C, 5-HT2A and 5-HT1A) muscarinic M3 and adrenergic(α2C, α2B, α2A and α1A) and histaminic H1 was correlated with pharmacokinetic parameters namely: the Solvent Accessible Surface Area (SASA), the molecular volume (V), the globularity(G), the Octanol/water partition coefficient (logP), the solubility(S), the dipole moment, the polarizability, and most important, the Blood/Brain barrier permeability. The statistical analysis was improved when we have considered the simultaneous contribution of logP, molecular volume and solvent accessible surface, polarizability and dipole moment (r^2 correlation = 0.90 for dopaminergic receptor D2) to the biological activity. Instead when the dipole moment was excluded the correlation coefficient r drastically decrease (r^2 correlation = 0.69). The best correlation between predicted and experimental biological activity was recorded when interaction between neuroleptics and membrane receptor D2 was analyzed.

C3-008P
Characterization, solubilization and purification of the rat Neurokinin A receptor produced in the methylotrophic yeast *Pichia pastoris*

N. Andre, C. Reinhart and H. Michel
Department of Molecular Membrane Biology, Max Planck Institut of Biophysics, Frankfurt, Germany.
E-mail: nicolas.andre@mpibp-frankfurt.mpg.de

The Neurokinin A receptor (NK2R) is a G-Protein Coupled Receptor (GPCR) involved in smooth muscle contractions. GPCRs form one of the largest protein superfamilies and are responsible for the transduction of extracellular signals into an intracellular response. NK2R agonist, Neurokinin A (NKA), is a polypeptide of the tachykinin family, which shares the common C-terminal sequence Phe-X-Gly-Leu-MetNH_2. Although much progress has been made in the pharmacological characterization of a large number of GPCRs, the only three-dimensional structure available is that of bovine rhodopsin. A 3D-structure of NK2R would increase our understanding of its molecular mechanism and of the signal transduction of all GPCRs. In order to produce the large and homogenous receptor preparations required for structural studies, heterologous production procedures have been established using the methylotrophic yeast *Pichia pastoris*. The receptor, produced as a fusion protein with both a Flag-tag and a His_{10} tag at its N-terminus and a Biotinylation

domain at its C-terminus for immuno-detection and purification, shows specific and saturable binding activity with its selective antagonist SR48968. The specific activity of the preparation was 200 pmol/mg, which corresponds to 1.25 mg of receptor per liter of culture. Detergent solubilization of functional receptor was achieved using a mixture of Decyl-Maltoside and Cholesteryl-HemiSuccinate. Pure receptor preparations were obtained via a combination of affinity purification steps using immobilized metal affinity chromatography (IMAC), Monomeric Avidin, and Anti-Flag M2 matrices. The large amount of receptor obtained from this procedure provides a starting point for tree-dimensional crystallization trials.

C3-009P
Identification of transferrin in mitochondria isolated from rat liver

M. Ani[1], A.-a. Moshtaghie[2] and M. Taher[3]
[1]*Laboratory of Protein Chem., Dept. of Clin. Biochem, Isfahan Univ. Med. Sci, Isfahan, Iran,* [2]*Laboratory of Trace elememts, Dept. of Clin. Biochem, Isfahan Univ. Med. Sci, Isfahan, Iran,* [3]*Laboratory of General Biochem, Dept of Clin. Biochem, Isfahan Univ. Med. Sci, Isfahan, Iran. E-mail: ani@pharm.mui.ac.ir*

A body of contradictory reports exist regarding the uptake of iron by the cell. The direct donation of iron from transferrin to the mitochondria has already been reported. The major aim of this study was to investigate the existence of transferrin on mitochondrial membrane for the uptake of iron.

Methods: Male rat livers were removed, homogenized and mitochondria were prepared. The mitochondrial homogenates were loaded on top of equilibrated column containing sepharose-4B activated CNBr in complex with anti transferrin. The column was first eluted with successive buffer solutions as follows: Wash-1; 200 ml of equilibration buffer (10 mM Phosphate saline buffer containing 0.1% Triton X100, pH 7.5). Wash-2; 100 ml of 10 mM potassium phosphate, pH 7.5, 500 mM NaCl. Wash-3; 100 ml of equilibration buffer. Wash-4; 200 ml of 20 mM glycine/NaOH pH 10, 500 mM NaCl and 0.5% Triton X 100. Wash-5; 100 ml of 10 mM glycine/HCl pH 2, 150 mM NaCl. Fractions (2 ml) were then collected.

Results: The protein bound to the column was eluted in dissociation cycle using glycine/HCl buffer pH2. The collected fractions were pooled and further dialyzed against appropriate buffer. The purified eluted protein was electrophoresed on SDS-PAGE in parallel with trasferrin standard as marker. A sharp band was found at approximately 80 KD molecular weight attributed to trnsferrin band .

Conclusion: The presence of transferrin in mitochondria is an explanation to the mechanism of iron transferred from cytosol to mitochondria.

C3-010P
The rat excitatory amino acid carrier 1 is modulated by an enriched environment

J. Andin[1], M. Mazya[1], A. Mohammed[2] and J. Marcusson[1]
[1]*Division of Geriatric Medicine, Department of Neuroscience and Locomotion, Linköping university, Linköping, Sweden,* [2]*Division of Experimental Geriatric Medicine, Neurontec, Karolinska Institute, Huddinge, Sweden. E-mail: josan@inr.liu.se*

The glutamate system is of great importance for neuronal plasticity, cell death, and cognitive functions such as memory and learning. In order to avoid excitotoxicity and optimize the transmitting signal, a strict regulation of the glutamate concentration and of the duration of the signal is required. An enriched environment is known to cause significant changes in brain biochemistry and anatomy in rodents. In this study, *in situ* hybridization, using digoxigenin-labeled cRNA probes, has been used to elucidate changes in the neuronal glutamate transporter, excitatory amino acid carrier (EAAC1) in rat, after exposure to different environments. Rats housed in an enriched laboratory environment showed a decrease in mRNA expression of EAAC1 in hippocampal areas cornu anterior 1 and cornu anterior 2, and in the parietal cortex, compared to rats housed in standard laboratory environments. These results indicate that environmental factors affect the transporter part of the glutamate system. Further studies on the importance of environmental factors for the glutamate system are warranted and could provide insight into mechanisms behind maintenance and development of brain functions.

C3-011P
Differential modulation of intracellular calcium by arachidonic acid in cultured cortical astrocytes

S. Alloisio[1], S. Ferroni[2] and M. Nobile[1]
[1]*Institute of Biophysics, National Research Council, Genoa, Italy,* [2]*Department of Human and General Physiology, University of Bologna, Bologna, Italy. E-mail: alloisio@ge.ibf.cnr.it*

Arachidonic acid (AA) and its eicosanoid metabolites (prostaglandins and leukotrienes) play critical roles in the initiation or modulation of a broad spectrum of biological responses, including inflammatory processes. Accordingly, the regulation of the activity of the AA cascades is thought to be of therapeutic relevance for the treatment of inflammation. We here investigated the action of extracellular AA in the modulation of the intracellular calcium signaling ($[Ca^{2+}]i$) on cultured neocortical type-1 astrocytes by using single-cell microfluorimetry. We present evidence that AA-induced $[Ca^{2+}]i$ rise is coupled to several signal transduction pathways, including depletion of intracellular Ca^{2+} stores, AA-dependent Ca^{2+} entry and AA-induced potentiation of the P2X7-evoked $[Ca^{2+}]i$ rise. Moreover, we show that the predominant mechanism for regulated entry of Ca^{2+} in non-excitable cells, the capacitative calcium entry (CCE), is potently depressed by AA. The AA-induced increase in $[Ca^{2+}]i$ was insensitive to inhibitors of lipoxygenase-, cyclo-oxygenase- and cytochrome P450 epoxygenase-dependent signal transduction cascades. The AA-dependent Ca^{2+} entry was not significantly inhibited by micromolar concentrations of ruthenium red, a blocker of plasma membrane cationic channels belonging to a subclass (TRPV) of the transient receptor potential channel family. Collectively, the results demonstrate that AA modulates $[Ca^{2+}]i$ through mechanisms that are independent of activity of CCE and TRPV channels, revealing a novel mechanism for controlling $[Ca^{2+}]i$ homeostasis in astroglial cells. Supported by MIUR (Italy)

C3-012P
Functional reconstitution of the Oxa1 complex, a protein integrase of the inner membrane of mitochondria

H. Bauerschmitt and J. M. Herrmann
Laboratory of JM Herrmann and W. Neupert, Department of Physiological Chemistry, Adolf Butenandt Institute, Ludwig-Maximilians-University Munich, Munich, Germany. E-mail: heike.bauerschmitt@bio.med.uni-muenchen.de

The Oxa1 protein forms a homooligomeric complex in the inner membrane of mitochondria. This complex is required for membrane integration of both nuclear and mitochondrially encoded

proteins. Oxa1 belongs to a large Alb3/Oxa1/YidC protein family, the members of which are involved in the biogenesis of membrane proteins in bacteria, mitochondria and chloroplasts. In order to characterize the molecular function of Oxa1 we purified the Oxa1 complex from mitochondria of the filamentous fungus *Neurospora crassa*. Upon reconstitution into proteoliposomes, the Oxa1 complex can catalyse the integration of radiolabeled membrane proteins *in vitro*. This protein insertion process is strongly stimulated when an artificial membrane potential is applied. Our observations suggest that Oxa1 functions as a "protein integrase" which catalyzes the insertion of hydrophobic sequences into membranes.

C3-013P
Functional characterization of temperature-gated ion channels using an improved technique for rapid heating and cooling of superfusing solutions in patch-clamp experiments

J. Benedikt, I. Dittert, L. Vyklicky and V. Vlachova
Department of Cellular Neurophysiology, Institute of Physiology AS CR, Prague, Czech Republic. E-mail: benedikt@biomed.cas.cz

Temperature is known to modulate functions of all ion channels, including their gating, conductance and ligand-binding affinities. Even though several channels exhibit a high temperature dependence, only few of them can be directly activated by temperature alone. Recent cloning efforts have identified six temperature-gated channels, interestingly all belonging to the transient receptor potential (TRP) family of excitatory channels. Among them, vanilloid receptor TRPV1 was the first found to respond to noxious heat stimulus >43 °C with exceptionally high temperature coefficient values $Q_{10} > 20$ [1, 2]. Another channel, cold-activated TRPM8, opens at ~28°C and saturates at 10 °C with Q_{10} ~24 [3]. Since a prominent characteristic for these channels is a rapid response to temperature changes, a sufficiently fast time course of the temperature stimulation is required for exploring the rate-limiting steps in channel gating kinetics. In order to analyze the temperature-induced responses from recombinant TRPV1 and TRPM8 receptors, we developed an improved technique that enables to apply fast temperature changes from 4 up to 60 °C (0.1 °C/ms) to solutions superfusing cultured cells. The principle of this technique is in pre-cooling and/or heating the common outlet of a manifold consisting of seven tubes connected to barrels containing different solutions. This technique significantly improves the time resolution in studying kinetics of temperature-gated channels and represents a promising tool for better understanding of the molecular mechanisms of channel gating.

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C3-014P
Solution NMR studies of the LA7-EGF-A pair of modules from the LDLR

N. Beglova and S. C. Blacklow
Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA.
E-mail: nbeglova@rics.bwh.harvard.edu

The low-density lipoprotein receptor (LDLR) normally carries lipoprotein particles into cells, and releases them upon delivery to the low pH milieu of the endosome. Loss-of-function mutations in the LDLR gene cause familial hypercholesterolemia, a common autosomal dominant genetic disorder, characterized clinically by elevated concentrations of plasma low-density lipoprotein (LDL) and cholesterol, and an increased risk of atherosclerosis and coronary heart disease. Recent structural and functional studies of the receptor, combined with the prior knowledge about normal receptor function and the effects of FH mutations on the LDLR function, revealed a detailed molecular model for how the acidic environment of the endosome triggers release of bound lipoprotein particles. The receptor dynamically interconverts between open (ligand-active) and closed (ligand-inactive) conformations in response to pH, relying on a specific arrangement of fixed and flexible interdomain connections to facilitate efficient binding and release of its lipoprotein ligands. We studied by solution NMR the LR7-EGF-A pair that comprises the junction between the two functional domains of the LDLR, the ligand-binding domain and the EGF-precursor homology domain (EGFP). Our findings revealed that the interface between LA7 and EGF-A is fixed and locked in virtually the same conformation at both neutral and endosomal pH. This fixed interdomain arrangement restricts the conformational search space allowing the closure of the two domains to proceed readily at acidic pH. To provide a more detailed understanding of the structural relationships in this interdomain junction, we investigated by solution NMR the backbone dynamics of the LA7-EGF-A pair.

C3-015P
ApoA-I mediated cholesterol efflux in primary human fibroblasts is linked to association of ABCA1 with membrane microdomains

S. Bandulik, G. Liebisch and G. Schmitz
Department Institute for Clinical Chemistry-University Hospital Regensburg, University of Regensburg, Regensburg, Germany.
E-mail: sascha.bandulik@klinik.uni-regensburg.de

Today it is well accepted that the ABC transporter ABCA1 is the major regulator of apoA-I mediated cholesterol efflux, which is essential for cell cholesterol homeostasis. The mechanism of apoA-I lipidation by ABCA1 is still controversial, but results from our and other groups indicate the involvement of membrane microdomains (Drobnik et al. *Traffic* 2002; Gaus et al. *FASEB J* 2004). Such microdomains, termed lipid rafts, are characterized by the enrichment of cholesterol, shpingolipids and saturated phospholipids, and in addition by their insolubility in different detergents such as Triton X-100 and Lubrol WX. Our data show that ABCA1 is partially located in Lubrol- but not in Triton-detergent-resistant membranes (DRM) of primary human fribroblasts. Analysis of Triton- and Lubrol-DRM by ESI-MS/MS revealed different lipid composition. Lubrol-DRM association of ABCA1 increased by cholesterol loading and decreased by deloading cells with apoA-I. Preliminary data indicate a time-dependent disappearance of ABCA1 from Lubrol-DRM upon apoA-I stimulation. Different authors proposed a two-step mechanism for apoA-I lipidation by a "fast" and a "slow" cholesterol pool (Gaus et al. *FASEB J* 2004). Saito et al. (*J Lipid Res* 1997) showed that apoAI binding capacity to phosphatidylcholine (PC) vesicles increased by addtion of cholesterol. Therefore, we propose a mechanism, in which ABCA1-DRM-association promotes apoA-I binding to cholesterol-rich membrane microdomains, which may form the small cholesterol pool for an initial "fast" efflux. Binding of apoA-I to ABCA1 within lipid rafts could also cluster a potential signaling complex to initiate apoA-I internalization and "slow" bulk efflux.

C3-016P
Altered signalling of an inhibitory VEGF-A splice variant results from a defect in heparin binding; implications for angiogenesis in development and tissue repair

K. Ballmer-Hofer[1], S. Cébe Suarez[1], U. Hoffmann[1], M. Pepper[2] and R. Nisato[3]
[1]*Molecular cell biology, Biomolecular research, Paul Scherrer Institut, Villigen, Switzerland,* [2]*Unitas Hospital, Pretoria, South Africa,* [3]*Department of Cell Physiology and Metabolism, University of Geneva, Geneva, Switzerland. E-mail: kurt.ballmer@psi.ch*

Angiogenesis is the process through which new blood vessels are formed from pre-existing ones. The development of functional vessels requires spatio-temporal coordination of the production and release of growth factors such as Vascular Endothelial Growth Factors (VEGFs) by stromal and haematopoietic cells. VEGF family members are cysteine crosslinked dimers and are produced in multiple isoforms upon alternative splicing giving rise to soluble and matrix-associated proteins. Although structurally very similar, the various VEGF isoforms display distinct biological properties upon binding to specific subtypes of VEGF receptors. Recently, a new VEGF-A splice variant, VEGF165b, has been isolated from kidney epithelial cells. Its sequence is identical to that of VEGF165 except for the last six amino acids at the carboxyterminal end. In the present study we characterized the signalling properties of this isoform in detail. VEGF165b efficiently blocked binding of VEGF165 to its receptors indicating normal receptor binding. Biological assays such as collagen invasion of endothelial cells and induction of angiogenesis on the chicken chorioallantois showed that VEGF165b has anti-angiogenic properties counteracting VEGF165. Strikingly, VEGF165b did not bind heparan sulphate glycosaminoglycans (HSPG) and only weakly activated MAP kinase signalling by VEGF receptor 2. Activation of VEGF receptor 2 was further stimulated by HSPG when added together with VEGF165 but not with the variant protein.

C3-017P
Receptorial characterization of a new delta opioid peptide antagonist, Tyr-Tic-(2S,3R)betaMePhe-Phe-OH

E. Birkás[1], G. Tóth[1], I. Kertész[1], L. Bakota[2], K. Gulya[2] and M. Szûcs[1]
[1]*Laboratory of Molecular Pharmacology, Institute of Biochemistry, Biological Research Center, Szeged, Hungary,* [2]*Department of Zoology and Cell Biology, University of Szeged, Szeged, Hungary. E-mail: szucsm@brc.hu*

Tyr-Tic-(2S,3R)betaMePhe-Phe-OH was synthesized and used in comparative analysis in rat, wild type and d opioid receptor knock-out (DOR-KO) mouse brain membranes. It was also radiolabeled yielding in [3H]Tyr-Tic-(2S,3R)betaMePhe-Phe-OH with a specific activity of 53.7 Ci/mmol. Saturation binding experiments revealed a dissociation constant, K_d of 0.28 ± 0.001 nM and receptor density, B_{max} of 155 ± 6.6 fmol × mg/protein in rat brain membranes. The binding affinity was increased in the presence of Na^+ in accordance with the antagonist character of the new ligand. There were fewer binding sites with higher affinity in wild type mouse brain membranes. No specific binding was detected in DOR-KO mouse brain membranes. In accordance with this result, no labeling was seen with receptor autoradiography after 3 months exposure in DOR-KO brains. While the prototypic delta ligands Ile5,6-deltorphine and naltrindol displaced the radioligand with high affinity, mu and kappa specific ligands showed poor affinity in competition binding assays. Interestingly, unlabelled Tyr-Tic-(2S,3R)betaMePhe-Phe-OH displaced more binding than the former two delta ligands in mice but not in rats. Naltrindol and Tyr-Tic-(2S,3R)betaMePhe-Phe-OH also differed in their ability to antagonize the stimulating effect of the delta agonist DTLET in mouse brain. These results support the existence of delta opioid receptors with distinct ligand binding profile.

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C3-018P
Electrostatic interactions link agonist binding to channel gating in the neuronal alpha7 nicotinic receptor

M. Criado, J. Mulet, G. Susana, S. Salvador and S. Francisco
Neurobiología Molecular, Instituto de Neurociencias, Universidad Miguel Hernandez, Sant Joan d'Alacant, Alicante Spain. E-mail: manuel.criado@umh.es

Ligand-gated ion channels mediate rapid synaptic transmission upon activation by the corresponding neurotransmitter. Channel opening is triggered upon conformational changes induced by agonist binding and through molecular mechanisms that are not well understood. Previously, we demonstrated that gating of the neuronal nicotinic alpha7 receptor depended on the negatively charged D266 residue located in the linker between the M2 and M3 transmembrane regions. Here we have explored the possibility that a network of electrostatic interactions between D266 and other charged residues controls receptor gating. For this purpose we mutated certain amino acids that, although far away in the primary structure, might be close to D266 according to current models of nicotinic receptor structure. Mutants receptors were then expressed in *Xenopus* oocytes and electrophysiologically characterized. Mutants at positions E45, K46 and D135 exhibited poor or null functional responses to different nicotinic agonists regardless of significant membrane expression, whereas D128A showed a gain-of-function effect. A gating mechanism controlled by a salt bridge between K46 and D266 does not appear likely, since the double reverse charge mutant K46D/D266K did not restore receptor function. An electrostatic network formed by residues E45, K46, D128, D135, D266 and possibly others, would rather links agonist binding to channel gating.

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C3-019P
Acute effects of β-endorphin on blood pressure and some hormones in healthy and hypertensive subjects. The role played by opioid receptor agonism

D. Cozzolino, D. Gruosso, A. A. Giammarco, D. Cataldo, C. Di Maggio, A. Cavalli, A. Pulcino, G. Renzo, I. Prevete, F. C. Sasso and R. Torella
Cardiovascular Research Center, II University of Naples, Naples, Italy. E-mail: domenico.cozzolino@unina2.it

Some evidences suggested an involvement of the opioid system in the regulation of blood pressure. Moreover, some opioid peptides are increased in plasma of patients (Pts) with essential hypertension. This study investigated the effects of a high dose infusion of β-endorphin, an opioid peptide, on blood pressure and

neurohormonal profile in 11 healthy normotensive subjects (C) and in 12 Pts (mean age: 38.9 and 40.4 years, respectively), and the mediation played by opioid receptor agonism. According to a randomized double-blind design, each subject received 1-h intravenous infusion of β-endorphin (250 μg/h) and, on a separate occasion, the same infusion protocol preceded by a bolus (8 mg) of opioid antagonist naloxone. Basal plasma levels of β-endorphin, norepinephrine, and endothelin-1 in Pts were higher than C. In C, β-endorphin produced a reduction of blood pressure ($P < 0.01$) and circulating norepinephrine ($P < 0.02$), and an increase in atrial natriuretic factor ($P < 0.003$) and growth hormone ($P < 0.0001$). In Pts, β-endorphin reduced systemic vascular resistance ($P < 0.0001$), blood pressure ($P < 0.0001$), and plasma levels of norepinephrine ($P < 0.0001$) and endothelin-1 ($P < 0.0001$), and increased plasma levels of atrial natriuretic factor ($P < 0.0001$), growth hormone ($P < 0.0001$) and insulin-like growth factor-1 ($P < 0.0001$). These hemodynamic and hormonal responses to β-endorphin in Pts were significantly ($P < 0.0001$) higher than C. Naloxone preceding β-endorphin infusion reversed all these hemodynamic and hormonal effects in both groups of subjects. In conclusion, β-endorphin induces hypotensive and beneficial hormonal effects in man. These effects are mediated by opioid receptor agonism and are enhanced in essential hypertension.

C3-020P
Involvement of Ca^{2+} signalling in VIP-induced VEGF and C-Fos expression induced by VIP in LNCaP cells

M. J. Carmena, B. Collado, A. B. Fernandez-Martinez, A. Valdehita, S. Sotomayor and J. C. Prieto-Villapun
Department of Biochemistry and Molecular Biology, University of Alcalá, Alcalá De Henares, Spain.
E-mail: mariajose.carmena@uah.es

Vasoactive intestinal peptide (VIP) is a pleiotropic neuropeptide that binds to VAPC1 and VAPC2 receptors that are preferentially coupled to Gas in the prostate gland and stimulate cAMP. The involvement of Ca^{2+} in VIP signalling has been less investigated. Both receptors can couple to the IP3/Ca^{2+} pathway through Gq and Gβi and thus enhancing $[Ca^{2+}]i$ levels. We have recently demonstrated that VIP increases the expression of the major angiogenic factor, vascular endothelial growth factor (VEGF) [1] in the human prostate LNCaP cell line. Here we have investigated the effect of VIP on: (a) intracellular Ca^{2+} levels, (b) expression of c-fos and (c) expression of vascular endothelial growth factor (VEGF) in LNCaP cells. RT-PCR experiments showed that VIP induced the expression of c-fos mRNA. Western blot analysis indicated that this feature was accompanied by VIP stimulation of c-fos protein synthesis. By means of the calcium probe fura-2, we observed that VIP enhanced intracellular Ca^{2+} levels. The regulatory effect of VIP on c-fos expression was dependent on the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) since BAPTA/AM (an intracellular calcium chelator) decreased c-fos expression to basal levels at both mRNA and protein steps. Real-time RT-PCR showed that VIP stimulated VEGF mRNA expression: the effect was Ca^{2+}-dependent since BAPTA/AM inhibited this VIP action by 43%. Present data suggests that: (a) VIP could act through both cAMP and $[Ca^{2+}]i$ increases in human prostate LNCaP cancer cells, and (b) c-Fos could be involved in the induction of VEGF since the promoter region of the VEGF gene possesses AP-1 response elements. It represents that an initiating signal acting upon VIP receptors may regulate the nuclear oncogene c-fos and angiogenesis.

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C3-021P
Association of plasminogen with dipeptidyl peptidase IV and Na^+–H^+ exchanger isoform NHE3 regulates invasion of human 1-LN prostate tumor cells

M. A. Gonzalez-Gronow, M. K. Uma, G. Gawdi and S. V. Pizzo
Department of Pathology, Duke University, Durham, NC, USA.
E-mail: gonza002@mc.duke.edu

Binding of plasminogen type II (Pg 2) to dipeptidyl peptidase IV (DPP IV) on the surface of the highly invasive 1-LN human prostate tumor cell line induces an intracellular Ca^{2+} ($[Ca^{2+}]i$) signaling cascade accompanied by a rise in intracellular pH (pHi). In endothelial cells Pg 2 regulates intracellular pH via Na^+/H^+ exchange (NHE) antiporters; however, this mechanism has not been demonstrated in any other cell type including prostate cancer cells. Since the Pg 2 receptor DPP IV is associated to NHE3 in kidney cell plasma membranes, we investigated a similar association in 1-LN cells and a mechanistic explanation for changes in $[Ca^{2+}]i$ or pHi induced by Pg 2. Our results suggest that the signaling cascade initiated by Pg 2 and its receptor proceeds via activation of phospholipase C which promotes formation of inositol 3, 4, 5-trisphosphate, an inducer of Ca^{2+} release from endoplasmic reticulum stores. Furthermore, our results suggest that Pg 2 may regulate pHi via an association with NHE3 linked to DPP IV in these cells. These associations suggest that Pg has the potential to regulate simultaneously calcium signaling pathways and Na^+/H^+ exchanges necessary for tumor cell proliferation and invasiveness.

C3-022P
A major clathrin-independent endocytic pathway in mammalian cells revealed by magnetic purification of endosomes

O. O. Glebov and B. J. Nichols
MRC Laboratory of Molecular Biology, Cambridge, UK.
E-mail: og@mrc-lmb.cam.ac.uk

Previous studies provide evidence for an endocytic mechanism in mammalian cells that is separate from both clathrin-coated pits and caveolae. This mechanism, however, has been defined largely in such negative terms, and the structures that mediate the relevant vesicle budding event at the plasma membrane have not been identified. We developed a ferro-fluid based magnetic purification assay to isolate the functionally active pool of early endosomal intermediates, and identified the proteins enriched in this preparation. Candidate proteins were tagged with GFP and were shown to be present in a specific population of early endosomes accumulating glycosylphosphatidylinositol (GPI)-linked proteins, fluid-phase markers and cholera toxin B-subunit (CTB), but not caveolin-1 or transferrin. Total Internal Reflection Fluorescence (TIRF) microscopy revealed the presence of dynamic microdomains in the plasma membrane that are distinct from both clathrin-coated pits and caveolae and are frequently budding into the cell. Downregulation of the pathway by siRNA or expression of a specific truncation mutant inhibited clathrin-independent uptake of cholera toxin and endocytosis of GPI-linked protein. Thus, we describe a previously uncharacterized major clathrin-independent endocytic pathway in mammalian cells.

C3-023P
Insights into the activation of EGF receptor under oxidative stress

T. Goldkorn, E. M. Khan, T. Ravid and J. M. Heidinger
Signal Transduction-Internal Medicine, University of California, Davis, CA, USA. E-mail: ttgoldkorn@ucdavis.edu

Recent crystallographic studies have offered a new understanding of how receptor tyrosine kinases from the ErbB family are regulated by their growth factor ligands. A large conformational change was shown to occur upon ligand binding, where a solely receptor-mediated mode of dimerization was documented. We have shown that oxidative stress, in the form of H_2O_2, activates the epidermal growth factor (EGF) receptor (EGFR) differently than its ligand. Most notably, H_2O_2 activation of EGFR resulted in aberrant EGFR phosphorylation as well as impaired trafficking and degradation of the receptor. Using various biochemical techniques, we now demonstrate that H_2O_2 activation of EGFR is ligand-independent and does not induce receptor dimerization. Thus, an unprecedented apparently activated state was found for the EGFR monomer under oxidative stress. Furthermore, H_2O_2 activation of EGFR is temperature-dependent and is inhibited by the addition of cholesterol, suggesting that EGFR activation by H_2O_2 is dependent upon membrane fluidity. Overall, our findings suggest that H_2O_2 activation of EGFR does not fit the current paradigm of EGFR activation by its cognate ligand, EGF. We are currently investigating the possibility that H_2O_2 activates EGFR by causing a change in membrane fluidity as well as a conformational change of the receptor itself. By combining the information gained from the recent biochemical studies, we hope to develop models for the allosteric regulation of EGFR under oxidative stress. These models will greatly improve our understanding of ErbB receptor signaling under oxidative stress, which will generate opportunities for the design of new anticancer agents.

C3-024P
How much do the different lipid raft markers overlap? A fluorescence imaging study on membrane organization

I. Gombos[1], A. Lõrincz[1], I. Pomozi[2], G. Steinbach[2], G. Garab[2], G. László[1] and J. Matkó[1]
[1]Department of Immunology, Eötvös Loránd University, Budapest, Hungary, [2]Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary. E-mail: jimx@elte.hu

Lipid raft microdomains enriched in glycosphingolipids and cholesterol are expressed in cellular plasma membranes (PM) and involved in compartmentation (recruitment/coupling or isolation) of many receptor and signal molecules. However, their size, lifetime and diversity are still highly debated. The choice of proper markers with high enough selectivity and minimal perturbation is also a prerequisite in visualizing rafts in live cells. Here we analyzed the spatial correlation between several widely used lipid raft markers on different cell types (human and mouse lymphoid and myeloid cells, fibroblasts and rat heart muscle tissue) using different fluorescence imaging techniques. We used monoclonal antibodies against GPI-achored protein markers, diIC18(3) and choleratoxin B subunit (CTXB) as lipid markers and an IgG3 type anti-cholesterol antibody (ACHA8/8) developed by us recently. We found a weak and cell type-dependent correlation (c: 0.3–0.6) between diIC18(3) and CTXB lipid markers. Intensity cross-correlation and FRET data have shown a much higher correlation of protein markers with CTXB than with diIC18(3). DiIC18(3)-enriched PM domains revealed by differential polarization microscopy (DP-LSM) also weakly correlated with the GPI-microdomains on lymphoid cells. Our novel ACHA probe has shown a patchy PM staining on all cell types studied, but only after epitope-exposition by limited papain digestion of the cell surface. The extent of staining correlated well with the ganglioside content of the cell membrane, assessed by flow cytometric analysis of CTXB binding. The ACHA and CTXB probes showed a highly correlated PM redistribution (polarization) upon activation of T-cells. We conclude from our data that diIC18(3), although known to enrich in ordered and gel phase PM domains, can be applied as raft marker only with precautions, due to its weaker selectivity than that of CTXB, while ACHA8/8, after a careful further characterization, could be a useful new tool in microdomain research.

C3-025P
The role of caveolae in insulin signal transduction in adipocyte

E. Gonzalez-Muñoz, M. Palacín, A. Zorzano and M. Camps
Laboratory of Molecular Patology, Department of Biochemistry and Molecular Biology, University of Barcelona, Barcelona, Spain Spain. E-mail: egonzalez@pcb.ub.es

Caveolae are a subclass of lipid raft that are characterized by the presence of caveolin1 protein. They represent a 30% of the plasma membrane surface of adipocytes. However, its function is still unclear. The existence of a second insulin signaling PI3K independent pathway in adipocyte defined by flotillin/Cbl/TC10 and associated to caveolae has been reported (Saltiel et al. *Nature* 2000). In our study we define the role of this pathway in other actions of insulin in addition to the stimulation of Glut4 translocation to the plasma membrane. We decided therefore, to modify caveolae structure (using Fylipin and Nystatin, that are membrane cholesterol chelators) or both structure and composition [using beta-methylcyclodextrin (MCD) and beta-cyclodextrin (CD) that remove both cholesterol and most of caveolin from cell membrane] and we studied how insulin-inhibited lipolysis, insulin-stimulated lipogenesis and insulin-stimulated glucose transport are affected. In addition, we studied which elements of the two insulin signaling pathways that stimulate Glut4 membrane translocation (PI3K and Cbl/TC10) are affected by these caveolae disrupting agents. Only MCD treatment, could slightly reduce Akt basal phosphorylation and insulin-stimulated Akt and Cbl phosphorylation. Insulin-stimulated lipogenesis was ablated by cholesterol-removing drugs (CD and MCD) however this ablation was mainly due to a basal stimulating effect and secondarily to a decrease in insulin-stimulated lipogenesis. These drugs also promoted an increase in the values of basal and insulin-inhibited lipolysis. Basal glucose transport also increased but not the insulin-stimulated one, therefore glucose transport stimulation was reduced in a 70% in relation to control.

C3-026P
Up-regulation of NPY Y2 receptors in the hippocampal CA3 region of alcohol-preferring (P) rats relative to alcohol-non-preferring (NP) rats: a potential role of hippocampal Y2 receptors in mediating alcohol intake

B. H. Hwang[1], Z.-h. Gu[1], N. W. Gilpin[2], N. E. Badia-Elder[2], R. B. Stewart[2], A. Hansson[3] and M. Heilig[3]
[1]*Anatomy & Cell Biology, Indiana University, Indianapolis, IN, USA,* [2]*Psychology, IUPUI, Indianapolis, IN, USA,* [3]*Laboratory of Clinical and Translational Studies, NIAAA, Rockville, MD, USA.*
E-mail: bhwang@iupui.edu

Neuropeptide Y (NPY) modulates alcohol drinking, and NPY knockout mice drink more ethanol than wild-type controls. We have previously shown that an NPY mRNA deficit in the dentate gyrus of the hippocampus is associated with high alcohol consumption in alcohol-preferring (P) rats, when compared with alcohol-non-preferring (NP) rats. The P and NP rats are selectively bred for high and low alcohol preference, with P rats accepted as an animal model for studying alcoholism. For this study, we used *in situ* hybridization and receptor binding to examine Y2 receptor mRNA expression and Y2 receptor binding density in the hippocampus of P and NP rats. The specific Y2 receptor antagonist, BIIE0246, was also infused into the CA3 region of P rats to assess how it affected alcohol intake. The results showed that P rats contained more Y2 receptor mRNA and a higher density of Y2 receptor binding sites in the CA3 region than NP rats. A preliminary study also showed that BIIE0246 microinjections tend to reduce alcohol intake in P rats. In conclusion, results from this study suggest that Y2 receptors in the CA3 region play a role in mediating alcohol intake. Together with the literature, this study supports the notion that an NPY deficit in the dentate gyrus (DG) in conjunction with subsequent up-regulation of Y2 receptors in the CA3 of the DG-CA3 pathway contributes to the high alcohol preference and high drinking phenotype in P rats.

C3-027P
The structure of ErbB2 receptor: an energy transfer and molecular modeling study

G. Horváth[1], P. Bagossi[2], G. Vereb[1], J. W. Park[3], J. Tõzsér[2] and J. Szöllõsi[1]
[1]*Deptartment of Biophysics and Cell Biology, Medical and Health Center, University of Debrecen, Debrecen, Hungary,* [2]*Deptartment of Biochemistry and Molecular Biology, Medical and Health Center, University of Debrecen, Debrecen, Hungary,* [3]*Department of Medicine, University of California, San Francisco, CA, USA.*
E-mail: horvathg@delfin.klte.hu

The epidermal growth factor receptor family (EGFR, erbB2, erbB3 and erbB4) plays an important role in breast cancer and other tumorous malignancies. The overexpression of erbB2 is correlated with poor prognosis. In recent years immunotherapies have been developed in which anti-erbB2 antibodies arrest malignant cell growth, and increasing the efficacy of other chemotherapies. The correlation between the antibody epitopes and the effect of antibodies may lead to important insights to develop new therapies in other systems as well. We mapped the epitopes of erbB2 using energy transfer measurements on a gastric tumor cell line (N87). The flow cytometric energy transfer method (FCET) was used enabling us to determine the proximity between two spectrally overlapping flurophores in the 1–10 nm range. Labeling ErbB2 we used monoclonal antibody Fab-s labeled with Cy3 and Cy5 dyes, and the cell membrane was doped with BODIPY lipid probes to determine the distances of epitopes from the membrane. We found that 4D5 and 2C4 antibodies were closest to each other, 7C2 antibody was closer to 2C4, but was further away from 4D5. The F5-cys antibody was far away from all the other three Fab-s. Their distances from the membrane: F5-cys was the closest, while the others were relatively far from the membrane. We constructed a new model for the whole structure of the erbB2 receptor, consisting of three conservative segments: the extracellular domain, the transmembrane region and the tyrosine-kinase region. These segments were connected with molecule modeling techniques and we were able to dock the 4D5 and 2C4 Fab antibodies onto this structure. We got two tetrameric structures that fulfilled the distance requirements imposed by FCET measurements and they were bound to each other by all three domains.

C3-028P
Mutations in the third extracellular loop of M3 muscarinic receptor induce positive cooperativity between N-Methylscopolamine and Wieland–Gumlich Aldehyde

J. Jakubík and V. Dole
Laboratory of Neurochemistry, Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic.
E-mail: jakubik@biomed.cas.cz

Individual amino acids were mutated or the entire third extracellular loop (o3) of the M_3 muscarinic receptor was replaced with the corresponding sequence of M_2 receptor. Despite both parental subtypes (M_2wt and M_3wt) display negative cooperativity between N-methylscopolamine (NMS) and Wieland–Gumlich Aldehyde (WGA) exchange of the o3 loop switches negative cooperativity of M_3wt to positive. Gradual replacement of individual amino acids revealed that only three residues N419, V421 and T423 (M_2 sequence) of the o3 loop are involved in this effect. This is the first evidence that switching sequences of the two parental receptors, both exhibiting negative cooperativity, constitutes positive cooperativity of muscarinic allosteric ligand.

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C3-029P
The effect of 17 beta-estradiol on the content of insulin signaling molecules in liver and uterus of ovariectomized rats

G. Koricanac, M. Vulovic, T. Milosavljevic, Z. Zakula and N. Ribarac-Stepic
Laboratory for Molecular Biology and Endocrinology, "Vinca" Institute of Nuclear Sciences, Belgrade, Serbia and Montenegro.
E-mail: gogi@rt270.vin.bg.ac.yu

Although the effect of 17 beta-estradiol on glucose homeostasis has been reported, its influence on insulin signaling remains an intriguing question. The recently published data indicate the changes of insulin action during pregnancy, as well as in women taking oral contraceptives or receiving estrogen replacement therapy. However, these observations could also point to the possible utilization of 17 beta-estradiol in therapy of diabetes. The effect of 17 beta-estradiol on protein content of insulin signaling

molecules in the liver and uterus, as well as plasma insulin, glucose and citrulline level of ovariectomized rats has been investigated in this study. Female Wistar rats were ovariectomized 2 weeks before experiment and estradiol was injected 6 h prior to sacrifice. Protein content of insulin receptor (IR), insulin receptor substrates 1 and 2 (IRS-1 and IRS-2), Shc protein, phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (PKB) was determined by Western blot in the liver and uterus. Estradiol treatment did not change plasma glucose, insulin and citrulline level. However, hepatic IRS-1 and PI3-K level was decreased after estradiol injection, while IRS-2 content was significantly increased. On the contrary, the uterine protein content of all analyzed molecules was elevated in estradiol-treated rats, except Shc amount that was diminished. Despite the lack of changes in blood glucose and insulin level, estradiol treatment caused tissue-specific changes in protein content of insulin signaling molecules that were more prominent and more consistent in the rat uterus than in liver.

C3-030P
The inhibitory mechanisms of tumor angiogenesis through reduction of expression of VEGF receptors by green tea extract

A. Kojima-Yuasa[1], J.-H. Jin[1], K. Kinoshita[1], D. O. Kennedy[2] and I. Matsui-Yuasa[1]

[1]*Laboratory of Science of Nutritional Functions, Department of Food and Human Health Sciences, Graduate School of Human Life Science, Osaka City University, Osaka, Japan,* [2]*Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, NY, USA. E-mail: kojima@life.osaka-cu.ac.jp*

Introduction: Vascular endothelial growth factor (VEGF) is a major regulator of both physiological and pathological angiogenesis and is an endothelial cell-specific mitogen that promotes many other events necessary for angiogenesis. Epidemiological and animal studies have indicated that consumption of green tea is associated with a reduced risk of developing certain forms of cancer. However, the inhibitory mechanism of green tea in angiogenesis, an important process in tumor growth, has not been well established. In the present study, we have investigated the inhibitory mechanism of tumor angiogenesis, especially expression of VEGF receptors (Flt-1 and KDR/Flk-1) in human umbilical vein endothelial cells (HUVECs) by green tea extract (GTE).

Methods: GTE (0–25 μg/ml) were dissolved in ethanol. GTE was tested for its ability to inhibit cell viability, cell proliferation, cell cycle dynamics. The expression of VEGF receptors was detected using immunohistochemical staining and Western blotting. Protein tyrosine phosphorylation and retinoblastoma protein (Rb) phosphorylation were examined by Western blotting.

Results: GTE in culture media did not affect cell viability but significantly reduced cell proliferation dose-dependently and caused a dose-dependent accumulation of cells in the G1 phase. GTE decreased VEGF receptors levels in a dose dependent manner using immunohistochemical staining and Western blotting methods. GTE also decreased the levels of protein tyrosine phosphorylations (76, 72, 70 and 44 kDa) and hyperphosphorylated Rb in a dose-dependent manner.

Conclusion: These results suggest that GTE may have preventive effects on tumor angiogenesis and metastasis through reduction of expression of VEGF receptors.

C3-031P
Probing membrane heterogeneity with quantum dots

B. C. Lagerholm[1], G. E. Weinreb[1], A. S. Waggoner[2], N. L. Thompson[3] and K. Jacobson[1]

[1]*Deptartment of Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC, USA,* [2]*Molecular Biosensor and Imaging Center, Carnegie Mellon University, Pittsburgh, PA, USA,* [3]*Department of Chemistry, University of North Carolina, Chapel Hill, NC, USA. E-mail: lagerholm@med.unc.edu*

Some of the more convincing evidence for the existence of membrane heterogeneities in live cells has been obtained by single particle tracking (SPT) of 40 nm diameter gold particles conjugated to appropriate cell membrane markers. This technique has shown that in particular glycosyl phosphatidyl inositol (GPI) anchored proteins exist in part in membrane domains on the order of tens of nanometers in size and with lifetimes that range from milliseconds to seconds. We are exploring a variety of methods designed to take advantage of the smaller size and fluorescence properties of quantum dots and that can be used to identify and characterize membrane domains similar to those described by SPT. In particular, we have found that quantum dot intermittency (''blinking'') can be taken advantage of to determine sub-pixel positions with nanometer precision of single quantum dots within apparent sub-diffraction limited clusters composed of multiple quantum dots. We have used this method to probe the distribution of a variety of membrane markers, primarily in fixed cells. It is our aspiration that this method will be able to provide spatial and statistical information of membrane organization analogous to that obtainable by immunogold transmission electron microscopy, albeit at lower resolution but with the eventual goal of being able to statistically analyze changes in membrane domain organization in live cells.

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C3-032P
Human MT2 melatonin receptor and its melatonin recognition site: a structural model

L. Luley[1], T. Stockner[2], Z. Sovova[1], P. Mazna[3], R. Ettrich[1] and J. Teisinger[3]

[1]*Laboratory of High Performance Computing, Institute of Physical Biology of USB and Institute of Landscape Ecology of AS CR, Nove Hrady, Czech Republic,* [2]*Department of Biological Sciences, University of Calgary, Calgary, Alberta Canada,* [3]*Institute of Physiology,Academy of Sciences of the Czech Republic, Prague, Czech Republic. E-mail: luley@greentech.cz*

The pineal hormone melatonin, is present in all vertebrate species including humans. Aside from being an important regulator of seasonal reproduction and circadian rhythms melatonin was reported to be potentially important immunomodulator, powerful free radical scavenger and exertsoncostatic activity. Melatonin binding to specific G protein-coupled receptors, designated as MT1, MT2 and Mel1c, modulates wide range of intracellular messengers mediating hormone effects. Homology modeling of the hMT2 melatonin receptor is reported. The deduced amino acid sequence shows high homology with bovine rhodopsin, whose tertiary structure has been solved at 2.6 Å resolution. The resulting structure contains seven putative transmembrane domains connected by three extracellular and three intracellular loops. Docking of melatonin into the protein structure was explored. We have identified that for high-affinity melatonin

binding to hMT2 receptor are essential Val 204 and Leu 272 in transmembrane domains (TM) V and VI respectively as well as Tyr 298 in TM VII. We have also demonstrated the importance of Gly 271 for high-affinity binding to the hMT2 melatonin receptor. Experimental results from site-directed mutagenesis and saturation binding assays with 2-iodomelatonin [1] have been confronted with our molecular modeling results. Behavior of melatonin binding is explored by 10 ns molecular dynamics runs of the receptor in an octane layer.

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C3-033P
Studies on cell specific expression, structure and membrane localization of a late lymphoid activation antigen Ly77 (GL7)

M. Ádori, A. Balogh, Z. Weiszhár, J. Prechl, J. Matkó and **G. László**

Department of Immunology, Eötvös Loránd University, Budapest, Hungary. E-mail: glorial@cerberus.elte.hu

Ly77 (GL7), a 35 kDa activation antigen was originally described on mouse T- and B lymphocytes after 36–48 h *in vitro* activation. *In vivo* its presence was detected on pre- and immature B-cells in the bone marrow, on certain thymocytes, and in the germinal centers. The aim of the work presented here was to study the chemical nature of the epitope, to compare the size of the molecule on cells of various origin and to see the localization of GL7 in the cell membrane. Studied cell lines of pre-B (1–305), immature B (38C-13) or activated mature B-cell (2PK-3, LK-35, A20, TA-3, M12-4.1) phenotype were all highly expressed this marker but B-cell line of mature spleen marginal zone (X16C) phenotype was GL7 negative. Monocyte/macrophage type cell lines (J774A.1, P338D1) expressing certain B-cell markers (CD19, CR2) could also be stained by the Ab but other macrophages (WEHI-3, RAW264.7) were negative. TH cell hybridomas varied by their expression profile. Immunoprecipitation of GL7 from B and T lymphoid or monocyte cell lines revealed identical molecular weight. The epitope recognized by our Ab could be removed of all types of cells by neurominidase and also by tunikamycin treatment indicating that sialic acid residues are involved in the antibody binding site. Confocal laser-scanning microscopic colocalization with choleratoxinB (c:0,4–0,6) revealed raft-association of GL7 in the membrane of cell lines in either B- or TH lymphocytes or monocyte/macrophage cell lines. The *in vivo* staining pattern of the Ab and the carbohydrate nature of the epitope suggest an adhesion role for this molecule. To overcome the technical difficulties caused by using the original Ab (rat IgM), an scFv construct of the Ab was successfully created. In our ongoing studies with the scFv construct we are looking at membrane localization and interaction with other proteins of GL7 on various cell lines. The possible role of GL7 in signal transduction and in cell adhesion is currently under investigation.

C3-034P
GABA-responses of receptors formed by GABA rho-1 and-gamma-2 (K289M)

F. d. M. Trejo-Medinilla[1], A. López-Chávez[1], G. García-Alcocer[2], R. Miledi[1] and **A. Martinez-Torres**[1]

[1]MCN, Molecular & Cellular Neurobiology, UNAM, Queretaro, QRO Mexico, [2]School of Chemistry, UAQ, Queretaro, QRO Mexico. E-mail: ataulfo@inb.unam.mx

γ Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the adult brain activating GABA-A and GABA-C receptor/chloride channels. In recent years some evidence suggests that the subunits forming these receptors co-assemble giving rise to hybrid receptors with peculiar electrophysiological and pharmacological properties. On the other hand, mutations on the GABA-A γ2 subunit are linked to hereditary epilepsy in humans. A single amino acid change (K289M) leads to GEFS+ condition. We first investigated if the ρ1 and γ2 subunits are co-expressed in the human brain. RT-PCR revealed that they are both present in the cerebellum and caudate nucleus and immunodetection indicated that they are expressed along the Purkinje cell-line of the cerebellum. HEK293 cells were co-transfected with ρ1 and γ2 and GABA-currents assessed by whole-cell patch-clamp. The GABA-currents generated by the hybrid receptor were constantly larger than those of homomeric ρ1, 571.4 and 140.6 pA, respectively. However, when the γ2 (K289M) mutant was co-expressed with ρ1 the GABA-currents decreased to 150.9 pA. GABA EC50 and nH was determined resulting in the following: ρ1 (1.07 μM, 2.93), ρ-γ-2 (1.53 μM, 2.1) and ρ1-γ-2 (K289M) (2.6 μM, 7.9). The mutant receptor decay rate (τ50) (16) was also different compared to the wild type receptors (26 and 27). In conclusion we determined that GABA-C and GABA-A subunits are co-expressed in several areas of the human CNS and that they form hybrid receptors when expressed in cultured cells. Finally, a mutant of the γ2 subunit (K289M) associated with hereditary epilepsy alters the properties of receptors formed in association with GABA-C ρ1.

C3-035P
How many calcium-binding sites are involved in facilitation of transmitter release?

M. A. Mukhamedyarov, S. N. Grishin and A. L. Zefirov

Department of Normal Physiology, Kazan State Medical University, Kazan, Russian Federation. E-mail: maratm80@list.ru

Many synapses can increase their strength during short time period in activity-dependent manner; this process is called facilitation. The key role in facilitation is given to accumulation of residual calcium in pre-synaptic terminal, but exact mechanisms remain unknown. One possible mechanism of facilitation is the existence of additional calcium-binding sites, which have higher affinity to Ca ions. The aim of present work is the title of abstract. The experiments were performed on frog neuromuscular preparations. The end-plate currents (EPCs) were registrated extracellularly and with voltage-clamp technique. Normal Ringer's solution (Ca 1.8 mM) and "magnesium" solution (Ca 0.6 mM, Mg 4 mM) were used. Paired-pulse facilitation (PPF) was examined in 5–500 ms period. In some series of experiments also intracellular Ca buffers BAPTA-AM and EGTA-AM (100 mkM) were used. At the control PPF was described by sum of two exponentially decayed components. In case of Ringer's solution first component had larger amplitude and decayed to 100 ms, the second one, less pronounced – to 300 ms. In BAPTA-AM preparations both components were greatly reduced. In EGTA-AM preparations first component was slightly affected, second one was absent. In case of "magnesium" solution in

control experiments first and second PPF components were more pronounced, the "early", most significant and short component appeared. In BAPTA-AM preparations "early" component was missed and both others were decreased. In EGTA-AM preparations "early" component was not affected, the first one was shorten and second component was completely oppressed. We make conclusion that different PPF components are caused by activation of distinct calcium-binding sites which characterized by different affinity to Ca ions and distance from Ca channel.

C3-036P
Caveolae play important role in the fast non-genomic effect of estrogen: an *in vivo* study

N. Müllner[1], Á. Turi[1], A. L. Kiss[2], E. Kovács[2], E. Botos[2] and D. Lásztity[3]
[1]*Department of Medical Chemistry, Molecular and Pathobiochemistry, Semmelweis University, Budapest, Hungary,* [2]*Department of Human Morphology, Semmelweis University, Budapest, Hungary,* [3]*Department of Plant Physiology and Molecular Biology, Eötvös Lóránd University, Budapest, Hungary.*
E-mail: Mullner@puskin.sote.hu

Recently it was showed that E2 can induce a rapid and transient activation of the Src ERK phosphorylation cascade, indicating that ERα can associate with the plasma membrane. Increasing number of evidences suggest that caveolae can play important role in E2 induced signal transduction. Caveolae are caveolin-1 containing highly hydrophobic membrane domains that can preferentially accumulate signaling molecules thus caveolae/caveolin-1 seem to play regulatory role in signaling procedures. Our previous results showed that E2 treatment has significantly decreased the number of the surface connected caveolae in uterine smooth muscle cells and caveolin-1 expression was also down-regulated. Our present study provides further evidences about the interaction of ERα with caveolin/caveolae in uterine smooth muscle cells. We showed that the interaction between caveolin-1 and ERα was facilitated by E2. Src kinase was also found to accumulate in caveolae, and the amount of Src kinase has simultaneously increased with the amount of ERα. E2 treatment has also resulted in the tyrosine phosphorylation of Src kinase causing phosphorylation of caveolin-1. Our results indicate that E2 can regulate the plasma membrane associated signaling cascade by facilitating the interaction between ERα and caveolin-1/caveolae. This interaction stimulates Src kinase that phosphorylates caveolin-1. Phosphorylation of caveolin-1 can drive caveolae to pinch off from the plasma membrane by which the amount of the plasma membrane associated caveolin-1 is decreasing. The loss of caveolin/caveolae drives to activate signaling cascade, which triggers cell proliferation.

C3-037P
Overexpression and characterization of an active GPCR-G-protein fusion complex

S. F. Marino and H. Michel
Department of Molecular Membrane Biology, Max Planck Institute of Biophysics, Frankfurt, Germany.
E-mail: stephen.marino@mpibp-frankfurt.mpg.de

G-protein coupled receptors (GPCRs) function as cell surface receptors for a broad diversity of ligands and comprise one of the largest protein families. Elucidation of the molecular details of signal transduction through GPCRs awaits the solution of high resolution structures of the receptor species involved in passing the extracellular information across the plasma membrane. The critical challenge in this effort is the production of sufficient quantities of stable receptor species amenable to crystallization screening. Here we describe the high level expression and characterization of an active fusion complex between the kappa opioid receptor (KOR) and its cognate G-protein alpha subunit, $G\alpha_i1$. The KOR is stabilized against degradation in the context of the fusion protein and is competent to bind both agonists and antagonists. Functional coupling of the KOR with the Gα subunit was demonstrated by the effect of agonist addition on both nucleotide binding and nucleotide hydrolysis, and the effect of nucleotide binding on agonist affinity. Growth optimization has resulted in the highest expression level reported to date for an opioid receptor construct, and makes large scale solubilization and purification feasible. In addition to representing a physiologically relevant signaling complex, the additional hydrophilic surface area provided by the G-protein may enhance the chances of producing well diffracting crystals.

C3-038P
The proteins of the Fc receptor complex as detected by LC-MS/MS and confirmed by confocal microscopy

A. Jankowski and **J. Marshall**
Department of Chemistry and Biology, Ryerson University, Toronto, Ontario Canada. E-mail: 4marshal@ryerson.ca

We prepared phagosomes from magnetic beads engulfed by RAW macrophages and human neutrophils with or without IgG opsonization. Over the time course of particle engulfment, neutrophils or macrophages were disrupted with a French press and phagosomes purified over sucrose gradients. As a negative control, crude cellular lysates and culture medium were incubated with polystyrene beads. The resulting proteins were prepared by micro chromatography followed by trypsin digestion and LC-MS/MS. We found that proteins of the endoplasmic reticulum, histones and cytoskeletal proteins were associated with the negative control and were apparently cellular contamination of the phagosomal preparations. No accumulation of the endoplasmic reticulum markers calnexin, KDEL or SEC61 were observed at the phagosome using transfection of GFP chimera fusion proteins and real-time microscopic measurements of RAW cells. In contrast, many receptor associated signaling proteins, including the Fc receptor, and many of the class of signal molecules associated with the vesicular model of phagocytosis were observed by LC-MS/MS and subsequent real-time microscopy including the src, syk, p110, p85, ship-1 PLC, PLD, and the ras superfamily. We collected MS/MS data mapping to specific ras, rac and rho proteins and their respective GTPase activating and exchange factors such as ELMO, dock 180, and crk homologs. Thus we found agreement between MS/MS and live cell confocal microscopic evidence in support of the vesicular model of phagocytosis.

C3-039P
VEGFR-1 mediated increase of pituitary tumor cell proliferation through PI3K pathway activation

C. Onofri, M. Theodoropoulou, G. K. Stalla and U. Renner
Endocrinology Department, Max Planck Institute of Psychiatry, Munich, Germany. E-mail: onofri@mpipsykl.mpg.de

Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen and belongs to the VEGF family together with VEGF-B, VEGF-C, VEGF-D, VEGF-E and Placenta growth factor (PlGF); it exerts its action upon binding to two different tyrosine kinase receptors: VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) which are expressed in endothelial cells.

However, it has been shown that VEGF receptors may also be expressed in different non-endothelial cell types. We detected the presence of VEGFR-1 mRNA and protein, in endocrine cells of both normal human pituitary and pituitary adenomas, by *in situ* hybridization and immunohistochemistry. Moreover, we observed that in MtT-S, a rat somatotroph cell line which expresses VEGFR-1 mRNA and protein, both VEGF and PlGF (which is a specific ligand of VEGFR-1) were able to induce a significant dose-dependent proliferation response. We know that in endothelial cells, VEGFR-1 acts through Phosphatydil Inositol-3 Kinase (PI3K) survival pathway, thus in order to see if the same way is employed in pituitary tumor cells, the phosphorylation level of PI3K/Akt pathway components was determined after treatment with PlGF. After 30 min treatment, PlGF increased phosphorylation levels of PDK1 and Akt; furthermore it phosphorylated and therefore inactivated GSK3-beta, which inhibits cell cycle progression. Although the exact mechanism by which VEGFR-1 mediates its proliferating effects in non-endothelial tumor cells is still unclear, these data point to a role of this receptor in aberrant tumor growth and constitute it as a pharmacological target.

C3-040P
Gangliosides play a critical role in the organization of the tetraspanin-enriched microdomains

E. Odintsova[1], T. D. Butters[2], R. L. Schnaar[3] and F. Berditchevski[1]
[1]*CRUK Institute for Cancer Studies, University of Birmingham, Birmingham, UK,* [2]*Glycobiology Institute, University of Oxford, Oxford, UK,* [3]*Departments of Pharmacology and Neuroscience, The Johns Hopkins School of Medicine, Baltimore, MD, USA. E-mail: e.odintsova@bham.ac.uk*

The metastasis suppressor CD82/KAI1 is a member of the tetraspanin superfamily of transmembrane proteins. Although biological function of the tetraspanins remains unclear, they are found in the complexes with a number of membrane receptors, including various integrins and receptors of the ErbB family. CD82/KAI-1 tetraspanin protein regulates activity of the associated receptors by tethering them into specific microdomains. CD82-enriched microdomains are characterized by a high content of gangliosides that may function as mediators of the CD82-induced attenuation of the receptors activity. Two approaches were used to analyze the effect of gangliosides on the organization of the tetraspanin-enriched microdomains: (a) biosynthesis of gangliosides was suppressed with a specific non-toxic inhibitor, NB-DGJ; (b) concentration of gangliosides on the plasma membrane was increased after incubation of the cells with the exogenously added gangliosides. We found that in the NB-DGJ-treated mammary epithelial cells the association of CD82 and some other tetraspanins with EGFR was decreased though the surface expression level of CD82 was elevated. In contrast, there was only minor effect of the ganglioside depletion on the interactions of tetraspanins with the a3b1 integrin. Furthermore, we found that the decrease in the total levels of gangliosides changed the fractional distribution of tetraspanins in the sucrose gradient. In contrast, incubation of the cells with the exogenously added GM1 and GD1a decreased surface expression of the tetraspanins CD82 and CD151 but did not have effect on the assembly of the tetraspanins' complexes. Thus, we concluded that the complexes within tetraspanin-enriched microdomains are assembled in Golgi and their organization is regulated by the lipid composition of the membrane.

C3-041P
The cholesterol absorption inhibitor Ezetimib influences the raft function through its receptor aminopeptidase N (CD13) in human macrophages

E. Orsó[1], T. Werner[1], Z. Wolf[1], K. Szakszon[1], M. Binder[1], S. Bandulik[1], M. Grandl[1], T. Köbling[1], G. Liebisch[1], W. Kramer[2] and G. Schmitz[1]
[1]*Institute for Clinical Chemistry and Laboratory Medicine, University of Regensburg, Regensburg, Germany,* [2]*Aventis Pharma Deutschland GmbH, A Group of Sanofi-Aventis, Frankfurt, Germany. E-mail: evelyn.orso@klinik.uni-regensburg.de*

Aminopeptidase N (CD13) was recently identified as a receptor for Ezetimib, a novel cholesterol absorption inhibitor, in enterocytic brush border. Since CD13 is expressed in cholesterol/sphingolipid-rich raft-microdomains of macrophages, it is tempting to speculate that Ezetimib may influence the raft function in these cells. Human monocyte-derived macrophages were loaded *in vitro* with enzymatically modified LDL (eLDL) or oxidized LDL (oxLDL), in order to induce a foam cell-like phenotype, in the presence or absence of Ezetimib. Cellular content of total cholesterol (TC), free cholesterol (FC) and cholesteryl esters (CE) was measured by mass spectrometry. Protein and mRNA expression of genes, intimately involved in cellular lipid traffic, were detected by flow cytometry and TaqMan real-time PCR, respectively. Detergent-resistant membrane fractions (DRMs) were isolated by Lubrol-WX and the DRM-localization of proteins were followed by immunoblot. Localization and cellular distribution of raft-associated antigens such as CD13, CD14, and CD18 were assessed by confocal imaging. Ezetimib decreased the cellular content of TC and CE, parallel with an increase in FC, and up-regulation of the mRNA expression of ABCA1 and ABCG1. The decreased CE was consistent with the down-regulation of ACAT1 mRNA and reduction of intracellular lipid droplets. The presence of ABCA1 protein in DRMs was enhanced upon eLDL-load, but diminished upon Ezetimib administration. Ezetimib significantly down-regulated the protein expression of raft-associated antigens, such as CD13, CD14, CD11b, and CD36. In conclusion, Ezetimib inhibits differentiation and lipid-accumulation of monocyte-derived macrophages, likely through a CD13-dependent, raft-associated receptor (dys)clustering mechanism.

C3-042P
Internalization of magnetoliposomes by 3T3 fibroblasts

C. Peleman[1], D. Caluwier[1], J. Cocquyt[2], P. Van der Meeren[2], J. Baert[1] and M. De Cuyper[1]
[1]*Subfaculty of Medicine, Interdisciplinary Research Center, Katholieke Universiteit Leuven – Campus Kortrijk, Kortrijk, Belgium,* [2]*Departement of Applied Analytical and Physical Chemistry, Faculty of Agricultural and Applied Biological Sciences, University of Ghent, Ghent, Belgium. E-mail: christa.peleman@kulak.ac.be*

Magnetoliposomes (MLs) consist of superparamagnetic iron oxide cores (diameter: 15 nm), covered with a bilayer of phospholipids. With the aim to elaborate successful applications in the biomedical field, it is often desirable that the particles are taken up within the cell envisaged. Engineering the surface of

the particles may drastically modulate this capacity. As a result, we prepared neutral, anionic and cationic MLs by incubating and dialysing laurate-stabilized Fe_3O_4 colloids with preformed vesicles made of either dimyristoylphosphatidylcholine (DMPC), or DMPC/dimyristoylphosphatidylglycerol, or DMPC/dioleoyltrimethylammonium propane. Excess lipids were removed by high-gradient magnetophoresis. The magnetite coating was checked by measuring the phosphate and fatty acyl content; the charge quality was derived from electrophoretic mobility measurements. 3T3 murine fibroblasts were taken as a representative model to qualitatively check the magnetoliposome's potency to become internalized. Incubation of the particles (0.1 mg Fe_3O_4/ml complete growth medium) for 24 h with the cells, grown on microscopy coverslips, and after staining with Prussian Blue, revealed that, in the experimental conditions applied, neutral and negatively charged MLs were only slightly internalized, whereas uptake of the cationic MLs was a few orders of magnitude higher. These results may be of relevance, for instance, for labeling and monitoring cell tracking by magnetic resonance imaging.

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C3-043P
Relative binding affinities of recombinant domain mutants of the human polymeric immunoglobulin receptor (pIgR) for IgM

E. Prinsloo and V. Oosthuizen
Immunoglobulin Receptor Research Group, Department of Biochemistry & Microbiology, Nelson Mandela Metropolitan University Summerstrand Campus (South), Port Elizabeth, Eastern Cape, South Africa. E-mail: earl.prinsloo@nmmu.ac.za

The membrane bound glycoprotein, polymeric immunoglobulin receptor (pIgR) (105 kDa), is the primary transport molecule of polymeric immunoglobulins (i.e. IgA and IgM) across epithelial cells via transcytosis in order to establish immunity at mucosal surfaces. During this process pIgR binds (via five homologous immunoglobulin-like domains of the ectodomain) to the polymeric immunoglobulin. Binding is covalent (to IgA) and non-covalent (to IgM). The ectodomain of pIgR is cleaved at Arginine-585 and released bound to the polymeric immunoglobulin. It is thereafter referred to as Secretory Component (75 kDa) and is responsible for protection of the mucosal surfaces against microorganisms. It is known that domain I of pIgR is the primary domain involved in the interaction with polymeric immunoglobulins. Binding of pIgR domain I to IgA and IgM has been characterized by ELISA and is believed to be the major contributor to total immunoglobulin binding. This study aimed to characterize the binding of recombinant human pIgR domain mutants to polymeric IgM using evanescent wave biosensor analysis on BIAcore X, allowing greater insight into the contribution of each of the five ectodomains towards ligand binding through analysis of association and dissociation rates. Recombinant domain mutants of human pIgR were amplified, cloned and expressed in *Escherichia coli* BL21 (DE3). Mutants were refolded (*in vitro*) and purified to homogeneity and the binding was analyzed using BIAcore X at varying flow rates and ligand concentrations. Results show the contribution of the individual domains to total binding is attributed to association rates.

C3-044P
Carbohydrate structures recognized by some C-type lectin receptors from NK cell gene complex

J. Pavlíèek[1], O. Vanìk[1], Z. Lejsková[1], R. Ettrich[2], V. Kopecký Jr[3], P. Man[4], M. Vrbacký[5] and K. Bezouška[1,2]
[1]Department of Biochemistry, Faculty of Science, Charles University, Hlavova 8, CZ-12840, Prague, Czech Republic, [2]Laboratory of High Performance Computing, Institute of Landscape Ecology, Czech Academy of Science, Zámek 136, CZ-37333, Nove Hrady, Czech Republic, [3]Institute of Physics, Faculty of Mathematics and Physics, Charles University, Ke Karlovu 5, CZ-12116, Prague, Czech Republic, [4]Institute of Microbiology, Czech Academy of Science, Vídeòská 1083, CZ-14220, Prague, Czech Republic, [5]Department of Pathological Physiology, 1st Medical Faculty, Charles University, U nemocnice 5, CZ-12853, Prague, Czech Republic. E-mail: pavlicek@biomed.cas.cz

The natural killer (NK) cell gene complex represents an important group of genes encoding surface receptors expressed predominantly on NK cells. All of them involve globular domains belonging to C-type lectins [CITACE]. Ligands for these domains are intensively investigated. It is already well established that some important ligands can have character of polypeptides, however the original lectin activity remains conserved in many cases. The aim of our work was to distinguish various monosaccharide specificity for CD69 receptor [1], for unique isoforms of NKR-P1 protein and for other receptors. It was observed that slight changes in the primary structure of the proteins can cause astonishing modifications of the binding specificity for ligands of more complex structures. We focused on the problem of very high-affinity oligosaccharide ligands, whereas its solution can lead to better understanding of the function of these lymphocyte receptors.

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C3-045P
Transport mechanisms responsible for the hepatic uptake of the bile acid derived diagnostic agent B22956: an '*in vitro*' study

A. Libra[1], C. Fernetti[2], M. Visigalli[3], L. Anelli[3], V. Lorusso[3], F. Štaud[1], C. Tiribelli[2] and **L. Pascolo**[2]
[1]Department of Pharmacology and Toxicology, Charles University, Hradec Králové, Czech Republic, [2]CSF-Area Science Park Basovizza, Department of Biochemistry, Biophysics and Macromolecules, University of Trieste, Trieste, Italy, [3]BRACCO-Centro Ricerche Milano, Milano, Italy. E-mail: lorella.pascolo@csf.units.it

Background: B22956 is a new gadolinium chelate which belongs to the class of intravascular contrast agents for magnetic resonance imaging (MRI). It is excreted predominantly by biliary excretion and could be potentially advantageous in hepatobiliary imaging.

Aim: To investigate the molecular mechanisms of hepatic transport of B22956 in models of human liver-derived cells.
Methods: B22956 uptake was measured in hepatic tumoral (HepG2) and non-tumoral (Chang liver) cell lines. Absolute quantitative real-time RT-PCR analyses, using cloned PCR products as standards, were performed on total RNA of human normal liver, HepG2 and Chang liver cells to evaluate the transcription of 12 drug transport genes possibly involved: OATP-A, OATP-B, OATP-C, OATP-D, OATP-E, OATP-8, OAT2, OAT3, OCT1, NTCP, PEPT-1, PEPT-2.
Results: A more efficient transport of B22956 was found in Chang liver that in HepG2 cells. The B22956 transport was inhibited by CCK8, a specific substrate of OATP-8. Real-time RT-PCR analyses revealed that all the genes, except OAT3, were expressed in normal liver. Different transcription profiles were observed in the cell lines. In HepG2 cells the expression of OATP-C, OATP-D and OATP-8 was greatly repressed, while OATP-B and OATs expression was maintained or increased. In Chang liver cells OAT genes were undetectable, while the expression of OATP-D, OATP-E and OATP-8 was similar to normal liver.
Conclusions: Transport studies and gene expression analyses indicate that B22956 is a good substrate for the liver specific OATP-8, which has been reported to be poorly expressed or absent in liver tumors. The additional involvement of OATP-D and OATP-E must be considered. It could be speculated that B22956 will be helpful in detecting hepatic tumoral lesions by CA-MRI.

C3-046P
PAR3 expression and function in mouse lymphocytes

Y. I. Petrova[1], O. V. Kameneva[1], L. Scudder[2], W. F. Bahou[2] and M. V. Skok[1]
[1]Molecular Immunology, Palladin Institute of Biochemistry, Kiev, Ukraine, [2]Haematology division, State University of New York at Stony Brook, Stony Brook, NY, USA.
E-mail: julia@biochem.kiev.ua

Protease-activated receptors (PARs) comprise a family of seven transmembrane G-protein coupled receptors, which mediate cell activation upon receptor cleavage. The role of PARs in blood coagulation system is well known. In contrast to the most explored PAR1, which plays a role in T lymphocyte activation, PAR3 functions in lymphocytes are currently unknown, although their expression was found in the human thymus and lymph nodes. The aim of the work was to study PAR3 expression and functions in mouse lymphocytes using cleavage site-specific monoclonal antibody (mAb) 8E8. By means of flow cytometry it was shown that up to 70% of mouse splenic B lymphocytes specifically bound mAb 8E8 demonstrating high PAR3 expression level in these cells. Statistically significant PAR3 expression was observed also in platelets, CD4/8 T lymphocytes, natural killers, macrophages, but not in granulocytes, the part of positive cells being much less than in B lymphocytes. MAb 8E8 partially inhibited thrombin-induced platelet aggregation. It didn't affect prothrombinase activation in either platelets or lymphocytes. The small increase of intracellular Ca^{2+} was observed in splenic cells in the presence of mAb 8E8. Thrombin dose-dependently enhanced mouse B lymphocyte activation stimulated with anti-CD40 Ab. MAb 8E8 neutralized the effect of thrombin, but did not influence proliferation itself. In contrast, thrombin did not affect anti-CD3-stimulated proliferation of T lymphocytes. These data correlated with those of flow cytometry and indicated that stimulating effect of thrombin on B lymphocyte activation is mediated by PAR3. In whole, the data obtained for the first time demonstrate the presence of PAR3 in mouse lymphocytes and the role of thrombin in lymphocyte activation. It denotes PARs as a possible link between blood coagulation and immunity.

C3-047P
Effects of ethanol ingestion on somatostatin-induced adenylate cyclase inhibition and on Gi protein levels in the frontoparietal cortex of virgin and parturient rats

L. Puebla-Jiménez[1], M. d. C. Boyano-Adánez[1], V. Barrios[2], D. Aguado[1], E. Burgos[1] and E. Arilla[1]
[1]Biochemistry and Molecular Biology, University of Alcalá, Alcalá De Henares, Madrid Spain, [2]Laboratory of Research, Hospital Infantil Universitario Niño Jesús, Madrid, Madrid Spain.
E-mail: lilian.puebla

Previous results from our group demonstrated that ethanol ingestion before and during pregnancy decreases the somatostatin (SRIF) receptor density in the rat frontoparietal cortex on the day of delivery with respect to control parturient rats as well as in virgin rats as compared to their controls. Since SRIF receptors are coupled to adenylate cyclase (AC) via inhibitory Gi proteins, in the present study we examined basal and forskolin (FK)-stimulated AC activity, SRIF-mediated inhibition of the enzyme and Gi protein levels in frontoparietal cortical membranes from control virgin, ethanol-treated virgin, control parturient and ethanol-treated parturient (0, 10 or 30 days postpartum) rats. Ethanol ingestion before and during gestation decreased basal and FK-stimulated AC activity in frontoparietal cortical membranes on the day of delivery as compared to control parturient rats. Although SRIF inhibited AC activity in all the experimental groups, chronic ethanol ingestion decreased the capacity of SRIF to inhibit basal and FK-stimulated AC activity in both virgin and parturient rats as compared with their respective controls. However, the capacity of SRIF to inhibit AC activity in membranes from alcoholic virgin rats was reduced to a greater extent than in alcoholic parturient rats, which may be related to the marked loss of SRIF receptors observed in this group. Western blot analyses revealed that ethanol ingestion during gestation does not alter the levels of Gialpha1, Gialpha2 or Gialpha3 proteins in the frontoparietal cortex. In conclusion, the present results show that ethanol exerts less effect on the SRIFergic/AC system of the frontoparietal cortex in gestational rats than in virgin rats, which suggests that gestation might confer partial resistance to the ethanol-induced effects.

C3-048P
Production, solubilization and purification of the human adenosine A_{2a} receptor in *Pichia pastoris*

C. Prual, C. Reinhart and H. Michel
Department of Molecular Membrane Biology, Max-Planck-Institute of Biophysics, Frankfurt, Germany.
E-mail: cecile.prual@mpibp-frankfurt.mpg.de

Among the membrane proteins, G protein-coupled receptors (GPCRs) correspond to the largest family and play a key role in signal transduction through the cell membrane. Around 50% of pharmaceuticals currently on the market target these receptors. Despite their profound importance, only a single high resolution GPCR structure, that of bovine rhodopsin, is currently available. As a member of the GPCR family, the human adenosine A_{2a} receptor is mainly expressed in the central nervous system. It is important for vasodilatation and stimulation of sensory nerves. It antagonizes dopaminergic activity and its study may thus be

applicable to the treatment of schizophrenia or Parkinson's disease. One of the many difficulties in obtaining membrane protein structures has been the limited amount of receptor available from native sources. In addition, the quality and quantity of receptor from recombinant sources is generally poor. We produced the human adenosine A_{2a} receptor in the methylotrophic yeast *Pichia pastoris* to more than 180 pmol/mg of membrane protein, which is among the highest heterologous production levels reported so far for a GPCR. Solubilization is often a very problematic step in obtaining a high yield of functional receptor. In our study, a yield of 70% of functional receptor was reached after membrane solubilization. Purification of the adenosine A_{2a} receptor was successfully performed and routinely provides mg amounts of pure, homogeneous and functional receptor. Crystallization trials of the adenosine A_{2a} receptor are underway.

C3-049P
Chimerical receptors whose independent fragments may stay apart but signal together

J.-P. Silva, K. E. Volynski, V. G. Lelianova, M. A. Rahman and Y. A. Ushkaryov
Biological Sciences, Imperial College London, London, UK.
E-mail: john.silva@imperial.ac.uk

Members of the unusual long N-terminus, group B (LNB) subfamily of G protein-coupled receptors (GPCRs) are cleaved constitutively into two pieces: N-terminal fragments (NTFs) that resemble cell-adhesion proteins without transmembrane regions (TMRs), and the C-terminal fragments (CTFs) that are typical GPCRs with 7 TMRs. However, the functional role of this cleavage remains unclear. We used latrophilin, a representative of this family, to investigate the cellular processing and molecular organization of LNB-GPCRs. Latrophilin, a neurone-specific receptor for α-latrotoxin (a potent secretagogue from the black widow spider), has been implicated in regulation of transmitter release. We demonstrate that NTF and CTF of latrophilin (and probably all LNB-GPCRs) are delivered to the plasma membrane only after intracellular cleavage. On the cell surface, the two fragments behave as independent membrane proteins; they are targeted to different sites and are recycled separately. Based on their structures and behaviours, NTF and CTF are proposed to function in cell contact and signalling, respectively. However, the two fragments are capable of interacting with each other. α-Latrotoxin binding to NTF promotes this interaction and leads to signalling via CTF to phospholipase C and Ca^{2+} stores. The NTF-CTF interaction depends on the state of CTF phosphorylation and can occur between the fragments of distinct members of the LNB family. These findings define a novel principle of GPCR architecture and introduce a new level of complexity and regulation in the network of signalling pathways.

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C3-050P
The clopidogrel active metabolite disrupts P2Y12 receptor oligomers and partitions them out of lipid rafts

P. Savi, J.-L. Zachayus, N. Delesque-Touchard, C. LabouretT, C. Herve, M.-F. Uzabiaga, J.-M. Pereillo, J.-M. Culouscou, F. Bono, P. Ferrara and J.-M. Herbert
Sanofi-Aventis, Department of Thrombosis and Angiogenesis, Toulouse, France. E-mail: pierre.savi@sanofi-aventis.com

P2Y12, a G-protein coupled receptor that plays a central role in platelet activation has been recently identified as the receptor targeted by the antithrombotic drug, clopidogrel. In this study, we further deciphered the mechanism of action of clopidogrel and of its active metabolite (Act-Met) on P2Y12 receptors. Using biochemical approaches, we demonstrated the existence of homo-oligomeric complexes of P2Y12 receptors at the surface of mammalian cells and in freshly isolated platelets. *In vitro* treatment with Act-Met or *in vivo* oral administration to rats with clopidogrel induced the breakdown of these oligomers into dimeric and monomeric entities in P2Y12 expressing HEK293 and platelets respectively. In addition, we showed the predominant association of P2Y12 oligomers to cell membrane lipid rafts and the partitioning of P2Y12 out of rafts in response to clopidogrel and Act-Met. The raft-associated P2Y12 oligomers represented the functional form of the receptor, as demonstrated by binding and signal transduction studies. Finally, using a series of receptors individually mutated on each cysteine residue and a chimeric P2Y12/P2Y13 receptor, we were able to show that mutation on cysteine 97 rendered P2Y12 fully insensitive to Act-Met activity. Finally, using a series of receptors individually mutated on each cysteine residue and a chimeric P2Y12/P2Y13 receptor, we were able to show that cysteine 97, within the first extracellular loop of P2Y12, was the molecular target of Act-Met.

C3-051P
Ox-LDL and E-LDL differentially regulate ceramide and cholesterol raft microdomain generation in human macrophages

G. Schmitz, M. Grandl, G. Liebisch, A. Boettcher, G. MaaBared, S. Bandulik, K. Szakszon and T. Langmann
Institute of Clinical Chemistry and Laboratory Medicine, University of Regensburg, Regensburg, Germany.
E-mail: gerd.schmitz@klinik.uni-regensburg.de

A hallmark in atherosclerosis is the generation of lipid loaded macrophage foam cells. In order to study the effects of different lipoprotein modifications, monocyte-derived macrophages from apolipoprotein E3 donors were incubated with two types of atherogenic lipoproteins, enzymatically modified LDL (E-LDL) and mildly oxidized LDL (Ox-LDL) followed by subsequent deloading with antiatherogenic HDL3 and apoA-I particles. Total cell analysis using lipidomics, genomics and proteomics was applied to unravel key mechanisms underlying the regulation of cellular lipid influx, efflux, storage and raft formation. The cellular lipid content of the cells was determined with ESI-MS/MS. The surface expression of cholesterol, ceramide and glycosphingolipids and the surface lipid distribution was analyzed by flow cytometry and confocal microscopy using different fluorescent labeled antibodies or toxins (e.g. antibodies against ceramide backbone lipids, theta-toxin, cholera-toxin). We also investigated gene expression profiles of sphingolipid and cholesterol metabolism by Affymetrix microarrays and quantitative TaqMan RT-PCR. We have identified that E-LDL loading predominantly increased the cellular cholesterol content, while Ox-LDL loading preferentially increased the cellular ceramide content. Ox-LDL in comparison to E-LDL also led to a higher cell surface expression of ceramide and glycosphingolipids. Confocal microscopy confirmed an induction of ceramide rafts with Ox-LDL loading while E-LDL generated cholesterol-rich membrane microdomains, which was directly related to changes in gene expression of sphingolipid and sterol metabolism. Using this approach we were also able to demonstrate distinct abnormal regulations of the cellular cholesterol and phospholipid influx/efflux rheostat in cells of three different lipid trafficking disorders: apoE4 homozygosity, ABCA1 deficiency and Niemann-Pick C disease.

C3-052P
Activation of MAPkinase pathway plays an essential role in the sympathetic regulation of inflammatory mediator production in macrophages

J. Szelényi[1], S. E. Vizi[1], Z. Selmeczy[1], A. Brózik[2] and M. Magócsi[2]
[1]*Neuroimmunology, Pharmacology, Institute of Experimental Medicine of the Hungarian Academy of Sciences, Budapest, Hungary,* [2]*Cell Biology, Institute of Hematology and Immunology of the National Medical Centre, Budapest, Hungary.*
E-mail: szelenyi@koki.hu

In this paper we demonstrate that in macrophages, isoproterenol is able to enhance the production of TNF-alpha, IL-12 and NO in combination with PKC activation in contrast to its known decreasing effect in LPS stimulated cells. These contrasting effects were accompanied by parallel changes in activation of ERK1/2 and p38 MAPKs. Thus, isoproterenol significantly increased MAPK activation (phosphorylation) in PMA-treated cells and, conversely, it decreased the activation of ERK1/2 and p38 in LPS-stimulated cells. The opposing effects of isoproterenol on LPS- vs. PMA-induced mediator production and the concurrent changes in MAPK activation highlight the role of this kinase pathway in macrophage activation and provide new insights regarding the flexible ways through which beta-adrenergic receptor stimulation can modulate the inflammatory response in macrophages. This dichotomy offers new viewpoints in the therapeutic approaches of inflammatory and autoimmune diseases, since the beta-adrenergic signal was considered till now only to be immunosuppressive.

C3-053P
Lateral organization of proteins in bacterial photosynthetic membranes

C. Mascle-Allemand[1], V. Prima[1], S. Scheuring[2] and **J. N. Sturgis**[1]
[1]*LISM UPR-9027, Institut de Biologie Structurale et Microbiologie, CNRS, Marseille, France,* [2]*UMR-168 LRC-CEA-34V, Institut Curie, Paris, France. E-mail: sturgis@ibsm.cnrs-mrs.fr*

Recent images that we have obtained of bacterial photosynthetic membranes of *Rhodospirillum photometricum* (Scheuring et al. *PNAS* **31:** 11293–11297; Scheuring et al. *EMBO J.* **23:** 4127–4133) have shown a heterogeneous organization of the different proteins within the bacterial intracytoplasmic membranes. In particular the clustering of core complexes, the formation of peripheral antennae domains and the apparent absence of the cytochrome bc_1, and ATP synthase from large regions of the membrane. We present here a structural and functional analysis of the lateral organization of the membranes. This analysis allows us to understand the overall organization of the membrane and the interplay between different structural and functional perogatives. We propose a role for specific interactions in driving the organization for the photosynthetic apparatus in *Rhodosprillum photometricum*. Generalization of our observations in this bacterium allows us to propose general rules for the development and differentiation of different types of membranes and the rules for the organization of proteins within them.

C3-054P
Biophysical properties of a lipid bilayer containing ceramide: relevance to the biochemical role of ceramide

L. Silva[1], R. F. de Almeida[2], A. Fedorov[1] and M. Prieto[1]
[1]*Centro de Química-Física Molecular, Instituto Superior Técnico, Lisbon, Portugal,* [2]*Centro de Química e Bioquímica, Faculdade Ciencias Universidade de Lisboa, Lisbon, Portugal.*
E-mail: lianacsilva@ist.utl.pt

Ceramide (Cer), a biosynthetic precursor of sphingomyelin that can be also generated by the action of sphingomyelinase in cell membranes, has recently emerged as a key molecule in the modulation of several cellular processes such as apoptosis and stress-signaling cascades, probably by inducing the formation of large raft platforms. Cer is considered to act as a second messenger in various signaling pathways and its action is commonly ascribed to changes in the membrane physical properties after Cer generation. Increase in membrane order, microdomain formation (gel-fluid phase separation) and formation of non-lamellar lipid phases are frequently reported for this lipid. In this work we applied fluorescence methodologies to binary POPC/Cer lipid mixtures containing either trans-parinaric acid (t-PNA) or diphenylhexatriene (DPH) probes. Both anisotropy and mean fluorescence lifetime of t-PnA sharply increase for Cer, reporting the formation of gel phase Cer rich domains. In contrast, the properties of DPH underwent only minor changes with increasing Cer content, showing that this probe is excluded from Cer rich phases, which are, therefore, highly ordered. The variation of t-PnA anisotropy with temperature allowed the determination of the POPC/Cer phase diagram, which is in agreement with a reported one [1]. t-PNA is a suitable probe to report the changes undergone by lipid bilayers in the presence of Cer, and its fluorescence properties are very sensitive to the presence of even low mole fractions of Cer-enriched domains. Fluorescence-resonance energy transfer studies with t-PnA for determination of Cer domain size are underway.

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C3-055P
TrkA-containing oligodendroglial microdomains and NGF signaling

M. Schmitz, S. Klopfleisch, S. Klöppner and H. H. Althaus
Max Planck Institute for Experimental Medicine, Göttingen, Germany. E-mail: Schmitz@em.mpg.de

Previous results have shown that pig oligodendrocytes (OL) express TrkA and respond to NGF [1]. Several reports have indicated, that tyrosine kinase (TK) response might depend on as to whether the receptor is targeted to microdomains. TrkA seems to belong to the groups of receptors, which is constantly present in caveolin-containing microdomains. Hence, we were interested to know as to whether NGF signaling is modulated by microdomain components such as caveolin and cholesterol. Western blotting (WB) revealed the presence of caveolin-1 and -2 whereas caveolin-3 could not be detected. Immunocytochemistry of cultured pig OL demonstrate the co-expression of caveolin and the 140 kDa NGF receptor TrkA. Caveolin containing microdomains were isolated via previously published buoyant density centrifugation methods (+/– Triton X-100) and by using MACS-technology. Western blotting showed a co-labeling of the caveolar protein, flotillin-1, in addition to TrkA, p75 NTR, and p21

Ras. However, by using MACS-technology an OL plasma membrane fraction was obtained, which was extracted by Triton X-100 at 4 °C as well as at 37 °C. Surprisingly, TrkA was not exclusively present in 4 °C Triton X-100 insoluble fraction. Cells exposed to PEG-cholesterol increased their process formation; PEG-cholesterol plus NGF accelerated the NGF response; under both conditions, an in-gel-kinase assay demonstrated an increased MAPK activity, a step of downstream TrkA signaling. Concomitantly, caveolin expression is significantly up-regulated within 48 h of NGF exposure. Yet, it has to be clarified if caveolin up-regulation leads to a functional interaction with TK activity [2]. Exposure to cyclodextrin (1.2 mM), which disrupts caveolar microdomains by removing cholesterol from the plasma membrane, resulted in a less effective NGF response.

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C3-056P
Cell surface pattern of PDGF receptors affects signalling in glioblastoma cells

L. Ujlaky-Nagy, Á. Szöõr, J. Szöllõsi and G. Vereb
Department of Biophysics and Cellbiology, University of Debrecen, Debrecen, Hungary. E-mail: lnagy@jaguar.unideb.hu

PDGF receptors are transmembrane tyrosine kinases that play an important role in the development and proliferation of glial tumors. Key events in their activation are di- and possibly oligomerization followed by trans-phosphorylation and downstream signaling cascades including transient elevation of the intracellular Ca^{2+} concentration. Lipid rafts are special microdomains of the cell surface membrane rich in glicosphyngolipids, cholesterol, GPI-linked proteins, and large number of signal transducing molecules. We have examined the cell surface organization of PDGFR in relation to these lipid rafts and its functional consequences. PDGF receptors α and β were labeled with indirect immunofluorescence. Their spatial arrangement and relationship to lipid rafts decorated with fluorescent choleratoxin B subunits (CTX-B) were determined by confocal laser scanning microscopy. Intracellular calcium levels as a measure of receptor activation in response to PDGF were measured with ratio videomicroscopy. The phosphorylation of receptors was assessed with anti-phosphotyrosine antibody in Western-blot and *in situ* immunofluorescence experiments. The glioblastoma cell lines investigated express primarily the β type PDGF receptors. The number of receptors in the cell membrane, indicated by the relative number of pixels over threshold fluorescence after labeling these receptors appeared to be increasing as cell cultures reached confluence (from 0.05 + 0.01 to 0.32 + 0.07). Receptors showed a non-random, clustered distribution in the cell membrane. The overlap of receptor clusters with CTX-B-labeled lipid rafts was substantial and also depended on the confluence of the cell culture. The cross-correlation coefficient (C) was calculated to statistically characterize the extent of overlap. As confluence of the culture increased, C also increased (from 0.31 + 0.04 to 0.49 + 0.04). Furthermore, receptors showed higher relative phosphorylation in rafts than outside rafts. Cross-linking of the lipid rafts by CTX-B at 37 °C led to the aggregation of lipid rafts and sequestration of PDGFR clusters from them. Reducing the cholesterol content of the cell membrane by methyl-beta-cyclodextrin dispersed lipid rafts and PDGFR clusters and almost completely abolished response to PDGF stimulus. We conclude that PDGFRβ is localized in membrane patches of glioblastoma cells that are rich in cholesterol and GM1 gangliozide and the surface density of the receptor and its overlap with these lipid rafts increases with the confluence of the cell culture. The raft localization of PDGFR has a functional consequence as destroying rafts disperses the receptor clusters and abolishes their physiological response to PDGF stimulus.

C3-057P
Prenatal saline but not morphine exposure alters glucocorticoid receptor binding in the hippocampus of adult rats

I. Vathy[1,2], A. Rimanóczy[1,3], R. Šlamberová[1,4], M. Riley[2] and N. Bar[1]
[1]Laboratory of Developmental Neurobiology, Department of Psychiatry and Behavioral Sciences, Albert Einstein College of Medicine, New York, NY, USA, [2]Department of Neurosciences, Albert Einstein College of Medicine, New York, NY, USA, [3]Department of Psychiatry, Albert Szent-Györgyi Center for Medical and Pharmaceutical Sciences, Faculty of Medicine, University, Szeged, Hungary, [4]Department of Normal, Pathological and Clinical Physiology, 3rd Faculty of Medicine, Charles University, Prague, Czech Republic. E-mail: vathy@aecom.yu.edu

The present study examined the binding characteristics of mineralocorticoid (MR) and glucocorticoid receptors (GR) in young adult male and female rats exposed prenatally (E11–E18) to morphine (10 mg/kg/2×/day), saline or no treatment at all (controls). One day after birth, on postnatal day 1, litters were cross-fostered such that control dams were paired and their litters were crossed; saline- and morphine-treated dams paired and half of each saline litter was crossed with half of each morphine litter. Thus, in each mother (control, saline and morphine) raised half of her own and half of the adopted litter. Animals were adrenalectomized 24 h prior to decapitation. The brains were quickly removed; hippocampus and hypothalamus were dissected and stored at –80 °C until assayed. Saturation binding assays were conducted, the binding site density (B_{max}) and affinity (K_d) were determined. The results demonstrates that prenatal stress due to maternal saline injection increases MR and GR binding in the hippocampus of adult male rats and this effect is prevented by prenatal morphine-exposure. In female rat hippocampus, the MR and GR binding sites are comparable in all three groups. However, there is a significant main effect of ovarian hormones on both MR and GR binding. Diestrous females have less GR and more MR binding sites than proestrous females in the hippocampus. Thus, prenatal morphine exposure sex specifically affects prenatal stress-induced alterations in MR and GR binding in the hippocampus of drug-exposed, young adult animals.

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C3-058P
5-HT receptors and arachidonic acid cascade: pharmacological evidence for functional coupling in the equine digital artery

C. Zizzadoro, M. Caruso, A. Tortora, C. Putignano, C. Belloli and P. Ormas
Laboratory of Veterinary Pharmacology and Toxicology, Department of Animal Health and Welfare, University of Bari, Valenzano (BA), Italy. E-mail: c.zizzadoro@veterinaria.uniba.it

Type 2 5-hydrossytryptamine (5-HT2) receptor coupling to phospholipase A2 (PLA2) activation has been documented in neurons of central nervous system. Moreover evidence exists for a contribution of arachidonic acid (AA) cyclooxygenase (COX)-dependent pathway to the vasomotor response elicited by 5-HT in vascular beds of various animal species. The present work was performed to evaluate the functional role of enzymes involved in

AA cascade in mediating the contractile effect of 5-HT in the equine digital artery (EDA). To this aim, endothelium-denuded rings isolated from digital arteries of healthy horses were mounted in organ baths for isometric tension recording and their responses to cumulative doses of 5-HT were assayed in the presence and absence of 10 μM concentration of different enzyme inhibitors. 5-HT evoked dose-related and predominantly 5-HT2 mediated contractions. Treatments with indomethacin (non selective COX inhibitor) and SC560 (COX-1 selective inhibitor) significantly reduced 5-HT maximum contractile response to a similar extent ($P < 0.01$, $n = 7$; $P < 0.001$, $n = 6$, respectively). Comparable results were observed also following exposure to the non-selective PLA2 inhibitor OBAA ($P < 0.001$, $n = 6$). In contrast, NS398 (COX-2 selective inhibitor; $n = 8$) and NDGA (non-selective lipoxygenase inhibitor; $n = 8$) produced variable effects, resulting in non-significant modifications of agonist efficacy. None of the tested drugs affected 5-HT potency, thus confirming their action being exerted at post-receptor level. These data suggest that the downstream signaling pathways involved in the vascular contractile effects of 5-HT in the normal EDA likely include 5-HT2 receptor-mediated activation of PLA2 and COX-1-dependent production of vasoconstrictor arachidonate metabolites.

C4–Lipid-protein Interactions in Membrane

C4-001
The ways peptide antibiotics can kill bacteria by interacting with specific lipids

B. de Kruijff
Department Biochemistry of Membranes, Utrecht University, Utrecht, the Netherlands. E-mail: b.dekruijff@chem.uu.nl

In this lecture the mode of membrane action of lantibiotics will be described. Lantibiotics are polypeptides that kill bacteria. They contain special ring structures that are closed by lanthionine residues. One well-studied lantibiotic is nisin that kills bacteria by targeted pore formation using Lipid II as receptor [1]. Lipid II consists of a bactoprenol chain that is linked via a pyrophosphate to a disaccharide containing a pentapeptide. The latter moiety is the building block for the peptidoglycan synthesis. The N-terminal part of nisin docks on the pyrophosphate unit of Lipid II [2] after which nisin becomes inserted into the membrane and assembles together with Lipid II in a transmembrane pore complex consisting of 8 nisin and 4 Lipid II molecules. Surprisingly, nisin variants and related lantibiotics that effectively kill bacteria and specifically dock on Lipid II do not form pores in the membrane and thus must use another mechanism to kill bacteria. We discovered by fluorescence microscopy that they remove Lipid II in the bacterial membranes from its functional location (septum, spiral zones) to other places in the membrane thereby inhibiting cell growth and division. These studies reveal that the lantibiotics are promising candidates for the development of new antibiotics that are highly needed with the alarming rise of drug resistant bacteria.

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C4-002
The role of the PI4P- and ARF-binding FAPPs at the Golgi complex

M. A. De Matteis[1], A. Godi[1], A. Di Campli[1], G. D'Angelo[1], R. Polishchuk[2], E. Polishchuk[2], D. Alessi[3], I. Ventura[4], J. Saus[4], F. Revert[4] and J. Lucocq[5]
[1]*Physiopathology of Secretion, Cell Biology and Oncology, Consorzio Mario Negri Sud, Santa Maria Imbaro (Chieti), Italy,* [2]*Membrane Sorting and Biogenesis, Cell Biology and Oncology, Consorzio Mario Negri Sud, Santa Maria Imbaro (Chieti), Italy,* [3]*Division of Cell Signalling, University of Dundee, Dundee, UK,* [4]*Molecular Pathology, Instituto de Investigaciones Citologicas, Valencia, Spain,* [5]*MSI/WTB Complex, University of Dundee, Dundee, UK. E-mail: dematteis@negrisud.it*

The Golgi complex is the main sorting station of the secretory pathway, where neosythesized proteins are targeted to their final destinations. Among the different exit pathways from the Golgi complex, the one directed to the plasma membrane (PM) is the least defined in terms of the molecular machineries involved. Nevertheless, the relevance of a lipid-based machinery in this transport step is well established both in yeast and mammals. This includes phosphatidylinositol 4-phosphate (PI4P), that as well as being the precursor for PI45P2, has a direct role in membrane trafficking. In mammals, the effectors of PI4P in the transport from the trans-Golgi network (TGN) to the PM are not known. Here, we report the identification of two of these, the four-phosphate-adaptor proteins 1 and 2 (FAPP1 and FAPP2). Both of these localize at specific domains of the TGN where the carriers destined to the PM emerge as tubular protrusions. FAPPs are recruited to these sites due to the dual interactions of their PH domain with PI4P and the small GTPase ARF. Displacement or knockdown of FAPPs inhibits cargo transfer to the PM. In polarized cells, FAPP-PH interferes with the generation of carriers directed to the basolateral (but not to the apical) PM. While the final mechanisms of action of FAPPs remain to be defined, it is interesting to note that FAPPs are members of a large family of proteins, which includes CERT and OSBP1, that are endowed with a PH domain that binds PI4P and is targeted to the Golgi complex. Here, we show that both CERT and OSBP1 localize to the TGN in an ARF-dependent way. We are currently investigating whether the different members of the FAPP family control different routes of transport out of the TGN.

C4-003
Why does nature form a two-dimensional membrane protein crystal?

D. Oesterhelt
Max Planck Institute of Biochemistry, Martinsried, Germany. E-mail: oesterhe@biochem.mpg.de

At few instances two-dimensional protein crystals occur naturally and *in vivo*. One spectacular example is the two-dimensional crystalline array of bacteriorhodopsin and lipids in the purple membrane covering up to 80% of the cell surface of halo-philic archaea upon induction by shortage of oxygen and the presence of light. By neutron diffraction we could localize glycolipid molecules at specific positions of the lipid lattice and by X-ray crystallography elucidate the atomar details of the glyco-lipid protein interaction. Subsequently all lipid-protein contacts were changed by point mutations one by one. Only the mutation at position 80 exchanging a tryptophan for an isoleucine would eliminate the lattice formation in the living cell. This allowed to address the question why the cell forms a 2D crystal. No differences in proton pumping efficiency of bacteriorhodopsin nor in the capacity for photo-phosphorylation mediating phototrophic growth of the

cell could be detected, but photosynthetic growth itself was prevented in the mutant cells unable to form the crystalline array. It could be established that this incapacity is due to the formation of a photochemical side product of the photochemical cycle of the molecule driving proton translocation. Instead of a continuous cycle between all-trans and 13-cis retinal molecules the non-crystalline form occasionally produces a 9-cis retinal, which does not fit the binding site and thereby inactivates the protein. Thus, on the long range of hours or days, as typical for generation times of these archaea in their natural habitats of salines and salt ponds, photosynthesis ceases. Thus, the crystalline array stabilizes the protein molecule in such a way that the photochemical precision is optimized and allows for a long time sunlight exposure without any photochemical inactivating side product.

C4-004
Computer simulations of lipids and transmembrane helices

D. P. Tieleman
Department of Biological Sciences, University of Calgary, Calgary, AB Canada. E-mail: tieleman@ucalgary.ca

Molecular dynamics simulations provide a computational approach to studying lipid–protein interactions in atomistic deal. With current computers and software, models containing hundreds of lipids can be simulated for hundreds of nanoseconds, bringing a range of biochemical problems within reach. We are developing a library of lipid structures for systematic investigations of the effect of the type of lipid on the structure and dynamics of membrane proteins. We are also working on a number of technical problems, including the choice of simulation parameters and boundary conditions. To validate our models, we are investigating a number of well-characterized transmembrane helices to understand what drives their aggregation behavior. MS1 is a designed membrane version of the GCN4-P1 leucine zipper. In simulations with up to 36, initially monomeric, peptides, MS1 rapidly aggregates into dimers and trimers with well-defined structures. WALP23 is a designed peptide that forms dimers under certain conditions. Molecular models help narrow down possible modes of interaction to a few that can be tested experimentally. In the last example, we are combining x-ray diffraction with molecular dynamics simulations to understand the partitioning of the antimicrobial peptide alamethicin.

C4-005
Uptake of beta-galactosidase mediated by the cell penetrating peptide pep-1 into large unilamellar vesicles and HeLa cells is driven by membrane potential

S. T. Henriques[1], J. Costa[2] and M. A. Castanho[1]
[1]Laboratory of Molecular Biophysics, Centro de Química e Bioquímica, Universidade de Lisboa, Lisbon, Portugal, [2]Laboratory of Glicobiology, Instituto de Tecnologic Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal.
E-mail: sthenriques@fc.ul.pt

The cell-penetrating peptide (CPP) pep-1 (Ac-KETWWETWWTEWSQPKKKRKV-cysteamine) is capable of introducing large proteins into different cell lines, maintaining their biological activity. Two possible mechanisms have been proposed to explain the entrance of other CPPs in cells, endosomal-dependent and independent. In the present work, we evaluated the molecular mechanisms of pep-1-mediated cellular uptake of beta-galactosidase (beta-Gal) from *E. coli*, in large unilamellar vesicles (LUV) and HeLa cells. Fluorescence spectroscopy was used to conclude on the biophysical foundations of the translocation process in model systems (LUV). Immunofluorescence microscopy was used to study the translocation in HeLa Cells. Enzymatic activity detection enabled monitoring the internalization of beta-Gal into LUV and the functionality of the protein in the interior of HeLa cells. Beta-Gal translocated into LUV in a transmembrane potential-dependent manner. Likewise, beta-Gal incorporation was extensively decreased in depolarized cells. Furthermore, beta-Gal uptake efficiency and kinetics were temperature-independent and beta-Gal did not co-localize with endosomes, lysosomes or caveosomes. Therefore, beta-Gal translocation was not associated with the endosomal pathway. Although, an excess of pep-1 was mandatory for beta-Gal translocation *in vivo*, transmembrane pores were not formed as concluded from the trypan blue exclusion method. These results altogether indicated that the protein uptake both *in vitro* with LUV and *in vivo* with HeLa cells was mainly, if not solely, dependent on negative transmembrane potential across the bilayer, which suggests a physical mechanism governed by electrostatic interactions between pep-1 (positively-charged) and membranes (negatively-charged).

C4-006
Requirement of phosphatidylethanolamine for the normal structure and activity of the multidrug transporter LmrP

B. Gbaguidi, J.-M. Ruysschaert and C. Vigano
Center for Structural Biology and Bioinformatics, Free University of Brussels, Brussels, Belgium. E-mail: cvigano@ulb.ac.be

LmrP is a *Lactococcus lactis* 45-kDa membrane protein that confers resistance to a wide variety of lipophilic compounds by acting as a proton motive force-driven efflux pump. Measurements of tryptophan fluorescence attenuation by acrylamide demonstrate that LmrP undergoes conformational changes related to the transport of substrates. When LmrP is reconstituted into liposomes made from *E. coli*/phosphatidylcholine lipids, the proton motive force alters the accessibility of the cytosolic loops towards the water phase. Photolabelling experiments with radioactive tetracycline demonstrate that this structural reorganization increases the affinity of LmrP for its substrates. Ligand binding mediates a subsequent conformational change in the cytosolic loops, which requires the proton motive force-mediated restructuring. This drug binding-mediated reorganization may be related to the transition between a high- and low-affinity drug binding site necessary for drug release into the extracellular medium. In the absence of phosphatidylethanolamine in the membrane, these conformational changes are inhibited. Attenuated Total Reflection Fourier Transform Infrared analysis demonstrates that the structure of LmrP is indeed modified when phosphatidylethanolamine is absent in the membrane. These data suggest that phosphatidylethanolamine is essential for the structure and activity of LmrP.

C4-007P
Studying of the N acylethanolamines binding to serum albumin

M. Artamonov[1], O. Zhukov[1], O. Savchuk[1], O. Yakovenko[2], S. Yarmolyuk[2] and N. M. Gula[1]
[1]Department of Lipid Biochemistry, Palladin Institute of Biochemistry, Kiev, Ukraine, [2]Department of Combinatorial Chemistry, Institute of Molecular Biology and Genetics, Kiev, Ukraine.
E-mail: artamon@biochem.kiev.ua

N-acylethanolamines (NAEs) are minor lipids, which have high biological activity. It is known that the blood circulation integrates metabolism. Based on wide distribution of exogenous NAEs

in the organism one can assume the existence of NAE transport in blood circulation. For the moment, nothing is known about such transport. The aim of study was: to estimate the probability of NAEs binding to human serum albumin by means of computer modelling of HSA-NAE18:0 complex; to investigate the binding of radiolabelled NAE16:0 to proteins containing in rat serum; to study of complex formation between HSA and N-palmitoylethanolamine (NAE16:0), N-stearoylethanolamine (NAE18:0), N-oleoylethanolamine (NAE18:1n9) and N-arachidonoylethanolamine (NAE20:4n6) by mass spectrometry and fluorimetry. It was shown that [9,10 3H] NAE16:0 was easily assimilated and found in the blood in 20 min after *per os* feeding of rats. The isolation of albumin by affinity chromatography followed by measurements of isolated fraction radioactivity showed that radioactivity was associated with albumin fraction. The measurement of intrinsic Trp-fluorescence of HSA showed NAE16:0, NAE18:0, NAE18:1n9 and NAE20:4n6 caused the quenching of fluorescence. In addition the NAE20:4n6 induced the small fluorescence shift, possibly due to four double bounds in the NAE20:4n6 molecule. MALDI-MS data show molecular weight shift between pure albumin and NAE-HSA complexes. Computer modelling was made by Dock and Autodock programmes. We found five sites on an albumin molecule, which were able to bind five NAE18:0 molecules. The principal site of the binding was localized near the Trp-214 residue and the energy of NAE18:0-HSA binding has following values: – 8.47 kcal/mol. The molecular dynamic simulations session by GROMACS program was performed for this complex. The results showed the significant complex stability without sizeable changes of conformation and pointed at junctions between NAE18:0 and HSA molecule. The potential energy of this complex was essentially lower than that for NAE18:0 molecules in water box.

Conclusions: Our findings allow to suppose that namely albumins are one of the most probable transport form for saturated NAEs in an organism.

C4-008P
Lipid-interacting amphipathic alpha helix mediates monotopic membrane interaction of a viral replication complex

T. Ahola[1], P. Spuul[1], A. Lampio[1], A. Merits[2] and L. Kaariainen[1]
[1]*Institute of Biotechnology, University of Helsinki, Helsinki, Finland,* [2]*Estonian Biocentre, Tartu, Estonia.*
E-mail: tero.ahola@helsinki.fi

The replication complexes of all positive-strand RNA viruses are associated with cellular membrane structures. The replication complex of Semliki Forest virus consists of four virus-encoded components, the nonstructural proteins nsP1-nsP4. NsP1 (537 aa) is the only replicase component with affinity for membranes. Membrane binding of nsP1 is mediated in a monotopic fashion by a 20 aa peptide located in the middle of the protein. The NMR structure of the peptide showed an amphipathic alpha helix with hydrophilic (positively charged) and hydrophobic surfaces (*J Biol Chem* **275**: 37853–37859). The peptide by itself was capable of binding liposomes containing anionic phospholipids, such as phosphatidylserine, and the peptide also competed with the full-length protein in liposome binding assays. Mutational analysis of the peptide confirmed that certain hydrophobic and positively charged residues within it are essential in attaching nsP1 to membranes and that the same residues are also necessary for viral RNA replication. One of the mutations changed two consecutive leucine residues into alanines. The virus containing this mutation was incapable of producing progeny as such, but reproducibly gave rise to a compensatory mutation, which changed the second alanine to valine, thus increasing the hydrophobic nature of the peptide. The virus with an ala-val dipeptide at this location resembled the wild type in its replication.

C4-009P
"Kiss and run" mechanism of transmitter release at the cold-blooded neuromuscular junction

M. M. Abdrakhmanov and P. N. Grigoryev
Department of Normal Physiology, Kazan State Medical University, Kazan, Russian Federation. E-mail: mmaratt1980@list.ru

The processes of exo- and endocytosis of synaptic vesicles have been studied at the frog motor nerve terminals by using optical and electrophysiological techniques for extracellular recording. The extracellular increase either of K^+ (40 mmol/l) or adding of sucrose (30 mmol/l) produced roughly the same increase of frequency of the miniature end-plate potentials. After enhancing of quantal release by elevating of the K^+ concentration at the presence styryl fluorescent dye (FM1-43) in nerve terminals the light spots – clusters of vesicles that past exo-endocytotic cycle – were observed. After enhance of quantal release by elevating of K concentration fluorescent spots disappeared, but remained at the presence of sucrose solution. In the case of stimulation of quantal release by sucrose (30 mmol/l) there were no spots. All these observations suggest that synaptic vesicles at the frog neuromuscular junction can be released by the full exocytosic cycle with the following endocytosis or/and by «kiss and run» mechanism (temporary pore formation).

C4-010P
Fluorescence confocal microscopy: Direct visualization of the lytic action of antibiotic peptides

E. E. Ambroggio[1], F. Separovic[2], J. Bowie[3], G. D. Fidelio[1] and L. A. Bagatolli[4]
[1]*Biofísica, Química Biológica-CIQUIBIC-CONICET, UNC-Facultad de Ciencias Químicas, Córdoba, Córdoba Argentina,* [2]*School of Chemistry, University of Melbourne, Melbourne, Australia,* [3]*Department of Chemistry, University of Adelaide, Adelaide, Australia,* [4]*MEMPHYS – Center for Biomembrane Physics, Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark.*
E-mail: ernesto@dqb.fcq.unc.edu.ar

Membrane lysis caused by antibiotic peptides is often rationalized by means of two different models: the so-called carpet model and the pore-forming model. We report the lytic activity of antibiotic peptides from Australian tree frogs: Maculatin 1.1, Citropin 1.1 and Aurein 1.2, on palmitoyloleoylposphatidylcholine (POPC) or POPC/POPG (phosphatidylglycerol) model membranes. Leakage experiments using fluorescence spectroscopy indicated that the peptide to lipid mole ratio necessary to induce a 50% of probe leakage was smaller for Maculatin compared with Aurein or Citropin, regardless of lipid membrane composition. To gain further insight into the lytic mechanism of these peptides we performed single vesicle experiments using confocal fluorescence microscopy. In these experiments, the time-course of leakage for different molecular weight (water soluble) fluorescent markers incorporated inside single giant unilamellar vesicles is observed after peptide exposure. We conclude that Maculatin and its related peptides demonstrate a pore-forming mechanism (differential leakage of small fluorescent probe compared with high molecular weight marker). Conversely, Citropin and Aurein provoke a total

membrane destabilization with vesicle burst without sequential probe leakage, an effect that can be assigned to a carpeting mechanism of lytic action. Additionally, in order to study the relevance of the proline residue on the membrane-action properties of Maculatin, the same experimental approach was used for Maculatin-Ala and Maculatin-Gly (Pro-15 was replaced by Ala or Gly, respectively). Although a similar peptide to lipid mole ratio was necessary to induce 50% of leakage for POPC membranes, the lytic activity of Maculatin-Ala and Maculatin-Gly decreased in POPC/POPG (1:1 mol) membranes compared with that observed for the naturally occurring Maculatin sequence. As observed for Maculatin, the lytic action of Maculatin-Ala and Maculatin-Gly is in keeping with the formation of pore-like structures at the membrane independently of lipid composition.

C4-011P
Cooperative adsorption of actin on syringomycin E modified planar lipid bilayers

A. N. Bessonov[1], L. V. Schagina[1], I. M. Kuznetsova[1], J. Y. Takemoto[2], K. K. Turoverov[1] and V. V. Malev[1]
[1]*Institute of Cytology RAS, Saint Petersburg, Russian Federation,* [2]*Utah State University, Logan, UT, USA.*
E-mail: abessonov@mail.cytspb.rssi.ru

Addition of G- or F-actin to the trans-side of bilayer lipid membranes (BLMs), modified by syringomycin E (SRE) from the cis-side, induces a significant increase in transmembrane current due to SRE channel-forming activity. The effect follows actin adsorption on BLMs that mainly results from hydrophobic interactions. A similar effect is observed with amphipathic Konig's polyanion (KP) [Gurnev et al. 2003, 2004, *Biol Membrany*; Bessonov et al. 2004, *Tsitologiia* (in Russian)]. The mechanism of the actin-induced increase in SRE channel-forming activity was unknown. Hydrophobic interactions cause the immersion of actin into BLMs to alter the local structure of the cis-monolayer. This immersion increases the affinity of the cis-monolayer to SRE-channel precursors since no changes occur in the energy barrier of SRE-channel closure at the transition from actin-free to actin-equilibrated systems. The dependence of SRE-channel number on the concentration of adsorbing species gives an S-shaped curve indicating cooperative actin adsorption. Analysis of the kinetics of SRE-channel number growth leads to the conclusion that actin molecules form aggregates (''spots'') on the membrane surface and that increasing the concentration of adsorbing molecules increases the radii of the ''spots'' rather than their number. It is hypothesized that SRE-channels are formed within the area of the cis-monolayer regions adjacent to ''spots'' thus explaining the side specificity of actin and KP effect on SRE channel-forming activity.

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C4-012P
Fine-tuning nicotinic receptor function through the lipid bilayer

J. E. Baenziger, C. J. B. da Costa and M. Goodreid
Biochemistry, Microbiology, and Immunology, Ottawa, Ontario Canada. E-mail: jebaenz@uottawa.ca

The ability of the nicotinic acetylcholine receptor (nAChR) to bind agonist and undergo agonist induced conformational change is sensitive to the surrounding lipid environment. In reconstituted membranes composed of phosphatidylcholine (PC), the nAChR adopts a desensitized-like state. Increasing levels of either cholesterol (Chol) or phosphatidic acid (PA) increasingly stabilizes the nAChR in a functional resting state, although PA is particularly effective in this regard. The inclusion of anionic lipids, such as PS, cardiolipin, and PI, does not shift nAChR conformational equilibrium towards the resting state, but PG (the closest structural analog to PA) is slightly effective. In all cases, there appears to be a link between the ability of lipid environment to stabilize a functional nAChR and a slowing of nAChR internal dynamics, as assessed by peptide hydrogen exchange kinetics. The internal dynamics of the nAChR are not sensitive to the physical properties of the membrane as both saturated PC and saturated PC/PA membranes are highly ordered and but stabilize a desensitized-like state that undergoes relatively rapid hydrogen exchange kinetics. Surprisingly, incorporation of the nAChR into membranes composed of anionic lipids has a dramatic effect on the physical properties of the lipid bilayer. Incorporation into PC/PA membranes leads to an increase in lateral packing density and gel to liquid crystal phase transition temperature of the bilayer, an effect suggestive of raft formation. In PC/PS, the nAChR alters the PS head group conformation as a result of concentrating divalent cations at the bilayer surface. Our results show that there are complex bi-directional interactions between the nAChR and its lipid environment. These interactions could play a significant role in fine-tuning post-synaptic membrane function.

C4-013P
Vesicle affinity capillary electrophoresis: a model system for the study of protein–lipid and drug-protein interactions

E. D. G. Breyer[1], K. Koticha[1], Y. Jia[1], W. V. Brown[2] and J. Strasters[3]
[1]*Department of Chemistry, Georgia State University, Atlanta, GA, USA,* [2]*VA Medical Center, Emory University, Atlanta, GA, USA,* [3]*Elan Corporation, King of Prussia, PA, USA.*
E-mail: ebreyer@gsu.edu

The value of VCE for structure–function studies and drug design for small drugs, peptides and proteins that are hydrophobic and/or strongly bound to lipids has been illustrated. VCE technique was designed to assess the effect of physico-chemical properties of apolipoprotein (apo) on the binding to lipoproteins, under physiological conditions (phosphate-saline buffer system at pH 7.4 and 37 °C) using vesicle as a model. Apolipoprotein AIV, CIII and their mutants binding properties to lipids have been evaluated using VCE. VCE is also applied to establish both specific and non-specific binding interaction between different drug analogs and the Paramyxoviridae family of viruses. These drug analogs are potential target for development of antiviral agents that would prevent or treat infection by the measles virus (MV) and other members of the paramyxovirus family. A non-competitive binding model is used to determine the multiple binding properties of antiviral agents to vesicle to study non-specific binding. Specific binding is investigated using two drug analogs to measles virus at various dilutions. These VCE binding constants are temperature and time dependent. The vesicles and measles virus were found to be stable under different temperature. The migration behavior of the different ligands (drugs or peptides) are reported in terms of relative mobility in order to correct for variability in viscosity at different vesicle (or virus) concentration. VCE was used to calculate both binding constants and the number of bound drugs to virus and apolipoproteins to lipoprotein-mimics.

C4-014P
Biophysical investigations of membrane polypeptides by solid-state NMR spectroscopy: Structure, dynamics, aggregation and topology

C. Aisenbrey and **B. Bechinger**
Laboratoire de RMN et Biophysique des Membranes, Faculté/ Institut de Chimie, University Louis Pasteur, Strasbourg, France. E-mail: bechinger@chimie.u-strasbg.fr

Solid-state NMR spectroscopy has a proven record during the investigation of the structure and dynamics of membrane-associated polypeptides. In particular, from oriented samples considerable details on the structure, dynamics and topology of the protein is obtained. The preparation of polypeptides labeled at single sites allows for their detailed structural investigation, even when considerable mosaicity, or conformational and topological equilibria do not allow the acquisition of multidimensional high-resolution solid-state NMR spectra. Here a solid-state NMR approach, which allows for the accurate determination of the tilt and rotational pitch angles of peptides reconstituted into uniaxially oriented membranes will be presented. The method works with transmembrane or in-plane oriented peptides that have been labeled with 3,3,3-2H3-alanine and 15N-leucine at two selected sites. Proton-decoupled 15N and 2H solid-state NMR spectroscopy at sample orientations of the membrane normal parallel to the magnetic field direction have been used to characterize the tilt and rotational pitch angle of several peptides in considerable detail. When many sample conditions have to be tested, e.g. in order to monitor the pH-dependent realignment of membrane-bound DNA transfectants, oriented ATR-FTIR measurements can provide very valuable complementary information. When the same samples are inserted into the magnetic field at a 90° tilted alignment, valuable information is obtained on the rotational diffusion constants in membranes. This parameter is directly correlated to the association and size of peptide complexes within the membrane environments. Whereas monomeric transmembrane peptides exhibit spectral averaging and well-defined resonances, larger complexes are characterized by broad spectral line shapes. In particular the deuterium line shape is sensitive to association of a few transmembrane helices. In contrast, the formation of much larger complexes affects the 15N chemical shift spectrum. Applications to some of the biological systems investigated by us will be presented. These include antibiotic peptides, viral or fungal channels, signal sequences, DNA transfectants, viral inhibitors of the intracellular TAP transporter, Alzheimer fibrils, colicins and proteins of the Bcl-family.

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C4-015P
Surfactant protein A interaction with the amphipatic peptide (KL4)4K inserted into two different surfactant-like liposomes

O. Cañadas, A. Saenz and C. Casals
Laboratory of Pulmonary Surfactant, Department of Biochemistry and Molecular Biology I, Complutense University of Madrid, Madrid, Spain. E-mail: olga@bbm1.ucm.es

Pulmonary surfactant, a lipid–protein complex that covers the alveolar surface, prevents alveolar collapse and contributes to lung defense. Alteration or deficiency of this system leads to respiratory distress syndrome (RDS). The 21-residue peptide KLLLLKLLLLKLLLLKLLLLK, (KL4)4K, has been clinically tested as a therapeutic agent for RDS. Surfactant protein A (SP-A), the most abundant lipoprotein of pulmonary surfactant, is implicated in multiple biological functions, including improvement of the biophysical activity of lung surfactant and host defense. Given the capability of SP-A to interact with bilayer surfaces, we explored the interaction of this protein with (KL4)4K-liposomes and analyzed SP-A's effects on the physical properties and surface activity of such liposomes. To that end we used dipalmitoylphosphatidylcholine/1-palmitoyl-2-oleoyl-phosphatidylglycerol/palmitic acid (DPPC/POPG/PA) and DPPC/1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC)/PA (28:9:5.6, w/w) vesicles, containing different concentrations of (KL4)4K. From steady-state emission anisotropy measurements of 1,6-diphenyl-1,3,5-hexatriene (DPH) embedded in both types of (KL4)4K-containing membranes, differential scanning calorimetry, vesicle aggregation assays, and interfacial adsorption studies in the absence and presence of SP-A, we conclude that SP-A interacts with (KL4)4K incorporated into both types of vesicles. This interaction is much stronger when (KL4)4K is inserted in DPPC/POPG/PA membranes, probably due to the outward location of the peptide in POPG-doped vesicles. Our study suggests that, at a SP-A/(KL4)4K weight ratio of 3.7, the peptide is withdrawn from DPPC/POPG/PA but not from DPPC/POPC/PA membranes as a consequence of SP-A/(KL4)4K interaction. These results are relevant for combined use of recombinant human SP-A and (KL4)4K-liposomes in RDS therapies.

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C4-016P
Characterization of β-amyloid(1-40) incorporated in model membranes: peptide conformation, acyl chain order, vesicle morphology, supported bilayer topography, and channel activity

M. R. de Planque[1], S. A. Contera[2], G. P. Mendes[1,2], D. T. Rijkers[3], J. F. Ryan[2] and A. Watts[1]
[1]Biomembrane Structure Unit, Department of Biochemistry, University of Oxford, Oxford, UK, [2]Bionanotechnology IRC, Department of Physics, University of Oxford, Oxford, UK, [3]Department of Medicinal Chemistry, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands. E-mail: maurits.deplanque@bioch.ox.ac.uk

The β-amyloid peptides Aβ(1–40) and Aβ(1–42) are abundant components of the fibrillar senile plaques associated with Alzheimer's disease, a neurodegenerative disorder of which the toxic mechanism remains to be elucidated. Non-fibrillar soluble Aβ

peptides have been shown to associate with (neuronal) cell membranes, leading either to surface-catalyzed aggregation or to membrane incorporation of the Aβ peptides, depending on the membrane composition. Membrane-incorporated Aβ peptides can fatally disrupt cellular homeostasis, either through the formation of discrete ion channels or pores, or through an indiscrete degradation of bilayer integrity, most likely depending on the oligomeric state of the Aβ peptides prior to membrane adsorption. Aβ-containing membranes have been investigated with a large number of biophysical techniques, but because of the variety in employed lipid compositions and Aβ incorporation protocols, these different methods do not necessarily yield complementary information. Therefore, we have characterized various aspects of Aβ(1–40)-containing model membranes while keeping the membranes as similar as experimentally feasible. Aβ(1–40) was incorporated by hydration of a mixed peptide-lipid film in order to control the effective concentration of Aβ in the bilayers. The conformation of Aβ(1–40) incorporated in unilamellar vesicles was studied by circular dichroism. Subsequently, these vesicles were either fused with a planar bilayer to record channel or channel-like activity, or were fused together on a mica support for AFM imaging of the bilayer topography. Additionally, multilamellar vesicles of similar lipid composition were used to characterize the effect of Aβ(1–40) on lipid acyl chain order and on vesicle morphology by solid-state ^{2}H and ^{31}P NMR.

C4-017P
The role of Islet Amyloid PolyPeptide (IAPP) – membrane interactions in type 2 diabetes mellitus

M. F. M. Engel[1,2], B. de Kruijff[2], J. W. M. Höppener[1] and J. A. Killian[2]
[1]*Department of Metabolic and Endocrine Diseases, University Medical Centre Utrecht, Utrecht, The Netherlands,* [2]*Department Biochemistry of Membranes, Utrecht University, Utrecht, The Netherlands. E-mail: m.f.m.engel@chem.uu.nl*

Amyloid fibril formation from the 37 amino acids human Islet Amyloid Polypeptide (hIAPP) is thought to be responsible for pancreatic islet β-cell death in patients with type 2 diabetes mellitus [1]. hIAPP is produced by the islet β-cells, and overproduced in states of insulin resistance, promoting its aggregation and fibril formation. It has recently been proposed that during hIAPP amyloid formation, lipid uptake from the cell membrane is responsible for hIAPP-induced membrane damage and subsequent β-cell death [2]. Here, we study the interaction of hIAPP with different types of model membranes. hIAPP strongly interacts with lipid monolayers, and even inserts in lipid monolayers at initial surface pressures above 35 mN/m, indicating that hIAPP might penetrate biological cell membranes *in vivo*. Vesicle leakage assays show that hIAPP-induced membrane disruption is surprisingly fast and seems to occur before hIAPP fibrils are detected. The presence of anionic lipids increases IAPP-membrane interactions. No vesicle disruption is observed when preformed hIAPP fibrils, or the non-amyloidogenic mouse IAPP are used. The obtained results further the understanding of the role of hIAPP-membrane interactions in amyloid-induced β-cell death, and may contribute to the development of novel therapeutic agents for type 2 diabetes mellitus.

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C4-018P
Folding and stability of aquaporin PM28A in lipid bilayer vesicles

H. E. Findlay and P. J. Booth
School of Medical Sciences, Department of Biochemistry, University of Bristol, Bristol, UK. E-mail: h.e.findlay@bristol.ac.uk

Aquaporins are small integral membrane proteins composed of six transmembrane α-helices with a common topology, that transport either water alone or water and small solutes across the lipid membrane. The spinach protein PM28A is a major aquaporin in the leaf plasma membrane, comprising around 10% of the total membrane protein. The study of membrane proteins *in vitro* remains challenging, partly due to problems encountered when working outside the native lipid environment. Maintaining protein stability and function can be difficult, therefore establishing optimal conditions is essential. We here investigate the unfolding of purified recombinant PM28A from detergent micelles and its subsequent refolding and reconstitution into different lipid systems. The effect of liposome composition on folding and activity are examined.

C4-019P
The endocytosis processes at the frog neuromuscular junction are initiated by calcium ions

P. N. Grigoryev and M. M. Abdrakhmanov
Department of Normal Physiology, Kazan State Medical University, Kazan, Russian Federation. E-mail: grigorievp@yahoo.com

As well known, Ca ions plays critical role in many neuronal processes, including the regulation and modulation of synaptic vesicles events, however influence at the enocytosis is less known. Processes of endocytosis have been studied at the frog neuromuscular terminals by using optical and electrophysiological techniques for extracellular recording. BAPTA and EGTA were introduced by incubating the cells in 50 mkmol of the AM-ester form of the drug dissolved in the normal external solutions. Electrophysiologically extracellular isoosmotic increasing of K ions resulted in enhancing of quantal release to 7–12 Hz, moreover frequency of transmitter release in norm was 0.5–0.7 Hz. After preliminary incubation at the BAPTA-AM, EGTA-AM K ions increased the exocytosis too. In normal Ringer solutions after enhancing of quantal release at the presence of styryl fluorestent dye (FM1–43) in nerve terminals the light spots – cluster of vesicles that past exo-endocytotic cycle – were observed, similar results for EGTA-AM action took place. Lack of light spots when using another chelator – Bapta-AM (similar-affinity but possessing is more rapid of the time constant for Ca binding than EGTA-AM) or blocker of Ca-channel – Cd ions – were observed. BAPTA-AM, EGTA-AM, and Cd ions did not prevent from disappearing of the fluorescent spots in the case of enhance of quantal release at the staining synapses. All these observations suggest the existence of intracellular Ca-sensitive site of endocytosis that was located of more distance from Ca channel, than exocytosis site was.

C4-020P
The intracellular parasite *Toxoplasma gondii* secretes a soluble phosphatidylserine decarboxylase

N. Gupta and D. R. Voelker
K601, Medicine, National Jewish Medical and Research Center, Denver, CO, USA. E-mail: guptan@njc.org

Toxoplasma gondii is an obligate intracellular protozoan parasite capable of causing fatal infections in immunocompromised

individuals and in developing fetuses. Our research focuses on multiple aspects of phospholipid metabolism of this parasite. Examination of PtdSer metabolism reveals that *T. gondii* secretes a soluble PtdSer decarboxylase (PSD). The free parasite, incubated in intracellular-type medium, secretes a PSD that decarboxylates exogenous liposomal PtdSer to PtdEtn as detected by 14CO2-trapping assay and thin layer chromatography. The enzyme activity remained in the supernatant after centrifuging the medium from parasite cultures at 150 000 ***g*** for 45 min. Quantitatively, axenic *T. gondii* can secrete up to 20% of its PSD pool in 2 h at 37 °C. At 4 °C, the secretion is inhibited by ~90% and the depletion of parasite ATP causes ~65% decrease in the process. The presence of intracellular calcium chelator BAPTA-AM can partially (~40%) inhibit the PSD secretion suggesting calcium dependence of the process. Exogenous PtdSer has no effect on the process indicating that this lipid does not induce the secretion of PSD. These findings demonstrate extremely novel feature of the parasite enzyme since neither soluble nor secreted forms of PSD have been previously described for any organism.

C4-021P
Improvement and immobilization of membrane bound glycohydrolase through co-solvent system

M. Habibi-Rezaei and S. Balazadeh
Biotechnology, Biology Department, University of Tehran, Tehran, Iran. E-mail: mhabibi@khayam.ut.ac.ir

Water content can influence protein structure and therefore function. In recent years there has been much interest in the use of water soluble enzymes in organic solvents. For most instances low moisture system results in increasing the solubility of lipophilic substrates, enantioselectivity and regioselectivity of the catalysis, protein stability, and provide us unusual catalytic properties. Here we report different approach on non-aqueous enzymology in which membrane bound enzyme in its natural phospholipid microenvironment is used in low moisture solvent to catalyze glycohydrolytic reaction. In this study we introduce application of low water content solvent as co-solvent to make shift in optimum pH, and improve enzyme activity. In addition the hydrophobic immobilization of such structures and further stabilization of glycohydrolytic enzyme have been included.

C4-022P
Study of the electrostatic interaction between charged lipids and peptides at the membrane surface using molecular dynamics simulation

A. Isvoran[1], P. Nedellec[2], V. Beswick[2] and A. Sanson[2]
[1]Department of Chemistry, University of the West Timisoara, Timisoara, Romania, [2]Department of Cellular and Molecular Biology, Centre of Atomic Energy, Saclay, France.
E-mail: aisvoran@yahoo.com

It has been experimentally proven that many peripheral proteins use electrostatic interactions to bind to biological membranes. The aim of this paper is to analyze the lipid–peptide electrostatic interactions at the surface of the cell membrane using a model at the atomic level and the simulation of the molecular dynamics technique. Some recent papers in the literature study this problem by using a phenomenological approach for the electrostatic interactions. This is correct for longer distances between peptides and membranes but at the interface membrane-solution the phenomenological approach is incorrect. In order to reveal the importance of the electrostatic interactions for the peptide binding we used two membrane systems; one of them contains only electrically neutral lipids, palmitoyl-oleoyl-phosphatidyl-choline (POPC) and a positively charged peptide (protonated glutamate and arginine). The other system contains electrically neutral lipids (POPC), negatively charged lipids (palmitoyl-oleoyl-phosphatidyl-serine, POPS) and the same peptide. In the paper we present the steps used for building the systems and we compare for the two membrane systems the results of the molecular dynamics simulation for 1 ns. We also compare our results with those obtained using the phenomenological approach.

C4-023P
Structure–activity relationships of diastereomeric lysine ring-size analogs of the antimicrobial peptide gramicidin S

M. Kiricsi, E. J. Prenner, R. N. A. H. Lewis,
M. Jelokhani-Niaraki, R. S. Hodges and R. N. McElhaney
Department of Biochemistry and Protein Engineering Network of Centers of Excellence, University of Alberta, Edmonton, Alberta, Canada. E-mail: kiricsim@hotmail.com

Antimicrobial peptides are promising candidates of current attempts to develop newer types of antibiotics and are particularly attractive because of their non-specific mode of action the evolution of bacterial resistance to these agents is difficult. Structure–activity relationships were examined in seven analogues of the naturally occurring antimicrobial peptide gramicidin S, where the ring-expanded GS14 was modified by single or multiple enantiomeric inversions of its four lysine residues. Peptide conformation and amphiphilicity were investigated by CD spectroscopy and RP-HPLC. 31P-NMR spectroscopic and dye leakage experiments were performed to evaluate their capacity to induce non-lamellar phases and to permeabilize model membranes, their growth inhibitory activity against the mollicute *Acholeplasma laidlawii* was also examined. Our results indicate that the changes in peptide facial amphiphilicity caused by lysine enantiomeric inversions are strongly correlated with the amount and stability of β-sheet structure, effective hydrophobicity, and propensity for self-association, ability to disrupt the integrity of phospholipid bilayers, and to inhibit the growth of *A. laidlawii*. The capacity of these peptides to differentiate between bacterial and animal cell membranes exhibits a biphasic relationship with peptide amphiphilicity, suggesting that there may only be a narrow range of peptide amphiphilicity within which it is possible to achieve high antibiotic effectiveness and low haemolytic activity. These results were rationalized by considerations of how enantiomeric inversions of lysine residues are likely to be reflected in the physico-chemical properties and partitioning of peptides into lipid bilayer membranes.

C4-024P
Detection and physiological significance of non-bilayer phases in chloroplast thylakoid membranes. A 31-P NMR study

S. Krumova[1,2], C. Dijkema[1], A. van Hoek[1], G. Garab[2] and H. van Amerongen[1]
[1]Laboratory of Biophysics, Wageningen University, Wageningen, The Netherlands, [2]1Biological Research Center, Szeged, Hungary.
E-mail: sashka.krumova@wur.nl

In thylakoid membranes non-bilayer lipids account for about half of the lipid content. This determines the high propensity of the whole lipid mixture to participate in different lipid phases. The ability of the lipid mixture to segregate into bilayer and non-bilayer phases has been proposed to play an important role in regulating the protein content; it was also hypothesized

that the non-bilayer structures are in dynamic equilibrium with the membrane bilayer and this contributes to the structural flexibility of membranes (Garab et al. *TIPS* 2000; **5**: 489). Non-bilayer structures were detected in thylakoid membranes under various treatments: low pH (Semenova et al. *J Plant Physiol* 1999; **55**: 669), high temperature (Gounaris et al. *BBA* 1984; **766**: 198) and high concentration of cosolutes (Williams et al. *BBA* 1999; **1099**: 137). However, their presence has not been shown at physiologically relevant conditions and their role and function are not well understood. Although in thylakoid membranes phosphatidylglycerol is present at low concentrations (about 10%), it was estimated that around 70% is in the bulk phase (Ivancich et al. *BBA* 1994; **1196**: 51). We used PG as an intrinsic label for 31-P NMR studies for detection of different lipid phases. Our data show that preparations of intact thylakoid membranes contain non-bilayer phases. The formation of these phases and the partition of PG molecules between the bilayer and non-bilayer domains depends on the temperature, the pH of the medium and is also modulated by strong light. Our results are in accordance with the hypotheses that the changes in the lipid phase behavior play important roles in the functioning of violaxanthin de-epoxidase (Latowski et al. *Eur J Biochem* 2002; **269**: 4656) and the structural flexibility of the membranes.

C4-025P
Modulation of ion channel conformation and organization by hydrophobic mismatch: a fluorescence approach

D. A. Kelkar and A. Chattopadhyay
Centre for Cellular and Molecular Biology, Hyderabad, India.
E-mail: dkelkar@ccmb.res.in

The matching of hydrophobic lengths of integral membrane proteins and the surrounding lipid bilayer is an important factor that influences both structure and function of integral membrane proteins (Jensen and Mouritsen. *Biochim Biophys Acta* 2004; **1666**: 205–226). Gramicidin is a multitryptophan pentadecapeptide that forms cation selective ion channels in membranes. Importantly, channel activity and gating has been shown to be uniquely sensitive to membrane properties such as bilayer thickness and membrane mechanical properties (Andersen et al. *Methods Enzymol* 1999; **294**: 208–224). The functionally important carboxy terminal tryptophan residues of gramicidin are located at the membrane interface (Mukherjee and Chattopadhyay. *Biochemistry* 1994: **33**: 5089–5097), and stabilize the channel conformation, however other non-channel conformations are also formed in membranes. We have examined the effects of hydrophobic mismatch on the conformation and organization of gramicidin in membrane bilayers of varying thickness utilizing the intrinsic conformation dependent fluorescence of the structurally important tryptophan residues of gramicidin (Rawat et al. *Biophys J* 2004; **87**: 831–843). Our results utilizing steady state as well as time-resolved fluorescence spectroscopic approaches and circular dichroism spectroscopy show that gramicidin remains predominantly in the channel conformation and gramicidin tryptophans are at the membrane interfacial region over a range of positive and negative mismatch conditions. Interestingly, gramicidin is not excluded from the membrane even under conditions of extreme negative mismatch. In addition, experiments utilizing tryptophan fluorescence self quenching indicate peptide aggregation under conditions of extreme negative mismatch.

C4-026P
Immunohistochemical localization of Coenzyme A synthase in normal rat tissues

V. V. Lyzogubov[1,2], V. S. Usenko[2], V. A. Gurtovy[2], I. O. Nemazanyy[3], I. T. Gout[3,4] and V. V. Filonenko[3]
[1]Institute of Experimental Pathology, Oncology and Radiobiology, Kiev, Ukraine, [2]Morphological Laboratory BIONTEC, Dnepropetrovsk, Ukraine, [3]Institute of Molecular Biology and Genetics, Kiev, Ukraine, [4]University College London, London, UK.
E-mail: aniko@a-teleport.com

CoA synthase (CoAsy) mediates the last two steps in the biosynthesis of CoA, which is a main carrier of acetyl and acyl groups in living cells. Molecular cloning of CoAsy has provided the base for the characterization of this bi-functional enzyme. The aim: to investigate the localization of CoAsy in normal rat tissues by light and electron immunohistochemistry (IHC). The analysis was carried out using tissues and organs from Wistar rats, including kidney, tongue, parotid salivary glands, thyroid, esophagus, trachea, laryngeal cartilages, striated muscles, fat and nerves, stomach, liver, small intestine (distal parts), colon, kidney, testis, skin, cerebellum, adrenal glands. Quantitative IHC with computer image analysis was used for the evaluation of CoAsy expression. CoAsy was clearly identified in all tissues studied. However, the quantity and the distribution of CoAsy was different depending on the type of tissue. High concentration of CoAsy was discovered in: (a) cells participated in transport of pantothenate – kidney proximal convoluted tubules, enterocytes and colonocytes; (b) tissues with intensive metabolic and energetic processes – liver, fat, muscles; (c) stratified squamous epithelium of epidermis, esophagus and tongue. Electron microscopy showed the association of CoAsy with membrane structures (outer and inner mitochondrial membrane, smooth and rough endoplasmic reticulum, nuclear membranes). In some tissues considerable amount of CoAsy was found inside peroxisomes and in mitochondria's matrix. In addition, the level of CoAsy was found to change at different stages of differentiation of colon epithelial cells. In conclusion, CoA synthase is a ubiquitously expressed protein, but the level of expression and its subcellular localization depends on the type of tissue, function of cells and distinctive features of metabolism in them.

C4-027P
Disturbed cellular lipid efflux and HDL maturation in caveolin-1 deficient mice

G. Liebisch[1], S. Le Lay[2], A. Böttcher[1], A.-S. Lintner[1], T. Kurzchalia[2], K. Simons[2] and G. Schmitz[1]
[1]Institute of Clinical Chemistry and Laboratory Medicine, University of Regensburg, Regensburg, Germany, [2]Max Planck Institute of Cellular Biology and Genetics, Dresden, Germany.
E-mail: gerhard.liebisch@klinik.uni-regensburg.de

Caveolin-1 (cav1) is the basic structure protein of caveolae and has been implicated in intracellular cholesterol transport. Aim of this study was to investigate the role of cav1 on the lipoprotein metabolism in cav1 deficient mice. Moreover, it should be evaluated whether cellular changes in the cholesterol uptake or apolipoprotein-mediated efflux may be related to disturbed lipoprotein profile. The HDL lipoprotein profile of cav1 deficient mice was analyzed in detail using FPLC separation followed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) as well as non-denaturing gradient gel electrophoresis. Thus we could find an increased pre-α-HDL fraction as well as a shift of slow migrating α-HDL to fast α-HDL fractions in cav1–/– mice compared to control. To assess the role of cav1 deficiency on cellular cholesterol homeostasis mouse embryonic fibroblasts (MEFs) were loaded with free cholesterol (FC) prior induction of cellular cholesterol efflux. Interestingly, cav1 deficient MEFs

contained only ~50% free cholesterol and cholesteryl ester after FC loading compared wild type. Moreover, cav1 deficient MEFs revealed decreased level of apo AI specific phospholipid and a tendency towards decreased apo AI specific cholesterol efflux compared to wild type. Because MEFs showed no high induction of the apo AI specific lipid efflux theses experiments are currently repeated in mouse skin fibroblast. In summary, these data suggest an impaired HDL maturation in cav1 deficient mice, which may be related to a disturbed cellular lipid uptake and efflux.

C4-028P
Searching after gelsolin amyloid formation

I. Liepina[1], P. A. Janmey[2], C. Czaplewski[3] and A. Liwo[3]
[1]*Molecular Biophysics Group, Latvian Institute of Organic Synthesis, Riga, Latvia,* [2]*Institute of Medicine and Engineering, University of Pennsylvania, Philadelphia, PA 19104 USA,* [3]*Faculty of Chemistry, University of Gdansk, Gdansk, Poland.*
E-mail: inta@osi.lv

Mutated gelsolin amyloidogenic fragments 173–243 (G173–243) and 173–202 (G173–202) cause Finnish familial amyloidosis disease (FAF). The mechanism of amyloidogenesis is not clear. In our previous work [1] we started to investigate the mechanism of amyloid formation. In this work, using molecular dynamics (MD), we investigate interactions of gelsolin amyloidogenic fragment G173–202 [cleaved from the X-ray structure of gelsolin (1DON)] with dimyristoyl-phosphatidylcholine (DMPC) membrane bilayers. The G173–202 fragment was placed 9 Å away from a DMPC bilayer at two different orientations and subjected to NTP MD simulations at T = 312 K. After 100 ps of MD, the G173–202 fragment at either initial orientation adhered to the DMPC membrane, retaining β-sheet structure, suggesting that a lipid membrane could bind G173–202 fragments and serve as a germ for amyloidogenesis. Afterwards one more amyloidogenic fragment G173–202 was placed in the vicinity of G173–202 adhered to the DMPC membrane and the new system was subjected to NTP MD simulations at T = 312 K. After 700 ps of MD, the newly added amyloidogenic fragment G173–202 was interacting with the membrane attached fragment G173–202, indicating the possible mechanism of membrane initiated amyloidosis. Subsequently all three systems were placed in a periodic lipid-water box and subjected to MD simulations to investigate of the stability of the structures.

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C4-029P
From β-sheets to a pH-induced α-helical structure in human calcitonin: an integrated investigation by Circular Dichroism, Raman Spectroscopy and channel formation in planar lipid membranes

D. Meleleo[1], S. Micelli[1], V. Capozzi[2], G. Perna[2], E. Mezzenga[2], V. Picciarelli[3] and E. Gallucci[1]
[1]*Physiology, Dept. Farmaco-Biologico, Università degli Studi di Bari, Bari, Italy,* [2]*Physics, Dept. di Scienze Biomediche, Università degli Studi di Foggia, Foggia, Italy,* [3]*Physics, Dept. Interateneo di Fisica, Università degli Studi di Bari, Bari, Italy.*
E-mail: micelli@farmbiol.uniba.it

Human Calcitonin (hCt) exerts a hypocalcemizing effect, and decreases bone resorption by inhibiting osteoclast activity. However, its use is limited by its propensity to form fibrils in solution due to a preponderance of β-sheet structures (Arvinte et al. *J Biol Chem* 1993; **26**: 6415; Bauer et al. *Biochem* 1994; **33**: 12276; Kamihira et al. *Prot Sci* 2000; **9**: 867), whereas an α-helical conformation is formed in aqueous acidic solution or when it interacts with negatively-charged lipids. This structure is reported to be important for interactions with receptors. However, hCt has been shown to form channels in planar lipid membranes (Stipani et al. *Biophys J* 2001; **81**: 3332; Micelli et al. *Biophys J* 2004; **87**: 1065) which could be a further mechanism for permeabilizing calcium in osteoclasts. In this study, we demonstrate that by lowering the pH from 7 to 4.5 (at which bone resorption takes place), hCt decreases its β-sheet content in favor of an α-helical conformation, as demonstrated by Circular Dichroism and Raman spectroscopy. Raman confocal microspectrometer apparatus, using the 632.8 nm line of a He-Ne as laser source and a notch filter (/200 cm line-with) to suppress the laser scattered light, was used. Besides, at pH 4.5, hCt increases its incorporation and assembly into membranes to form channels, supporting the notion that α-helical structures are a prerequisite for peptide incorporation into membranes. A model is proposed in which, depending on the protonation/deprotonation of His and Asp residues, hCt forms amphipathic α-helices necessary for the insertion and formation of a transmembrane channel in at least four assembled molecules.

C4-030P
Valine to glycine substitution in dolichol binding site of the yeast dolichol kinase affects the cell wall integrity

K. Machula, G. Sosinska and G. Palamarczyk
Laboratory of Fungal Glycobiology, Institute Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland.
E-mail: kmachula@ibb.waw.pl

SEC59, an essential gene, encodes CTP-dependent dolichol kinase. Phosphorylation of dolichol is the last and rate-limiting step in the synthesis of glycosyl carrier lipid dolichyl phosphate, required for N-glycosylation, O-mannosylation and GPI anchor biosynthesis. We identified the point mutation (Val332 to Gly) in the dolichol binding region of dolichol kinase resulting in the thermosensitive fenotype. Described erlier biochemical characterization of this mutant revealed decrease in the enzyme activity up to 5% of wild type level. As glycosylated proteins account for a major component of the cell wall, we expected that the sec59-1 mutation affects the cell wall composition. Calcofluor sensitivity, chitin content and phosphorylation status of MAPK indicated up regulation of the cell integrity pathway. In order to determine proteins interacting genetically with dolichol kinase we screened the yeast genomic library for the multicopy suppressors of the thermosensitive sec59-1 phenotype. Our screen resulted in isolation of the RER2 and SRT1-genes encoding cis-prenyltransferase, the enzyme acting upstream of Sec59p, catalyzing synthesis of dolichol backbone, and ROT1 gene encoding protein involved in β 1,6-glucan synthesis. Focusing on the mechanism of sec59-1 suppression at the restrictive temperature we analyzed possible changes in the mutant cells bearing multicopy supressors:
(i) chitin, β 1,3 and β 1,6-glucan content in the cell wall,
(ii) changes in glycosylation and stability of GPI-anchored plasma membrane and cell wall Gas1 protein responsible for the β 1,3-glucan remodeling as well as the degree of glycosylation of the vacuolar protein carboxypeptidase Y. In summary, our results point to the role of dolichol pathway in the maintenance of cell wall integrity.

C4-031P
Influence of lipids on the reconstitution into liposomes and activity of GPI-anchored proteins

S. Sesana, V. Sangiorgio, A. Bulbarelli, M. Pitto and **M. Masserini**
Department Experimental Medicine, University of Milano-Bicocca, Monza, Italy. E-mail: massimo.masserini@unimib.it

Inclusion into rafts and subsequental apical sorting has been shown to be important for proteins anchored to the plasma membrane via a glycosylphosphatidylinositol (GPI)-anchor, such as alkaline phosphatase and Prion protein. To investigate the interaction of GPI-anchored with lipids or rafts, we have utilized a model system consisting of alkaline phosphatase from human placenta (PLAP) incorporated into liposomes mimicking the enrichment of cholesterol and sphingolipids present in lipid rafts. Different mixtures of lipids were used: palmitoyl-oleyl-phosphatidylcholine (POPC), sphingomyelin (SM), GM1 ganglioside and cholesterol (Chol). The GPI-anchored form of PLAP was purified from a commercial preparation. Liposomes and proteoliposomes were obtained by dialysis of lipid dispersions in octylglucoside (with or without PLAP at different protein/lipid ratio), and purified by discontinuous sucrose gradient centrifugation. The proteoliposome fractions were defined as the fractions containing both the enzyme and lipids. The protein reconstitution efficiency was defined as the ratio of the activity (using p-nitrophenylphosphate as the substrate) in proteoliposomal fraction/total activity in the sucrose gradient. Our results showed that the reconstitution efficiency (RE) depend on the lipid mixture. Best result in term of RE were obtained in presence of POPC plus GM1 (RE 53 %). The highest values of protein specific activity was obtained in presence of GM1 (1.66 DO/μg). Moreover, the specific activity showed a dependence on the protein/lipid molar ratio, decreasing at higher molar ratios. These results show that biological activity of GPI-anchored proteins is affected by their lipid environment.

C4-032P
Two forms of a tail-anchored protein, differing in transmembrane domain length and intracellular sorting, interact differently with lipids

P. Ceppi[1], S. Colombo[2,4], M. Francolini[2,4], F. Raimondo[1], M. Pitto[1], N. Borgese[2,3,4] and **M. Masserini**[1]
[1]Department of Experimental, Environmental Medicine and Biotechnologies (DIMESAB), Medical School, University of Milano-Bicocca, Monza, Italy, [2]Cell and Molecular Pharmacology Section, C.N.R. Instittute for Neuroscience, Milan, Italy, [3]Department of Pharmacobiological Science, University of Catanzaro 'Magna Graecia', Catanzaro, Italy, [4]Department of Medical Pharmacology, University of Milan, Milan, Italy.
E-mail: massimo.masserini@unimib.it

A number of C-tail-anchored (TA) proteins require a short transmembrane domain (TMD) of moderate hydrophobicity to maintain their ER residence, but whether lipids are involved in this sorting mechanism is nor clear. To investigate this issue, we studied lipid–protein interaction by differential scanning calorimetry (DSC) and fluorescence techniques, using liposomes embedding cytochrome b_5 wild-type ($b_5$wt, an ER resident) or mutant ($b_5$ext, having five extra non-polar amino acids in the TMD, transported to the plasma membrane). The proteins were incorporated into liposomes of palmitoyl-oleyl-phosphatidylcholine (POPC) plus 5–20% either palmitoyl-oleyl-phosphatidylserine (POPS), distearoyl-phosphatidylcholine (DSPC), distearoyl-phosphatidylserine (DSPS), distearoyl-phosphatidic acid (DSPA) or C-16 ceramide (CER). Any of these lipids was forming a phase-separated domain, as suggested by the appearance of an additional higher temperature thermotropic transition besides that of POPC. Whereas the insertion of $b_5$wt in the bilayer affected the POPC component, $b_5$ext caused a decrease of the higher temperature, phase-separated component in all mixtures, except POPC/DSPC. These data have also been confirmed using fluorescence spectroscopy. The fluorescence excimer:monomer (E/M) intensity ratio of pyrene-PC embedded in POPC containing either POPS, DSPS or DSPA was significantly increased in the presence of $b_5$wt. Accordingly, also the E/M ratio of ceramide-BODIPY probe in POPC/CER liposomes, increased. These results suggest that $b_5$wt and $b_5$ext have opposite behavior with respect to the laterally segregated domains that form in these mixed liposomes; in particular, the extended, exported mutant, interacts preferentially with the domain melting at higher temperature.

C4-033P
A homogeneous HTS assay for Acyl-CoA:cholesterol Acyltransferase 2, ACAT2

L. Redaelli, P. Arioli, L. Iuzzolino, **V. Nardese** and D. Carettoni
Biochemistry, Axxam, Milan, Italy.
E-mail: vanessa.nardese.vn@axxam.com

Acyl-CoA:cholesterol Acyltransferase 2 (ACAT2) is an integral transmembrane enzyme which catalyzes the formation of cholesterol esters secreted as apoB-containing lipoprotein particles. ACAT2-mediated cholesterol esterification has been demonstrated to be crucial for atherogenesis, but the complexity of the protein and the currently available assay format based on TLC have restricted so far the accessibility of this important target enzyme to the high-throughput drug discovery process. Bioinformatic analysis of the human ACAT2 sequence allowed us to design a mutant of ACAT2, named Enhanced Activity-ACAT2 (EA-ACAT2) predicted to display an increased transferase activity. Recombinant wtACAT2 and EA-ACAT2 were expressed in insect cells and purified in the active form by membrane fractionation and chromatographic separation. Their activities were assayed both in crude membrane fraction and in the purified form. Despite a similar production yield, EA-ACAT2 resulted four times more active than wtACAT2. Gel filtration chromatography was used to characterize the quaternary structure of the mutant, which was markedly different in respect with wtACAT2. Finally, a homogeneous assay with a fluorescent readout was developed to follow cholesterol esterification in 384 MTP format. The kinetic parameters of EA-ACAT2 were calculated and the possibility to detect a specific inhibition was demonstrated by using a reference inhibitor.

C4-034P
Increased synthesis of dolichol compensates for defect in yeast cells defective in dolichylphosphate mannose synthase activity

J. Orłowski, A. Janik and G. Palamarczyk
Laboratory of fungal Glycobiology, Institute of Biochemistry and Biophysics Polish Academy of Sciences, Warsaw, Poland.
E-mail: jacekorlowski@ibb.waw.pl

Yeast dolichyl phosphate mannose synthase (Dpm1p), DPM1 gene encoded, a pivotal protein, is required for protein N-glycosylation, O-and C-mannosylation and glycosylphosphatidyl inositol (GPI) membrane anchoring of proteins. Impairment in Dpm1p activity results in conditionally lethal thermosensitive

phenotype. Overexpression of the yeast RER2 gene in dpm1–6 mutant restores growth of the cells at a non-permisive temperature. The RER2 gene encodes cis-prenyltransferase (cis-PT-ase), the first enzyme of the mevalonate pathway involved in dolichol biosynthesis. Its overexpression lead to the increase of cis-PT-ase activity, dolichol and dolichyl phosphate content as well as to 20-fold increase of Dpm1p affinity towards DolP. Simultaneously, although on the transcriptional level we observed over threefold increase of DPM1 mRNA in the RER2 transformed dpm1–6 cells Dpm1 protein level and enzymatic activity remained unchanged. On the other hand elevated dolichol level in the dpm1–6 was concomitant with the increase of the Alg7 protein activity thus we observed almost three fold increase in DolPPGlcNAc2 synthesis. dpm1–6 mutant compared to the wild type control, appeared more sensitive to Calcofluor White. This effect was, however, suppressed in the RER2-transformed dpm1–6. The latter suggest that the mutation in Dpm1p leads also to alteration in the cell wall composition which can be overcome by the elevated dosage of the substrate for Dpm1p. In summary, our results point to the role of dolichol in the maintenance of cell wall integrity.

C4-035P
Comparative proteomics of membrane microdomains from renal cell carcinoma and adiacent normal kidney

F. Raimondo[1], P. Ceppi[1], A. Di Fonzo[1], F. Magni[1], N. Cordani[5], S. Ferrero[2], R. Perego[1], C. Sarto[4], P. Favini[3], F. Rocco[3], **M. Pitto**[1] and P. Mocarelli[1,4]

[1]Dept of Exp., Env. Medicine & Biotechnology, Univ. Milano Bicocca, Monza, Italy, [2]Pathology Unit, Dept of Medicine, Surgery and Dental Sciences, Univ Milano, Milano, Italy, [3]Dept. Urology, Hospital Maggiore IRCCS, Univ Milano, Milano, Italy, [4]Hospital of Desio, Univ. Dept. of Laboratory Medicine, Univ. Milano Bicocca, Desio, Italy, [5]Dept of Science and Biomedical Technologies, Univ Milano, Segrate (MI), Italy.
E-mail: marina.pitto@unimib.it

Recent studies suggest that subcellular comparative proteomics could help in finding tumor specific proteins to be used as peripheral markers and/or targets for therapy. In particular caveolin, the characteristic protein of caveolae – plasma-membrane lipidic microdomains involved in several human pathologies – has been recently described as a putative tumor marker of prostatic cancer. Aim of this research is to characterize the protein composition of caveolin-enriched microdomains prepared from surgical samples of renal cell carcinoma (RCC) and adiacent normal kidney (ANK) by a proteomic approach, in order to set up new markers for this neoplasm. To this purpose, plasma-membrane-enriched fractions were prepared from surgical samples of RCC and ANK, following nephrectomy. After treatment with Triton X-100, in which caveolar microdomains are insoluble, and floatation in a sucrose density gradient, the fractions displaying the highest caveolin enrichment were analyzed by two dimensional electrophoresis, using an optimized micro-scaled protocol. Typical caveolar proteins were detected by immunoblotting and the corresponding spots were identified by mass spectrometry. Comparison between RCC and ANK shows that higher amount of caveolin-1 and flotillin-1, known markers of caveolar microdomains, are recovered in tumoral samples. Other proteins, such as renal dipeptidase and aquaporin-1, are down-regulated in RCC microdomains. In conclusion, our results show that some caveolar proteins are differentially expressed in RCC compared to ANK. Since surface proteins are likely to be released by tumors in extracellular fluids, this approach looks promising in view of providing potential diagnostic and/or prognostic tumor markers.

C4-036P
A colorimetric assay of membrane lipids

M. A. Rodriguez and L. Beletskaya
Department of BMI, University of Ottawa, Ottawa, Ontario, Canada. E-mail: mrodrig@science.uottawa.ca

Brilliant blue's visible spectrum in basic aqueous medium is characterized by a slow quenching and right shifting of its maximum absorption peak around 560 nm. Membrane vesicles and polar lipids have been found to largely increase the rate of this quenching. By optimizing the pH and the time of incubation, we have developed a colorimetric assay for membrane preparations, membrane lipids, and pure polar lipids in aqueous suspension. The assay is usually linear in a range of one unit of absorbance and has a sensitivity of about 5 μg for pure lipids and extracts of membrane lipids and about 0.5 μg for lipids in membrane vesicles or in lipoproteins. Pure phospholipids and sphingomyelin show a similar level of response whereas neutral lipids and fatty acids have no effect on the quenching of the dye's absorption. Some soluble proteins show a level of response similar to the lipids alone, thus the application of the assay to tissue extracts will require a correction for the contribution to the quenching by the proteins. This assay is more specific than the measurement of the absorption at 325 nm due to the light dispersion by the lipid vesicles, and it is about 10 times more sensitive than the latter when applied to membrane vesicles or lipoproteins. When applied to the measurement of membrane suspensions, the assay is from two to five times more sensitive than the determination of membrane proteins.

C4-037P
Lipoprotein(a) level and lipid tetrad index in patients with Acute Myocardial Infarction in Myanmar

H. K. Sunn
Laboratory of Biochemistry, Biochemistry Department, University of Medicine(1), Yangon, Yangon Myanmar.
E-mail: htarkyisun@myanmar.com.mm

Lipoprotein(a)[Lp(a)] is a plasma particle closely related to low density lipoprotein.Lp(a) contains a cholesterol rich core that is covered by a complex of apoprotein B-100 covalently linked to apoprotein(a). Serum Lp(a) is involved in atherogenesis and thrombogenesis, and serves as a crucial link between these processes. In my study, the parameters measured were serum Lp(a), serum lipid profile and lipid tetrad index(LTI).The mean serum Lp(a) level of AMI patients was 14.91 mg/dl which was significantly higher than the control value of 4.78 mg/dl ($P < 0.0001$).The mean LTI of AMI patients was 16365.6 which was significantly higher than the control value of 765.2 ($P < 0.0001$). The LTI correlated with serum Lp(a) level both in control with $r = 18706$ and in AMI patients with $r = 0.26681$. Thus, determination of serum Lp(a) level and LTI in AMI serve as assessing the risk of AMI. Serum Lp(a) level and its correlation to AMI had never been studied in Myanmar. My study established serum Lp(a) level and LTI in AMI patients and relationship in AMI. The cut-off value of serum Lp(a) and LTI for Myanmar population was also established from my study. The outcome of the research data may be useful and applicable for the clinicians to determine the cardiovascular risk in Myanmar.

C4-038P
Activity of *Pichia pastoris* alternative cis-prenyltransferase is correlated with methanol or oleic acid induced proliferation of peroxisomes

M. Skoneczny[1], E. Swiezewska[2] and **A. Szkopinska**[2]
[1]*Department of Genetics, Institute of Biochemistry and Biophysics PAS, Warsaw, Poland,* [2]*Department of Biochemistry of Lipids, Institute of Biochemistry and Biophysics PAS, Warsaw, Poland.*
E-mail: babel@ibb.waw,pl

Taking a hint from our finding that disruption in yeast strain W303 of PEX1 gene responsible for peroxisome biogenesis blocks synthesis of longer chain dolichols in oleate induced pex1 disruptant we investigated dolichol synthesis in yeast *Pichia pastoris*. Growth of these cells on methanol as the only carbon and energy source causes peroxisome proliferation and induction of peroxisomal enzymes. 24 h methanol treatment was sufficient for the appearance of longer-chain dolichols. Moreover, it was practically the only dolichol family. Less specific oleic acid induction needed 48 h for the synthesis of longer dolichol family with typical one still present. Control cells cultured on glucose for 48 h did not reveal the presence of additional dolichol family. Peroxisomes purified from oleic acid treated cells synthesize *in vitro* polyprenols longer by two isoprene residues than those synthesized by microsomal fraction from glucose culture. These observations allow to suggest that chain length of dolichols synthesized in yeast cell may depend on the carbon and energy source supply of which mobilizes metabolic pathways localized to different cellular compartments. In case of methanol or oleic acid that may be peroxisomes.

C4-039P
Spontaneous membrane insertion of the cycling receptor Pex5p implies a new mechanistical model for peroxisomal protein import

E. Hambruch[1], D. Kerssen[1], W. Klaas[1], W.-H. Kunau[1], B. de Kruijff[2] and W. Schliebs[1]
[1]*Institute of Physiological Chemistry, Ruhr-University Bochum, Bochum, Germany,* [2]*Department of Biochemistry of Membranes, Utrecht University, Utrecht, The Netherlands.*
E-mail: wolfgang.schliebs@rub.de

Peroxisomal proteins carrying a peroxisome targeting signal type 1 (PTS1) are recognized in the cytosol by the mobile import receptor Pex5p. According to the extended shuttle model the soluble PTS1-receptor binds and enters the peroxisome, facilitates the cargo release and exits the peroxisome to allow for another round of import. Cycling of the receptor requires the combined action of several membrane-bound proteins, collectively termed peroxins. In order to study the sequential events at the membrane we generated yeast mutants in which Pex5p's interactions with other peroxins are blocked. Biochemical analysis of these mutants revealed that binding to either Pex13p or Pex14p is a prerequisite for the association of Pex5p with other known binding partners at the peroxisomal membrane, e.g. the RING-finger peroxins. Consequently, a Pex5p variant with single-point mutations in Pex13p and Pex14p -binding sites does not associate with membrane peroxins. However, the amount of membrane-associated Pex5p-cargo complex in such mutants is not reduced when compared with wild-type suggesting that the mutated PTS1-receptor can associate with the peroxisomal membrane in a peroxin-independent way. In order to test Pex5p for lipid-binding activity we applied purified yeast and human PTS1-receptor to monolayer- and bilayer binding experiments. Our *in vitro* analysis clearly shows that both soluble PTS1-receptors have the potential to insert spontaneously into phospholipid membranes. Therefore we suggest that the peroxisomal fraction of peroxin-uncoupled Pex5p in our mutants represents a lipid-bound state of the PTS1-receptor. Interestingly, PTS1-proteins are still imported into peroxisomes in these mutants. These observations led us to propose a peroxisome import model in which a transient lipid-bound form of Pex5p drives the translocation of PTS1-proteins across the peroxisomal membrane before it gets processed by other peroxins for recycling.

C4-040P
Characterization of the amphipatic peptide (KL4)4K, a therapeutic agent for respiratory distress syndrome, in two different surfactant-like liposomes

A. Saenz, O. Cañadas and C. Casals
Laboratory of Pulmonary Surfactant, Department of Biochemistry and Molecular Biology I, Complutense University of Madrid, Madrid, Spain. E-mail: jana@bbm1.ucm.es

The 21-residue peptide KLLLLKLLLLKLLLLKLLLLK, (KL4)4K, mimics some aspects of pulmonary surfactant protein B and has been clinically tested as a therapeutic agent for respiratory distress syndrome in premature infants. The objective of this study was to evaluate the optimal incorporation of (KL4)4K in two types of surfactant-like liposomes, and analyze (KL4)4K effects on the physical properties and surface activity of these liposomes. To that end, dipalmitoylphosphatidylcholine/1-palmitoyl-2-oleoyl-phosphatidylglycerol/palmitic acid (DPPC/POPG/PA) and DPPC/1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC)/PA (28:9:5.6, w/w) vesicles were used. From fluorescence anisotropy measurements of 1,6-diphenyl-1,3,5-hexatriene (DPH) we determined that (KL4)4K was incorporated into DPPC/POPG/PA vesicles up to a peptide concentration of 5.8% (w/w), whereas in POPC-doped membranes the peptide showed a limited accessibility to the membrane that might be due to the extended configuration that the peptide adopts in these liposomes as determined by CD. (KL4)4K promoted an increase of the Tm of both types of membranes as determined by DPH anisotropy and DSC. Incorporation of (KL4)4K into DPPC/POPG/PA, but not into POPC-doped vesicles, induced phase separation and enhanced calcium-induced vesicle aggregation, which was not reversed by EDTA. The different behavior of (KL4)4K in POPG- and POPC-doped vesicles might be related to the different conformation adopted by the peptide in these membranes, as well as to the large number of electrostatic interactions between the charged lysine residues of (KL4)4K and the anionic headgroups of POPG-containing membranes. In spite of these differences, (KL4)4K improves the surface activity of both types of membranes.

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C4-041P
Comparison between insect cell and yeast in structural requirement of signal sequence

Y. Tsuchiya, K. Morioka, J. Shirai and K. Yoshida
Laboratory of Epizootiology, Department of Exotic Diseases, National Institute of Animal Health, Kodaira, Tokyo Japan.
E-mail: tsuchiyayoshi24@yahoo.co.jp

Signal sequence plays an important role in the translocation of newly synthesized proteins across the membrane of the

endoplasmic reticulum (eukaryotic cells) or across the cell membrane (prokaryotic cells). These sequences are composed of three structurally and functionally distinct regions: a positively charged N-terminal region, a central core region, and a C-terminal region recognized by signal peptidase. In order to elucidate the structural requirements and functions of signal sequence in eukaryotic cells (yeast or insect cell), the N-terminal and the central core region of the chicken lysozyme signal peptide (CLSP) was altered, and the effect of mutations on secretion of human lysozyme (HLY) were studied. A hybrid gene composed of CLSP gene and HLY gene synthesized from short DNA oligomers was used for the production of recombinant HLY in insect cell using vacuole virus expression system, or in yeast. The existence of basic residue (Arg) in the N-terminal region was important and increased the secretion of HLY in yeast, but not in insect cell. In contrast with this, the increase of hydrophobicity in the central core region enhanced the secretion of HLY in both insect cell and yeast. Although they are eukaryotes, the structural requirements of signal sequence have some differences in yeast and insect cell in the N-terminal region, but those of the central core region may be common over species.

C4-042P
T-cadherin is an atypical LDL-receptor: LDL binding causes intracellular signaling

E. Talovskaya, K. Rubina, V. Cherenkov, T. Storozhevikh, V. Pinelis, Y. Parfyonova and V. Tkachuk
Molecular Endocrinology, Russian Cardiology Research Center, Moscow, Russian Federation. E-mail: e-tal@yandex.ru

Cadherins are a family of adhesion molecules that mediate Ca^{2+}-dependent cell–cell adhesion in solid tissues: their adhesions are formed as a result of interactions between extracellular domains of identical cadherins in neighboring cells. Binding of the intracellular domain to the actin cytoskeleton ensures the stability of cadherin-mediated adhesive junctions. T-cadherin (Tcad) is a unique member of cadherins; it has five tandem repeats in its extracellular portion, it is anchored to the cell surface membrane via a glycosyl phosfatydil inositol (GPI) moiety. Subcellular distribution of Tcad is restricted to lipid rafts on the cell membrane where it co-localizes with signal-transducing molecules. The function of Tcad has not been revealed yet. A comparative study on the Tcad expression in human organs and tissues revealed that Tcad content is maximal in the aorta, carotid, iliac and kidney arteries, heart. We showed that its expression in the smooth muscle cells depends on the cell phenotype and proliferate activity and increases in sclerotic lesion of vascular walls and restenosis. As a result of our last experiments, expression of Tcad in stably transfected cells increases low density lipoproteins (LDL) binding to the cell membrane. Binding LDL with Tcad positive cells membranes increases the level of intracellular Ca^{2+} and stimulates Ca^{2+} dependent signaling. The amplitude of Ca^{2+} response in Tcad positive cells significantly exceeds similar response in control. Boyden chamber migration assay demonstrates that Tcad expression facilitates cell migration towards 0.1% BSA gradient; LDL-induced migration is greater for T-cad expressing cells than in control. Thus Tcad, the new LDL receptor mediates the effects of the LDL on signal transduction and induced cell migration.

C4-043P
HIV fusion inhibitor peptide T-1249 is able to insert or adsorb to lipidic bilayers. Putative correlation with improved efficiency

A. S. Veiga[1], N. C. Santos[2], L. M. S. Loura[3,4], A. Fedorov[3] and M. A. R. B. Castanho[1]
[1]Laboratory of Molecular Biophysics, Centro de Química e Bioquímica, University of Lisbon, Lisbon, Portugal, [2]Instituto de Bioquímica/Faculdade de Medicina de Lisboa and Unidade de Biopatologia Vascular, University of Lisbon, Lisbon, Portugal, [3]Centro de Química Física Molecular, Instituto Superior Técnico, Lisbon, Portugal, [4]Centro de Química e Departamento de Química, University of Évora, Évora, Portugal. E-mail: asveiga@fc.ul.pt

T-1249 is a HIV fusion inhibitor peptide under clinical trials. Its interaction with biological membranes models (large unilamellar vesicles) was studied using fluorescence spectroscopy. A gp41 peptide that includes one of the hydrophobic terminals of T-1249, was also studied. Both peptides partition extensively to liquid-crystalline POPC (ΔG = –7.0 and –8.7 kcal/mol, for T-1249 and terminal peptide, respectively) and located at the interface of the membrane. T-1249 is essentially in a random coil conformation in this lipidic medium, although a small α-helix contribution is present. When other lipid compositions are used (DPPC, POPG + POPC, and POPC + cholesterol) partition decreases, the most severe effect being the presence of cholesterol. Partition experiments and fluorescence resonance energy transfer analysis show that T-1249 adsorbs to cholesterol-rich membranes. The improved clinical efficiency of T-1249 relative to enfuvirtide (T20) may be related to its bigger partition coefficient and ability to adsorb to rigid lipidic areas on the cell surface, where most receptors are inserted. Moreover, adsorption to the sterol-rich viral membrane helps to increase the local concentration of the inhibitor peptide at the fusion site.

C4-044P
Functionalized specialty natural rubber synthesis by *Hevea latex* membrane proteins-enzymes micellar microreactor using bacterial C55-Isoprene UPP as allylic initiator

D. Wititsuwannakul[1], A. Rattanapittyaporn[2] and R. Wititsuwannakul[2]
[1]Natural Rubber Enzymes Research Lab, Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Bangkok 10400 Thailand, [2]PSU Natural Rubber Research Centre, Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat-Yai, Songkla 90112 Thailand.
E-mail: scdwt@mahidol.ac.th , dhirayosw@yahoo.com

Bacterial C55-isoprene UPP was found effective in synthesis of new natural rubber. Active Rubber biosynthesis (RB) was shown in bottom fraction (BF) particles, membrane organelles of centrifuged fresh latex. Washed BF membrane (WBM) was unequivocally shown with proteins or enzymes system of high RB activity [1, 2]. *Hevea* rubber transferase (HRT) gene recently cloned was shown RB active with WBM [3] in the synthesis of new rubber molecules. Anionic surfactants (SDS, DOC) at conc. above CMC strongly activated new rubber formation by WBM. Proteins fractionated from WBM by a serial acetone extraction was with high RB activity in SDS or DOC mixed micelles. WBM proteins were also with RB regulator in reconstited assays. WBM enzymes system showed wide ranges of initiating allylic isoprenes for new rubber synthesis. C55-Isoprene UPP of

M. luteus is effectively used by WBM for new rubbers synthesis [4]. Comparisons of allylic isoprenes different chain length showed that UPP was most effective as initiator for new rubber formed compared to the shorter ones. WBM positive correlation between RB levels and allylic chain lengths are UPP > > GGPP > FPP > > IPP. However, shorter allylics formed more oligoisoprene intermediates than the longer UPP in a reverse order. Polyprenols of C50–C65 were the predominant isoprenes in latex. It's therefore of no surprise that *Hevea* WBM enzymes could effectively use bacterial C55-UPP in this new found microreactor for novel *in vitro* functionalized specialty RB.

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C4-045P
Antibacterial peptidomimetics and their influence on membrane structure

R. Willumeit[1], S. Linser[1], M. Kumpugdee[1], D. Svergun[2] and G. Tew[3]

[1]*Department of Macromolecular Structure Research, GKSS Research Center, Geesthacht, Germany,* [2]*EMBL Outstation, Hamburg, Germany,* [3]*Department of Polymer Science and Engineering, University of Massachusetts, Amherst, MA 01003, USA. E-mail: regine.willumeit@gkss.de*

Learning to mimic the essential physiochemical properties of natural macromolecules would foster unprecedented properties in synthetic polymers. Our first target has been a class of membrane active peptides, commonly referred to as host defense peptides that display broad spectrum antimicrobial activity and are non-toxic to mammalian cells. This class of natural molecules displays a rather unique architecture which is a facially amphiphilic (FA) structure in which positively charged polar and non-polar groups extend from opposite sides of the structure. This occurs regardless of whether the structure is a simple α-helix or a more complicated tertiary fold like that of a defensin peptide. We started to study cationic, FA poly(phenyleneethynylene)s in order to mimic the essential features of the natural host defense peptides. Previous Langmuir data confirmed an extended FA architecture. Antimicrobial assays showed that these polymers were reasonably active against several bacteria. A small molecule analogue (total molecular weight 590 g/mol) showed extremely potent and broad spectrum activity as well as significant selectivity so that it was ~100 times more active toward bacteria than mammalian red blood cells. This molecule contains a strict hydrocarbon backbone with two cationic amines appended. These compounds do not show resistance of pathogenic bacteria like classical antibiotics such as cipro. At the same time, the ability to create these mimics is an important scientific challenge since the mechanism of interaction and destruction of bacteria are not well understood. In this work first results measured by X-ray small angle scattering concerning the interaction of a promising oligomer (LA-03-149) with phospholipid vesicles are presented.

C4-046P
Membrane proteolipids identified as novel major latex protein allergens with high fidelity: the most updated sensitive allergens detectable in medical and household natural rubber products

R. Wititsuwannakul[1], K. Meng-umpun[1], K. Yoonram[1], P. Jewtragoon[1], D. Wititsuwannakul[2], R. G. Hamilton[3], P. Puvabanditsin[4] and J. Wititsuwannakul[4]

[1]*Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat-Yai, Songkhla 90112 Thailand,* [2]*Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, 10400 Thailand,* [3]*Allergy and Clinical Immunology Division, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21221, USA,* [4]*Dermatology Division, Department of Medicine, Medical School, Chulalongkorn University, Bangkok, 10330 Thailand. E-mail: rapepun.w@psu.ac.th, wrapepun@yahoo.com*

Current available serological assays for IgE specific latex protein allergens are still inadequate, displaying below the required diagnostic sensitivity. Certain allergen epitopes are evaded and missed from detection by these multiple Hev b test kits. Our recent study revealed the presence of new found novel latex allergen [1] as the bottom fraction membrane proteolipids (BMPL) of centrifuged fresh latex as verified via Floch & Lee protocol [*JBC* **191**: 807–817]. Immunoblots revealed highly significant IgE reactive BMPL bands (of 17#, 20, 22, 30*, 33 and 43 kD) with both Thailand and US allergic sera. Positive frequencies of IgE reactive to these BMPL detected by the sera are of high fidelity. Sera of US allergic patients, positive clinical history [Hx] and skin prick test [SPT], are 100% (44/44)# and 95% (40/42)* for 17 and 30 kD and slightly lower for other BMPL. Sera of Thai with Hx positive are 98% (52/53)# and 87% (46/53)* for 17 & 30 kD and lower for others. N-terminal amino acids data of these two most highly IgE reactive BMPL (17 & 30 kD) were different from other known Hev b 1-13 allergens. Results as obtained from our reliable tracing study, using both Western immunoblot and ELISA, clearly indicated that allergenic extractable proteins in the ultra-low protein gloves (< 50 μg/g) still detectable are proteolipids of BMPL. These missed detections by multiple Hev b kit showed that our new allergens assays are superior with sensitivity of higher accuracy. High fidelity of the two new allergens (95–100% frequency) is thus more reliably safer entity for development as new universal latex allergens test kit with highest safety of most accuracy.

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C4-047P
Influence of lipid membrane composition on its interaction with Pluronic

A. E. Zhirnov[1], T. V. Demina[1], O. O. Krylova[2], I. D. Grozdova[1] and N. S. Melik-Nubarov[1]

[1]*Macromolecules for Medicine and Biotechnology Lab, Polymers Chair, Chemistry Department, M.V. Lomonosov Moscow State University, Moscow, Russian Federation,* [2]*Institute for Biophysics, Kepler University, Linz, Austria. E-mail: jyrnoff@yandex.ru*

Copolymers of ethylene oxide (EO) and propylene oxide (PO) of $EO_{n/2}PO_mEO_{n/2}$ type (Pluronics) are able to reverse multi-drug resistance of tumor cells. Obviously, first step of Pluronics action on cells is their interaction with cell plasma membranes.

Previously we have shown that Pluronics bind to artificial membranes and enhance accumulation of anti-tumor drug doxorubicin (DOX) inside pH-gradient liposomes and flip–flop of NBD-labeled phosphatidylethanolamine in liposomes composed from lecithin only. Taking into account different lipid composition of cell membranes it seemed relevant to evaluate its significance for membrane sensitivity to Pluronic. We made binary liposomes containing, besides the lecithin, different amounts of other lipids of natural origin [cholesterol, phosphatidylethanolamine (PE), cardiolipin (CL), ganglioside GM1, sphingomyelin (SM), phosphatidic acid (PA)]. The influence of the additives on membrane microviscosity; Pluronic adsorption; the copolymer effect on flip-flop and DOX permeation was studied. The results showed that insertion of cholesterol increased microviscosity of the membrane and decreased adsorption of Pluronic and diminished Pluronic's capacity to enhance flip–flop and DOX accumulation. Similar tendencies were revealed upon incorporation of PE or GM1. On the contrary, addition of PA decreased microviscosity of the bilayer and provoked its destabilization in the presence of the copolymer. SM and CL did not induce any detectable changes in membrane permeability and microviscosity. Thus, a reverse dependence between the microviscosity of membranes and their sensitivity to Pluronic effects was demonstrated. This data may be relevant to mechanisms of Pluronic interaction with cells.

C5 – Lysophospholipids in Cell Signaling

C5-001
The ins and outs of lysophosphatidic acid signaling

W. Moolenaar[1], L. van Meeteren[1], P. Ruurs[1] and J. Jonkers[2]
[1]*Cellular Biochemistry, The Netherlands Cancer Institute, Amsterdam, The Netherlands,* [2]*Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands.*
E-mail: w.moolenaar@nki.nl

Lysophosphosphatidic acid (LPA; monoacyl-glycero-3-phosphate) is a lipid mediator with numerous biological actions, particularly as an inducer of cell proliferation, migration and survival. In addition, LPA induces growth cone collapse, neurite retraction and membrane depolarization as well as endothelial hyperpermeability. LPA signaling has been implicated in such diverse processes as wound healing, brain development and tumor progression. LPA binds to specific G protein-coupled receptors and thereby activates multiple signaling pathways, including those initiated by the small GTPases Ras, RhoA and Rac. LPA is produced from lysophosphatidylcholine by lysophospholipase D, also known as 'autotaxin' (ATX), a secreted phosphodiesterase originally identified as an autocrine motility factor for tumor cells. Using a sensitive fluorescence-based phosphodiesterase sensor, we find that ATX activity is negatively regulated by LPA and sphingosine-1-phosphate (S1P), implying that LPA can regulate its own biosynthesis in the extracellular environment and defining a novel role for S1P as a repressor of LPA production. Our functional studies in mice reveal that ATX deletion results in embryonic lethality, apparently due (in part) to impaired vascular development. This lecture will address selected topics on LPA receptor signaling and the action of ATX (lysophospholipase D). Finally, we will present evidence that undue production of LPA may contribute to the pathophysiological effects of exogenous secreted phospholipases D.

C5-002
Ceramide kinase is required for degranulation pathway in mast cell and regulated by Calmodulin/Ca^{2+} and PIP2 through specific interaction with their recognition domains

Y. Igarashi, T. J. Kim and S. Mitsutake
Department of Biomembrane and Biofunctional Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan. E-mail: yigarash@pharm.hokudai.ac.jp

Recently, we demonstrated that activation of ceramide kinase (CERK) and the formation of its product ceramide 1-phosphate (C1P) are necessary for the degranulation pathway in mast cell, and the kinase activity of this enzyme completely depended on intracellular concentration of Ca^{2+} [Mitsutake S *et. al. J Biol Chem* 2004; **279**: 17570]. These facts indicate that the regulation of CERK activity by Ca^{2+} is significant for their functions. However the regulation mechanism has been less understood. First, we found that calmodulin (CaM) was involved in Ca^{2+} dependent activation of CERK. The CaM-antagonist, W-7 decreased both CERK activity and intracellular C1P formation. And exogenously added CaM enhanced CERK activity even in low concentration of Ca^{2+}. CERK protein was co-immunoprecipitated with anit-CaM antibody, indicating the formation of CaM/CERK complexes intracellularly. *In vitro* CaM-binding assay also showed the Ca^{2+}-dependent binding of CaM to CERK. These results strongly suggested that CaM act as a Ca^{2+} sensor of CERK. Furthermore, CaM binding assay using various mutants of CERK revealed that CaM binding site of CERK was located in aa422–435, and this region seemed like CaM binding motif, Type 1-8-14B, and was predicted to form antipathic helical wheel that need CaM recognition. At last, in mast cell degranulation, we revealed that CERK act in down stream of CaM, but independent of calmodulin dependent protein kinase II, which assumed as main target of CaM in mast cell. Secondly, ceramide kinase has an extra long N-terminal sequence containing pleckstrin homology (PH) domain, compared to other lipid kinases such as sphingosine kinases. We found that the N-terminal PH domain play an important role in plasma membrane targeting through its specific interaction with PI (4,5) P2, by utilizing various deletion mutants. This membrane localization of creamed kinas is crucial for degranulation of mast cells probably by facilitating the membrane fusion. We additionally found Leu 10 adjoining the N- terminal PH domain plays an important role for its catalytic activity, although the precise mechanism is unknown yet.

C5-003
Lipid phosphate and growth factor receptor signaling in mammalian cells

N. J. Pyne, C. Waters, N. Moughal and S. Pyne
Department of Physiology and Pharmacology, Strathclyde Institute for Biomedical Sciences, University of Strathclyde, Glasgow, UK.
E-mail: n.j.pyne@strath.ac.uk

Several lines of evidence support a *functional* interaction between endogenous Trk A receptor (binds nerve growth factor) and the lysophosphatidic acid receptor, LPA_1 receptor signaling systems in cells. First, the treatment of cells with a sub-maximal concentration of LPA and NGF induced synergistic activation of p42/p44 MAPK. Second, the transfection of cells with LPA_1 receptor

anti-sense construct, which eliminated the expression of endogenous LPA_1, reduced *both* LPA- *and* NGF-stimulated activation of p42/p44 MAPK. Third, the over-expression of recombinant LPA_1 receptor potentiated LPA and NGF-dependent activation of p42/p44 MAPK. Collectively, these observations demonstrate *integrative* signaling between endogenous LPA_1 and Trk A receptor in mammalian cells. Comparison will be made with a related lipid phosphate receptor for sphingosine 1-phosphate, $S1P_1$ and integration with the PDGFβ receptor signaling systems. We have also demonstrated trafficking of the LPA_1 to the nucleus of mammalian cells. Several lines of evidence suggest an important role for the cell-matrix interaction in regulating nuclear localization of LPA_1 receptors. Indeed, nuclear localization of the LPA_1 receptor is correlated with actin stress fiber formation, suggesting that actomyosin contractility is required for LPA_1 receptor trafficking to the nucleus. These latter findings highlight a potential novel role for nuclear LPA_1 receptor signaling in mammalian cells.

C5-004
Computationally-guided structure discovery of lysophosphatidic acid receptors in designing novel ligands

G. Tigyi
Department of Physiology, University of Tennessee, Memphis, TN, USA. E-mail: gtigyi@physio1.utmem.edu

The lysophospholipid growth factors sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) are ligands for the S1P1 and LPA1 receptors, respectively. In addition, LPA is ligand for the lipid-regulated nuclear transcription factor PPARgamma. Computational models were developed and experimentally validated for the S1P1, S1P2, S1P4, LPA1, LPA2, LPA3 and PPARg receptor-ligand complexes. These validated models have been instrumental in understanding the differences in ligand recognition between each receptor subtype. Furthermore, LPA was docked computationally to the crystal structure of PPARg and this model revealed unique interactions, which distinguish LPA from the thiazolidine agonist Rosiglitazone. The modeling studies of S1P and LPA GPCR indicate that glutamate and glutamine (3.29) control the ligand specificity in the EDG receptor family between the two types of ligands. This specificity prediction was validated using site-directed mutants of S1P1 and LPA1 through radioligand binding, ligand-induced GTP-γ-35S binding, and receptor internalization assays. In PPARg, R288 is important for LPA binding but not of Rosiglitazone whereas, H323 and 449 each are essential for Rosiglitazone recognition without affecting that of LPA. The validated computational model of LPA receptors led us to derive a two point-of-contact model for ligand binding in the EDG family GPCR. This hypothesis has aided the identification of short-chain phosphatidates, acetalphosphates and fatty alcohol phosphates as novel subtype-selective ligands of the LPA receptors. Thus, computational mapping of the ligand-binding pocket can provide valid information necessary for understanding the molecular pharmacology of this receptor family.

C5-005
Calcium mobilization by sphingosine kinase and sphingosine-1-phosphate

D. Meyer zu Heringdorf
Institut für Pharmakologie, Universitätsklinikum Essen, Essen, Germany. E-mail: meyer-heringdorf@uni-essen.de

Changes in intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) form a signal that is involved in the regulation of many cellular functions. Cells express a toolkit of proteins and messenger molecules that work together to generate spatially and temporally organized Ca^{2+} signals. During the past years it has been demonstrated that sphingosine kinase and its product sphingosine-1-phosphate (S1P) play a role in Ca^{2+} mobilization by plasma membrane receptors, complementing the phospholipase C/inositol-1,4,5-trisphosphate pathway. Sphingosine kinase activity and S1P production can be stimulated by a variety of membrane receptors, among them G protein-coupled receptors, receptor tyrosine kinases, antigen and cytokine receptors. Inhibition of sphingosine kinase or sphingosine kinase antisense attenuated $[Ca^{2+}]_i$ increases induced by these receptors. However, the mechanism by which intracellularly formed S1P releases stored Ca^{2+} is still unknown. S1P is also an abundant extracellular mediator that activates specific G protein-coupled receptors. Several of these receptors induce $[Ca^{2+}]_i$ increases in response to extracellular S1P. Nevertheless, there is strong functional evidence that intracellular S1P mobilizes stored Ca^{2+} independently of G protein-coupled plasma membrane receptors, probably by directly acting on the Ca^{2+} stores. Intracellular application of S1P induced a Ca^{2+} mobilization from thapsigargin-sensitive stores even when Ca^{2+} signalling by G protein-coupled S1P receptors was blocked or when these receptors were absent. Identification of the protein(s) involved in this process will be an important issue in future research.

C5-006
How sphingosine-1-phosphate is exported through the plasma membrane?

K. Liliom
Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary. E-mail: liliom@enzim.hu

The lipid mediator sphingosine-1-phosphate (S1P) regulates fundamental cellular processes including proliferation, survival/apoptosis and migration. It is produced inside cells by the action of sphingosine kinases. It can act intracellularly as a second messenger as well as extracellularly as the natural ligand of five G protein-coupled receptors. To act as an autocrine/paracrine mediator, S1P have to cross the plasma membrane. Although the efflux of S1P has been demonstrated for several processes, the mechanism of transport is still unknown. For example, platelets are capable of storing S1P at micromolar concentration and release it to blood in response to thrombin stimulation. Activated mast cells generate S1P intracellularly which then leads to the activation of its cell-surface receptors. The secretion of S1P in HEK293 cells and other cell types has also been shown. It has been observed in yeast that the oligomycin resistance gene YOR1 endowed resistance to sphingosine. YOR1 is the yeast ortholog of the human multidrug resistance-associated proteins (MRP). The 13 human MRP belong to the superfamily of ATP-binding cassette proteins (ABCC subgroup). MRP transport organic anions, including leukotrienes, steroid hormones, gluthatione and glucuronide conjugates and bile salts. At least MRP4 and MRP5 have been shown to transport nucleoside monophosphates. The common feature of MRP is their broad substrate specificity.In this study, the possible involvement of MRP transporters in the export of S1P has been investigated. The intrinsic ATPase activity of MRP1, MRP2 and MRP6 is enhanced in the presence of S1P, indicative for its ATP-dependent transport. In a vesicular transport assay, the transport rate of known transported substrates of MRP1 is modified by the addition of S1P. The ATPase and transport activity measurements of the other MRP proteins are in progress.

C5-007P
Phospatidylinositol transfer protein β expression and phosphorylation is regulated by TNF-α in human neutrophils and myeloid cell lines

D. Avram, G. T. Snoek and K. W. Wirtz
Department of Biochemistry of Lipids, University of Utrecht, Institute of Biomembranes, Utrecht, the Netherlands.
E-mail: d.avram@chem.uu.nl

Phospatidylinositol transfer proteins (PI-TPs) play an important role in phospatidylinositol (PI) traffic between subcellular organelles. In mammalian cells two isoforms, PI-TPα and PI-TPβ, are expressed. Despite their high sequence homology and common biological properties, studies *in vivo* revealed distinct functions of these PI-TPs isoforms. Deletion of the PI-TPα gene leads to neurodegeneration and early death, while PI-TPβ gene deletion is lethal during murine embryonic development. Yet, the physiological pathways controlling PI-TPα / PI-TPβ expression remain elusive. Our studies show that short-lived mature human neutrophils are deficient in PI-TPα whilst PI-TPβ is expressed at high levels. 2D-PAGE followed by immunoblot analyses of the neutrophil cytoplasmic proteome revealed a second PI-TPβ form with a more acidic pI (6.3) most likely representing the isomer phosphorylated on Ser262 [1]. Activation of neutrophils by the proinflammatory cytokine TNF-α induced PI-TPβ over-expression and phosphorylation most likely via PKC-δ-dependent NF-kB transcription factor. Concomitant with TNF-α-controlled upregulation of the pI 6.3 form a third form with a more acidic pI (6.1) was detected probably representing phosphorylation of the second site Ser165. In the presence of the PKC-δ inhibitor rottlerin known to inhibit NF-kB nuclear translocation, the expression of PI-TPβ and its phosphorylated forms were entirely down-regulated. These results were confirmed also in other myeloid lineage cells such as promyelocytic HL-60 cells and THP-1 monocytes. Studies are in progress to demonstrate that upregulation and phosphorylation of PI-TPβ are part of the apoptosis program.

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C5-008P
Calcium signaling induced by sphingosylphosphorylcholine in thyroid cancer cells

E. Afrasiabi[1] and K. Törnquist[1,2]
[1]*Department of Biology, Åbo Akademi University, Turku, Finland,* [2]*The Minerva Foundation Institute for Medical Research, Helsinki, Finland. E-mail: emad.afrasiabi@abo.fi*

Several sphingolipid derivatives, including sphingosylphosphorylcholine (SPC), regulate a multitude of biologic processes. In the present study we show that human thyroid cancer cells (FRO cells) expresses G protein-coupled receptor 4 (GPR4), a putative SPC-specific receptor. In these cells SPC evoked a concentration-dependent increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) in a calcium containing, but not in a calcium-free buffer. Sphingosine 1-phosphate (S1P) evoked an increase in $[Ca^{2+}]_i$ in both a calcium-containing and a calcium-free buffer. The phospholipase C (PLC) inhibitor U 73122 potently attenuated the effect of SPC, suggesting that effects of SPC were mediated by a G-protein coupled receptor. Overnight pretreatment of the cells with pertussis toxin did not affect the SPC-evoked response. Interestingly, SPC did not evoke an increase in inositol phosphates, although S1P did so. Furthermore, in cells pretreated with thapsigargin to deplete intracellular calcium stores, SPC still evoked an increase in $[Ca^{2+}]_i$, suggesting that SPC mainly evoked entry of extracellular calcium. When the cells were pretreated with the protein kinase C (PKC) inhibitor GF 109203X, or when the cells were pretreated with PMA for 24 h, the SPC-evoked calcium entry was attenuated. Thus, the SPC-evoked calcium entry was apparently dependent on PKC. In sharp contrast, the increase in $[Ca^{2+}]_i$ evoked by S1P was not sensitive to GF109203X. Furthermore, the calcium entry evoked by the diacylglycerol analog OAG was not inhibited by GF109203X. Our results thus suggest, that in thyroid FRO cancer cells, the SPC-evoked entry of calcium was mediated by the PLC-PKC pathway.

C5-009P
Lysophosphatidic acid is a mediator of Trp-Lys-Tyr-Met-Val-D-Met-induced calcium influx

H.-Y. Lee[1], H.-K. Kang[1], J. Yun[1], J.-I. Park[1], J.-Y. Kwak[1], D. S. Min[2], J.-S. Chang[3] and **Y.-S. Bae**[1]
[1]*Department of Biochemistry, Dong-A University, Busan, South Korea,* [2]*Department of Molecular Biology, Pusan National University, Pusan, South Korea,* [3]*Department of Life Science, Daejin University, Pochun, Kyeonggido South Korea.*
E-mail: yoesik@donga.ac.kr

Intracellular calcium homeostasis is very strictly regulated, and the activation of G-protein-coupled receptor (GPCR) can cause two different calcium changes, intracellular calcium release and calcium influx. In this study, we investigated the possible role of lysophosphatidic acid (LPA) on GPCR-induced calcium signaling. The addition of exogenous LPA induced dramatic calcium influx but not intracellular calcium release in U937 cells. LPA-induced calcium influx was not affected by pertussis toxin and phospholipase C inhibitor (U73122), ruling out the involvement of pertussis toxin-sensitive G-proteins, and phospholipase C. Stimulation of U937 cells with Trp-Lys-Tyr-Met-Val-D-Met (WKYMVm), which binds to formyl peptide receptor like 1, enhanced phospholipase A2 and phospholipase D activation, indicating LPA formation. The inhibition of LPA synthesis by phospholipase A2-specific inhibitor (MAFP) or n-butanol significantly inhibited WKYMVm-induced calcium influx, suggesting a crucial role for LPA in the process. Taken together, we suggest that LPA mediates WKYMVm-induced calcium influx.

C5-010P
H_2O_2 is a signal molecule in the genesis of gallbladder tonic contraction

J. Behar, P. Cong and P. Biancani
Motility Research, Medicine, Brown University Medical School, Providence, RI, USA. E-mail: Jose_Behar@brown.edu

The gallbladder (GB) muscle contraction is due to myogenic mechanisms. We showed that indomethacin and an EP1receptor antagonist inhibit GB muscle contraction and that endogenous H_2O_2 stimulates PGE2. We examined whether the H_2O_2 generated by the cytosolic NAD[P]H contribute to the tonic

contraction by stimulating PGE2.The muscle tension was abolished by NAD[P]H inhibitor apocynin and by catalase (H_2O_2scavengers). The contraction decreased dose-dependently by 80 ± 1.2% suggesting that it is dependent on H_2O_2 generated by NADPH oxidase. Stretching denervated muscle strips by 1, 2, 3 and 4 g for 1 h caused a tension dependent increase in H_2O_2 and PGE2 levels compared to strips without stretch. The increase in tension, H_2O_2 and PGE2 levels were blocked by apocynin and tiron. Western blotting showed the presence of NAD[P]H subunits P47, P67, Rac1 and Nox1 but not Rac2 in GB muscle cells. Permeabilized GB muscle cells pretreated with antibodies against P47, P67 and Rac1 decreased H_2O_2 and PGE2 levels ($P < 0.05$). These antibodies blocked the expected rise of H_2O_2 and PGE2 levels induced by hydrophobic bile acids. They also blocked the bile acid induced contraction of GB muscle cells but had no effect on the CCK-8 induced muscle contraction. These data suggest that ROS, generated by cytosolic NAD[P]H oxidase, contribute to the maintenance of tonic muscle contraction by stimulating PGE2 synthesis.

C5-011P
Expression of the human sphingosine kinases (huSPHKs) evokes intracellular Ca^{2+} release in yeast cells

G. Grósz[1], K. Liliom[2], L. Takács[3] and **Z. Fehér**[1]
[1]Department of Human Genetics, University of Debrecen, Medical and Health Science Center, Debrecen, Hungary, [2]Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, Hungary, [3]Biosystems International, Paris, France. E-mail: feher@jaguar.dote.hu

Sphigosine-1-phosphate (S1P), the product of sphingosine kinase, is a novel lipid messenger involved in a wide variety of mammalian signal transduction processes. S1P stimulates degranulation in mast cells, this way playing a role in the development of allergic reactions. Earlier, huSPHK1 and 2 were cloned and characterized. Genes encoding two further isoforms huSPHK3 and 4 were also cloned and sequenced (Takács et al., unpublished). Isoforms 1 and 2 were successfully expressed in mammalian cells, but expression of huSPHK3 and 4 have been unsuccessful. The aim of our experiments is to investigate if huSPHK3 and 4 code for proteins, which are functional sphingosine kinase enzymes. The four different cDNAs were cloned into the yeast expression vector, pYES2/CT with an inducible GAL promoter. Yeast transformants harboring the four recombinant plasmids were constructed. Presence of intact plasmids was proved by reisolating them from the transformants. We have constructed the sphingosine kinase deficient Δlcb4Δlcb5 and Δlcb4Δdpl1 double yeast mutants using genetic crosses. Double mutants have been identified by PCR. These mutants would be useful hosts in the gene expression experiments. We plan to use the Δlcb4Δlcb5 double mutant lacking yeast sphingosine kinase activity for the expression and activity measurement of the foreign gene products. Δdpl1 mutant yeast strains lack the enzyme dihydro-sphingosine-phosphate lyase, and are sensitive to exogenous sphingosine. We used yeast spheroplasts of the double mutant Δlcb4Δdpl1 strain, to detect Ca^{2+}-release evoked by the human sphingosine kinases using Fluo-4 as Ca^{2+}-indicator in a fluorometric assay. SPHK1 activity was characterized by a large peak whereas SPHK2 gave a small but solid peak. In the case of SPHK3 and 4 we observed activity that cannot be characterized with regular peaks, but with a sustained elevation of fluorescence. We also plan to demonstrate the expression of the foreign proteins with Western-blot analysis.

C5-012P
Sphingosylphosphorylcholine induces the proliferation of human adipose tissue-derived mesenchymal stem cells by JNK-dependent pathway

J. H. Kim, E. S. Jeon and H. Y. Song
Laboratory of Signal Transduction, Department of Physiology, Pusan National University College of Medicine, Pusan, South Korea. E-mail: jhkimst@pusan.ac.kr

Sphingosylphosphorylcholine (SPC) has been implicated in a variety of cellular responses, including proliferation and differentiation. In this study, we showed that D-erythro, but not L-threo-SPC, stereo-selectively stimulated the proliferation of human adipose tissue-derived mesenchymal stem cells (hATSCs) with a maximal increase at 5 μM and that increased the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in hATSCs. In addition, SPC treatment induced the phosphorylation of c-Jun and ERK, however, pretreatment with the Jun N-terminal kinase (JNK) inhibitor, SP600125, specifically blocked the SPC-induced phosphorylation of c-Jun but not ERK. The SPC-induced proliferation was completely prevented by pretreatment with SP600125, but not with a p38 MAP Kinase inhibitor, SB202190, and a specific inhibitor of the upstream kinase of ERK1/2 kinase, U0126. Adenoviral over-expression of a dominant negative mutant of JNK2 attenuated the SPC-induced proliferation and phosphorylation of c-Jun. From these results, we suggest that JNK plays a crucial role in the SPC-induced proliferation by inducing the phosphorylation of c-Jun.

C5-013P
RNAi-mediated p85 β-PIX silencing results in reduced cell motility through dephosphorylation of FAK

J. Lee[1], W. K. Chang[1], C. G. Park[1], W. S. Choi[2], J. W. Han[3] and **H. Y. Lee**[1]
[1]Department of Pharmacology, College of Medicine, Konyang University, Nonsan, Chungnam South Korea, [2]Department of Immunology, College of Medicine, Konkuk University, Chungju, Chungbuk South Korea, [3]Department of Biochemistry and Molecular Biology, College of Pharmacy, Sungkyunkwan University, Suwon, Kyungkido South Korea. E-mail: hoi@konyang.ac.kr

Lysophosphatidic acid (LPA) mediates diverse biological responses including cell migration and invasion. Recently, we have shown that LPA stimulates p21-activated kinase (PAK) that is critical for focal adhesion kinase (FAK) phosphorylation and cell motility. Here we demonstrate the direct evidence that p85 β-PIX is necessary for cell motility by LPA through FAK phosphorylation. LPA induced p85 β-PIX binding to FAK, and transfection of siRNA specific to p85 β-PIX led to drastic inhibition of FAK phosphorylation and cell motility. In addition, LPA-induced co-localization and peripheral redistribution of p85 β-PIX with FAK and GIT-1 were significantly inhibited by introducing p85 β-PIX siRNA. Finally, p85 β-PIX siRNA- or N19Rho-transfected cells did not affect PAK activation, while the cells stably transfected with N17Rac1 showed the reduced LPA-induced PAK activation. Taken together, the present data suggest that p85 β-PIX, located downstream of Rac1, is a key regulator for the activations of FAK and plays a pivotal role in focal complex formation and cell motility by LPA

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C5-014P
Transmembrane export of sphingosine-1-phosphate by multidrug resistance-associated proteins

E. Nagy, F. Szeri, A. Váradi and K. Liliom
Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary. E-mail: nagy_era@freemail.hu

Sphingosine-1-phosphate (S1P) is a novel lipid mediator capable of regulating such fundamental cellular processes like proliferation, survival, apoptosis and migration. It is produced intracellularly by sphingosine kinases. It acts as a second messenger as well as on the cell surface being the high-affinity ligand of five G protein-coupled receptors. The intracellularly generated S1P have to be released from cells to act as an autocrine/paracrine mediator. Although the efflux of S1P has been demonstrated, the mechanism of transport is still unknown. For example, platelets store S1P inside at micromolar concentration and release it to blood in response to thrombin stimulation. Activated mast cells generate S1P intracellularly which then leads to the activation of the S1P-selective G protein-coupled receptors. The secretion of S1P in HEK293 cells has also been shown. The oligomycin resistance gene YOR1 in yeast endowed resistance to sphingosine, possibly by exporting S1P out of cells. YOR1 is the yeast ortholog of the human multidrug resistance-associated proteins (MRP). The 13 human MRP belong to the ABCC subgroup of ATP-binding cassette proteins. MRP transport organic anions, including leukotrienes, steroid hormones, gluthatione and glucuronide conjugates and bile salts. At least MRP4 and MRP5 have been shown to transport nucleoside monophosphates. The common feature of MRP is their broad substrate specificity. In this study, the possible involvement of MRP transporters in the export of S1P has been investigated. Human MRP1, MRP2 and MRP6 were expressed in the Sf9/baculovirus expression system and membrane preparations were made. The ATPase activity of these transporters were monitored in a complexation assay system measuring the released inorganic phosphate. The intrinsic ATPase activity of MRP1, MRP2 and MRP6 is enhanced in the presence of S1P, indicative for its ATP-dependent transport. In a vesicular transport assay using inside-out membrane vesicles, the uptake rate of known transported substrates of MRP1 was measured in the presence and absence of S1P. The transport rate of LTC4 was attenuated while that of NEM-GS was enhanced by S1P. The ATPase and transport activity measurements of MRP4 and other MRP proteins are in progress.

C6 – Physical methods for studying protein–protein interactions

C6-001
Data-driven docking for the study of biomolecular complexes.

A. M. Bonvin
Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, The Netherlands. E-mail: a.m.j.j.bonvin@chem.uu.nl

With the presently available amount of genetic information, a lot of attention focuses on systems biology and in particular on biomolecular interactions. Considering the huge number of such interactions, and their often weak and transient nature, conventional experimental methods such as X-ray crystallography and NMR spectroscopy will not be sufficient to gain structural insight into those. A wealth of biochemical and/or biophysical data can however easily be obtained for biomolecular complexes. Combining these data with docking, the process of modeling the 3D structure of a complex from its known constituents, should provide valuable structural information and complement the classical structural methods. We have developed for this purpose a data-driven docking approach called HADDOCK (High Ambiguity Driven protein–protein DOCKing) (http://www.nmr.chem.uu.nl/haddock). HADDOCK distinguishes itself from ab-initio docking methods in the fact that it encodes information from identified or predicted protein interfaces in ambiguous interaction restraints (AIRs) to drive the docking process. The AIRs are defined as ambiguous distance restraints between any identified residue at the interface of one protein (''active residues'') and all identified (''active'') plus surface neighbor (''passive'') residues on the other protein and *vice versa*. The AIRs only enforce that the identified, ''active'' residues make contacts with active or passive residues of the partner protein but do not define the relative orientation of the molecules. Flexibility is accounted for in different ways during the docking which allows to model (small) conformational changes taking place during complex formation.In my talk I will discuss the various sources of data that can be used to map interactions and illustrate their use in HADDOCK with examples from our laboratory together with results from our participation to the blind docking experiment CAPRI (Critical Assessment of PRedicted Interactions) (http://capri.ebi.ac.uk).

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C6-002
Efficient NMR methods for identifying inhibitors of protein–protein interactions

C. Dalvit
Chemistry, Nerviano Medical Sciences, Nerviano (Milano), Italy. E-mail: claudio.dalvit@nervianoms.com

Protein–protein interactions govern most of the cellular activity. Often the inhibition of these interactions represents the target of a drug discovery project. High Throughput Screening (HTS) performed on these systems results in the identification of a very limited number of hits. This is due to the intrinsic difficulty for a small molecule to compete with a protein. The protein binding interfaces are typically very large made of extensive hydrogen bond networks and hydrophobic interactions. However, another likely source for the unsuccessful HTS results can be ascribed to the failure of the assay at detecting weak potential inhibitors. Primary HTS can fail at identifying inhibitors due to the complexity of the assay, the large experimental errors in the measurements, the presence of bovine serum albumin (BSA) and the impossibility of directly characterizing the concentration, purity, stability, and aggregation state of the screened compounds. Over the last few years NMR-based screening has emerged as a reliable methodology for the detection of inhibitors of protein–protein interactions. Two of the NMR approaches, recently introduced,

use fluorine NMR spectroscopy. FAXS (Fluorine chemical shift Anisotropy and eXchange for Screening) and 3-FABS (three Fluorine Atoms for Biochemical Screening) allow performing binding and functional HTS, respectively and determine with accuracy the dissociation binding constant (KD) and the 50% mean inhibition concentration (IC50) of the identified binders and inhibitors, respectively. The presentation will provide an insight into the theory and practical aspects of these two experiments and present applications to the NMR screening of different biomolecular targets using an unprecedented low protein concentration.

C6-003
Atomic resolution definition of protein–protein interactions

I. D. Campbell
Department of Biochemistry, University of Oxford, Oxford, UK. E-mail: idc@bioch.ox.ac.uk

Networks of protein–protein interactions, including signal transduction cascades are essential for the function of biological cells. Unraveling these networks has become a major focus of attention and the 'interactome' of some organisms is being mapped using the yeast two hybrid method [1]. A wide range of methods are also used for *in vitro* detection, including bioinformatics, surface plasmon resonance, analytical ultracentrifugation co-immunoprecipitation, pull-down assays, protein-array chips, calorimetry, mass spectrometry and surface plasmon resonance. There is, however, a continuing need, not least in the pharmaceutical industry, to obtain atomic resolution definition of protein–protein complexes. X-ray crystallography gives the best definition of structure but NMR is also a very powerful tool for accessing interactions between macromolecules and various ligands. These interactions can be assessed at a wide variety of levels, including qualitative screening of libraries of pharmaceuticals and complete structure determination of a protein–peptide complex. This talk will concentrate on the study of complexes, especially in situations where NMR has advantages over X-ray crystallography. Examples will include complex formation between an intrinsically unfolded peptide and a protein (host protein–pathogen protein [2] and cytoplasmic tail-signaling protein [3]) and interactions involving several protein domains [4].

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C6-004
Automation in NMR investigations of protein structure and interactions

J. L. Markley[1,2,3], A. Bahrami[1,2,3], G. Cornilescu[1,2], H. Eghbalnia[1,2], K. Hallenga[1], M. Kainosho[4], C. S. Newman[1,2], M. Tonelli[1], R. C. Tyler[2], E. L. Ulrich[2], D. A. Vinarov[2], B. F. Volkman[5], L. Wang[1,2,3], W. M. Westler[1] and Z. Zolnai[2]
[1]*National Magnetic Resonance Facility at Madison, Biochemistry, University of Wisconsin, Madison, WI, USA,* [2]*Center for Eukaryotic Structural Genomics, Biochemistry, University of Wisconsin, Madison, WI, USA,* [3]*Graduate Program in Biophysics, University of Wisconsin, Madison, WI, USA,* [4]*Center for Eukaryotic Structural Genomics, Chemistry, Tokyo Metropolitan University, Tokyo, Japan,* [5]*Center for Eukaryotic Structural Genomics, Biochemistry, Medical College of Wisconsin, Milwaukee, WI, USA. E-mail: markley@nmrfam.wisc.edu*

We are developing an integrated platform for automating, insofar as possible, the various steps in protein structure determinations by NMR spectroscopy. This platform is designed to run under a laboratory information management system ("Sesame"). The overall strategy and operational parts of this platform will be described, along with pilot studies demonstrating proof of principle of other components. Protein production and labeling makes use of *in vitro* transcription and translation by a wheat germ cell-free system. This protein production platform also supports the incorporation of a variety of labeled amino acids, including stereo array isotope labeled (SAIL) amino acids, which will simplify automation with larger proteins and protein-protein complexes. The NMR platform will make use of a fast strategy for data collection and peak identification ('HIFI-NMR', High-resolution Iterative Frequency Identification for NMR), automated software packages for backbone and sidechain assignments ('PISTACHIO', Probabilistic Identification of Spin systems and Their Assignments including Coil-Helix Inference as Output), secondary structure determinations ('PECAN', Protein Energetic Conformational Analysis from NMR chemical shifts), and data validation ('LACS' Linear Analysis of Chemical Shifts). Strategies for structure determination, refinement, validation, and investigations of molecular interactions will be discussed, along with novel structures and interactions determined.

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C6-005
Protease inhibitors of the grasshopper family: structure, molecular flexibility, specificity and mechanism of action

L. Gráf[1,2], K. Fodor[2], B. Szenthe[1], A. Perczel[3], Z. Gáspári[3], V. Harmat[4], C. Hetényi[1], J. Antal[1], J. Kardos[1], G. Katona[5] and A. Patthy[2]
[1]*Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary,* [2]*Biotechnology Research Group of the HAS, Eötvös Loránd University, Budapest, Hungary,* [3]*Department of Organic Chemistry, Eötvös Loránd University, Budapest, Hungary,* [4]*Protein Modelling Group of the HAS, Eötvös Loránd University, Budapest, Hungary,* [5]*Department of Biochemistry, University of Leicester, Leicester, UK. E-mail: graf@ludens.elte.hu*

We have previously shown that a trypsin inhibitor from desert locust, Schistocerca gregaria (SGTI) is a taxon specific inhibitor that inhibits arthropod trypsins, such as crayfish trypsin, five orders of magnitude more effectively than mammalian trypsins. A chymotrypsin inhibitor from the same source (SGCI), with a structure homologous to SGTI, however, is an equally potent inhibitor of mammalian and arthropod enzymes. Thermal denaturation experiments confirmed the inhibition kinetics studies on SGTI: upon addition of the inhibitor the melting temperatures of crayfish and bovine trypsins increased with 27°C and 4.5°C, respectively. Our studies on H–D exchange and ^{15}N bacbone dynamics of recombinant SGCI and SGTI indicate that the internal dynamics of these two inhibitors significantly differ from each other. On this basis we suggest that the unprecedented taxon specificity of SGTI might be due to its unique dynamic properties. To further explore the structural cause of this specificity we crystallized natural crayfish trypsin in complex with chemically synthesized SGTI. Structural data show that in addition to the primary binding loop, residues P3–P3' of SGTI, the interactions between SGTI and the crayfish enzyme are also extended over the P12–P4 and P4'–P5' regions. In contrast to other serine protease – protein inhibitor complexes where secondary interactions

are mostly van der Waals contacts and do not affect specificity, our present study shows that the extension of the binding surface leads, in addition to the stabilization of the complex, to an increased specificity.

C6-006
You do not need to have 'shape' to get recognized: Story of natively unfolded gamma-synuclein interaction with ankyrin repeat

V. K. Singh and Z. Jia
Department of Biochemistry, Queen's University, Kingston, ON, Canada. E-mail: singhv@post.queensu.ca

Gamma-synuclein (SNCG), a natively unfolded or intrinsically unstructured phosphoprotein is implicated in breast cancer or ovarian cancer progression. So far, downstream interacting partners of elevated SNCG in breast cancer have not been identified. Here we present first evidence of effective interaction of SNCG with ankyrin repeat, a most frequently observed protein interaction motif. The ankyrin repeat protein–protein interaction module is involved in a diverse set of cellular functions, and consequently, defects in ankyrin repeat proteins have been found in a number of human diseases. We have characterized SNCG and ankyrin interaction using circular dichroism, fluorometry, differential scanning calorimetry, gel filtration and BIAcore. Surprisingly, strong binding of single ankyrin peptide does not induce any conformational change of SNCG which still remains largely unstructured within the tight complex. Fluorescence titration using single tyrosine present in SNCG revealed involvement of N-terminal region in complex formation. The biological significance of these observations in context of molecular recognition of SNCG in breast cancer will be discussed.

C6-007P
Subcellular localization of human exonuclease 1

N. Ø. Knudsen[1], L. Vinther[1], R. Bertelsen[1], S. Holten-Andersen[1], **S. D. Andersen**[1], S. E. Liberti[1], F. C. Nielsen[2], R. Hofstra[3] and L. J. Rasmussen[1]
[1]Department of Life Sciences and Chemistry, Roskilde University, Roskilde, Denmark, [2]Department of Clinical Biochemistry, University Hospital of Copenhagen, Copenhagen, Denmark, [3]Department of Medical Genetics, University of Groningen, Groningen, The Netherlands. E-mail: sda@ruc.dk

The human exonuclease 1 (hEXO1) belongs to the RAD2 family of endo- and exonucleases and is generally described as a 5'-3' exonuclease. The exact functional and biological roles of hEXO1 remain unclear, but this exonuclease has been implicated in DNA replication, recombination, and repair. In DNA mismatch repair (MMR), hEXO1 functions downstream the initial recognition step and is the only exonuclease identified as part of the human MMR system. It is believed to function both as a 5'-3' and a 3'-5' nuclease, as well as contributing to the stability of the MMR complex. The finding that Hereditary Nonpolyposis Colorectal Cancer (HNPCC) families harbour mutations in MMR genes has generated interest in this research area. Germline mutations in at least four genes, hMSH2, hMSH6, hMLH1, and hPMS2 have been found in HNPCC patients. However, because some HNPCC families fail to display mutations in the known MMR genes, it is possible that changes in other components of the MMR pathway may be responsible. One such candidate gene is hEXO1. The recent discovery that hEXO1 plays a role in MMR and the identification of germline mutations in hEXO1 in patients with atypical HNPCC suggest a possible nexus between hEXO1 mutations and HNPCC. To characterize the biological role of hEXO1 in these processes, we studied the subcellular localization as well as complex formation between hEXO1 and other MMR proteins including mutant alleles found in HNPCC patients. We present data showing that hEXO1 localizes to nucleus, colocalizes with PCNA, and that the C-terminal region of hEXO1 is sufficient for this localization. Using site-directed mutagenesis and confocal laser-scanning microscopy, we have identified the nuclear localization signal responsible for translocation of hEXO1 into nucleus.

C6-008P
A new method for identifying protein–protein interactions in bacteria

J. Borloo, L. De Smet and J. J. Van Beeumen
Protein Biochemistry and Protein Engineering, Biochemistry, Ghent University, Ghent, Belgium.
E-mail: Jimmy.Borloo@Ugent.be

Characterizing protein–protein interactions are of crucial importance for our understanding of cellular processes and several analytical methods have become available, some monitoring the interactions *in vitro* (tandem affinity purification, GST-pull down analysis, co-immunoprecipitation,…) while others can be categorized as *in vivo* approaches (cross-linking, yeast-2-hybrid, bacterial-2-hybrid, fluorescence resonance energy transfer). These methods all have proven successful, although bearing their minuses, such as harsh elution steps, low resolution, significant background, labour intensive, specific antibody requirements, etc. One of the most popular techniques is the yeast-2-hybrid system. It allows the selection of genes encoding potential interacting proteins without the need for protein purification. The coding sequence of the first of the two proteins (bait-protein) of interest is fused to the activation-domain of a transcriptional activator while the other (prey-protein encoding sequence) is fused to the DNA-binding-domain. Protein interaction then causes the formation of the functional assembled transcriptional activator, which ultimately results in the transcription of a reporter gene, leading to an easily observable phenotype. The biggest disadvantage of this method is that the protein interactions have to occur in the nucleus (of eukaryotes). Protein–protein interactions in other cellular compartments (i.e. periplasm, membrane) are not suited to be determined by the yeast-2-hybrid method. The 2-hybrid method presented and evaluated here has been described already for eukaryotes but is modified for its application in the identification process of protein–protein interactions in bacteria. Its main advantage, compared to the general yeast 2-hybrid system, is that it can be applied to interactions occurring in the membrane/periplasm.

C6-009P
Studies on the binding interaction between peptostreptococcal protein L and a recombinant Fv

A. J. Cossins, S. L. Harrison and M. G. Gore
Department of Biomolecular Sciences, School of Biological Sciences, University of Southampton, Southampton, Hampshire, UK.
E-mail: ajc2@soton.ac.uk

Protein L is a multidomain cell wall protein from *Peptostreptococcus magnus* that is one of a group of proteins capable of binding to antibodies without producing an immune response. In contrast to other immunoglobulin (Ig) binding proteins, such as protein A from *Staphylococcus aureus* and protein G from group C and G *streptococci*, which bind at the C_H2–C_H3 interface, protein L binds exclusively to the V_L domain of κ-chains. It has previously been shown that a single Ig binding domain of protein L (PpL) has two sites of interaction with the V_L domain, with the affinity

of the second site up to 50-fold less than that of the first, depending on the nature of the κ-chain. The two sites on PpL are distinct, with only one common residue implicated in both interactions. In contrast, the binding sites on the V_L domain appear to have 10 residues common to both PpL interactions.The binding interaction between PpL and a 12.6 kDa variable light chain fragment (Fv) has been investigated. Production of a recombinant PpL and recombinant Fv fragment allows the structure of each species and the specific nature of the binding interaction to be examined using a range of techniques, including stopped-flow fluorimetry, circular dichrosism, isothermal titration calorimetry and NMR. To further understand the interaction between PpL and Fv, in particular at the second binding site, mutagenesis of both species has been undertaken and here we discuss the effect of those changes on the affinity of PpL for Fv and *vice versa*.

C6-010P
Atomic force microscopy measuring of the sizes of ''beds-on-string''-like protein-nucleic acid complexes

E. V. Dubrovin, O. I. Kiselyova and I. V. Yaminsky
Laboratory of scanning probe microscopy, Physics Department, Moscow State University, Moscow, Russian Federation.
E-mail: dubrovin@polly.phys.msu.ru

The knowledge of sizes and structure of protein-nucleic acid complexes is often the key for understanding of their functioning. For complexes with ''beds-on-string''-like structure the task of the direct complex size measurement becomes complicated due to the fact that it is not always possible to use the methods of electron microscopy that require metal treatment which may lead to the destruction of the complex or to its change in sizes. The most adequate method for such measuring is atomic force microscopy (AFM) technique, which allows the measurements to be made in conditions that are nearer to the native ones and also in liquid environment. In this work we propose the method of definition of the size of such complexes using AFM images analysis and taking into account typical peculiarities of this technique, e.g. such as broadening effect. All our discussions are illustrated by means of an example of size definition of the complex of mRNA with major core mRNP protein YB-1.

C6-011P
A cell-free synthesis system for accelerated screening and production of recombinant proteins

U. von Groll, **J. Drees**, F. Schäfer, A. Zacharias, C. Krüsemann and K. Steinert
Protein Expression and Purification, Proteomics and Protein Expression and Purification, QIAGEN GmbH, Hilden, Germany.
E-mail: jutta.drees@qiagen.com

Linking genes to protein function and characterization of proteins' roles in cellular processes are major challenges in the post-genomic era. *In vitro* translation systems are proving to be an invaluable tool for proteomics studies as they provide protein in a procedure that is quick and does not require a great deal of time or investment in specialized equipment. Such systems have the added advantage that affinity-tag motifs can be introduced at either terminus of the protein coding sequence to facilitate purification and identification. Performing a systematic determination of the optimal combination and location of affinity tags in protein expression constructs can increase the proportion and final yield of soluble, active protein. Once optimal expression conditions have been determined, proteins can be produced in larger quantities and used in more advanced studies, such as protein–protein interaction studies, interaction mapping, and protein network studies. One system finding increasing use in the field of protein–protein interactions is the bead-based xMAP system. Using this system, proteins are immobilized on microscopic polystyrene beads and added to test samples containing potential interaction partners. Protein–protein interactions that take place at the beads' surface are detected by fluorescently labeled antibodies directed against interaction partners. Here we present data showing the robustness and efficiency of the EasyXpress system for the *in vitro* synthesis of recombinant proteins. The flexibility offered by the EasyXpress Linear Template Kit enables multiple expression constructs to be screened in parallel for optimal expression of soluble protein. We also demonstrate a protein–protein interaction mapping procedure using *in vitro* translated proteins and the LiquiChip xMAP-based assay system.

C6-012P
An NMR structural analysis of the transient complexes formed by Cytochrome f with Plastocyanin and Cytochrome c6

I. Díaz-Moreno[1], A. Díaz-Quintana[1], M. Ubbink[2] and M. A. De la Rosa[1]
[1]*Instituto de Bioquímica Vegetal y Fotosíntesis, University of Sevilla, Seville, Spain,* [2]*Gorlaeus Laboratory, Leiden Institute of Chemistry, University of Leiden, Leiden, The Netherlands.*
E-mail: irene@ibvf.csic.es

Plastocyanin (Pc) and Cytochrome c6 (Cc6) are two soluble metalloproteins acting as alternative redox carriers between cytochrome b6-f and photosystem I (PSI). The redox reaction of cytochrome f (Cf) with Pc and Cc6 has thus been used as an excellent case to study, in a comparative way, the nature of biological transient interactions. At present, there is a large amount of functional data regarding the interaction of Cf with Pc, but not on that with Cc6. We have determined the NMR structure of the Pc–Cf complex from the cyanobacterium Nostoc by dissecting the diamagnetic and paramagnetic contributions to the chemical-shift perturbations of Pc resonances upon Cf binding. The experimental restraints have been used to determine the complex structure by rigid body dynamics. For the Nostoc Cc6–Cf complex, only the diamagnetic contributions to the chemical shifts of Cc6 signals have been used as ambiguous restraints in docking calculations. Such NMR restraints have made it possible to solve the structure of the two complexes. Our data is consistent with a single conformation in which the hydrophobic patch surrounding tyrosine 1 in Cf docks the hydrophobic patch of Pc and Cc6, whereas electrostatic charge complementarities are established between the rims of the respective recognition sites. The orientation of Pc and Cc6 relative to Cf in Nostoc corresponds to a novel conformation, which is significantly distinct to those reported for other organisms (Díaz-Moreno et al. 2005; *JBC*, in press).

C6-013P
Structural characterization of the lactoferrin/ceruloplasmin complex by Small Angle X-Ray Scattering

A. Sabatucci[1], P. Vachette[2], C. Angelucci[1], V. Vasyliev[3], M. Beltramini[4], I. Cozzani[1] and **E. Dainese**[1]
[1]*Department of Biomedical Sciences, University of Teramo, Teramo, Italy,* [2]*IBBMC, UMR 8619 CNRS, Université Paris-Sud, Orsay, France,* [3]*Department of Molecular Genetics, Institute for Experimental Medicine, Saint Petersburg, Russian Federation.,* [4]*Department of Biology, University of Padua, Padova, Italy,*
E-zmail: edainese@unite.it

Ceruloplasmin (ferro-O2-oxidoreductase, EC 1.16.3.1) is a copper protein found in vertebrate plasma, which belongs to the family

of multicopper oxidases together with ascorbate oxidase, and laccases. In humans it accounts for 95% of plasma copper and seems to play an important role in iron metabolism and homeostasis by virtue of its capacity to oxidize Fe^{2+} to Fe^{3+}, which allows the subsequent incorporation of the latter into apo-transferrin. Like transferrin of the blood plasma, lactoferrin, the iron-containing protein of the milk and of human saliva, tightly binds two Fe^{3+}. Human ceruloplasmin and lactoferrin have been shown to interact both *in vivo* and *in vitro* forming a complex. Here we describe a structural study of this complex by Small Angle X-ray Scattering (SAXS). Our structural analysis shows that ceruloplasmin interacts with lactoferrin forming a complex via an interaction between the region containing the trinuclear copper cluster responsible for its ferroxidase activity and the region containing the iron binding sites of lactoferrin.The complex of lactoferrin with ceruloplamin we analyzed *in vitro* has possible physiologic implications on account that such proteins are known to be involved in acute inflammation processes and in antimicrobial activities. Thus, taking into account that apo-lactoferrin is able to specifically bound Fe^{3+} ions preventing their utilization by pathogenic bacteria, we propose that the assistance of ceruloplasmin ferroxidase activity can play a key role for its efficient function as it is believed for the iron incorporation into plasma transferrin.

C6-014P
A new mode of recognition: an entropically favoured T-cell receptor

L. K. Ely[1], T. Beddoe[1], J. M. Matthews[2], L. Kjer-Nielsen[3], J. McCluskey[3] and J. Rossjohn[1]
[1]*The Protein Crystallography Unit, Department of Biochemistry and Molecular Biology, Monash Universtiy, Clayton, Victoria, Australia,* [2]*School of Molecular and Microbial Biosciences, The University of Sydney, Sydney, New South Wales Australia,* [3]*Department of Microbiology and Immunology, The University of Melbourne, Parkville, Victoria Australia.*
E-mail: Lauren.Ely@med.monash.edu.au

T-cell receptors (TcRs) are heterodimeric cell-surface receptors that selectively recognize antigen presented as peptides by major histocompatibility complex molecules (MHC/peptide). The ligation of the TcR to the MHC/peptide complex has been characterized by typically low affinity interactions with slow association and fast dissociation rates. It has been proposed that these slow association rates and thus high activation energies are due to large conformational adjustments that the TcR makes in order to bind the MHC/peptide complex. Previous studies have suggested that the low affinity of this interaction is due to unfavourable entropic forces created by a flexible interface being stabilized upon binding. We have previously determined high-resolution crystal structures of the TcR clone LC13 in both the unliganded and liganded states. This wealth of structural knowledge renders this an ideal system to further explore the energetic basis of TcR – MHC/peptide interactions. A thermodynamic analysis was undertaken using surface plasmon resonance (Biacore) and isothermal titration calorimetry. The studies found the ligation of the LC13 TcR to the HLA-B8/peptide complex is both entropically and enthalpically favourable with a ΔH of –3.6 kcal/mol and a $T\Delta S$ of 3.4 kcal/mol. Both of these parameters lie outside the range of those previously reported for TcR – MHC/peptide interactions and most interestingly render this interaction the first example of an entropically favourable TcR binding event. This result reveals an important additional layer of complexity within the typical docking framework of TcR – MHC/peptide complex and furthermore has implications for our broader understanding of receptor-antigen recognition.

C6-015P
Studies on Pdx1 and Pdx2 – two proteins responsible for de novo vitamin B6 biosynthesis in the human malaria parasite, Plasmodium falciparum

K. Flicker[1], M. Gengenbacher[2], B. Kappes[2] and P. Macheroux[1]
[1]*Institute of Biochemistry, Universtiy of Technology Graz, Graz, Austria,* [2]*Institute of Parasitology, University Heidelberg, Heidelberg, Germany. E-mail: karlheinz.flicker@tugraz.at*

Vitamin B6 (pyridoxine) in its biologically active form pyridoxal-5'-phospate (PLP) plays an essential role as a cofactor of various enzymes involved in amino acid metabolism. Bacteria, fungi and plants are the only organisms capable to synthesize pyridoxine. Therefore, vitamin B6 is essential for both animals and humans. Until recently the biosynthetic pathway operating in *Escherichia coli* has been thought to be ubiquitous but has now been shown to be generally limited to the gamma-branch of proteobacteria. Pdx1 and Pdx2 from Plasmodium falciparum are orthologs of the extremely conserved but functionally poorly characterized family of SNO and SNZ proteins. These proteins are constituents of an alternative *de novo* vitamin B6 pathway. Neither of the proteins shows homology to proteins involved in vitamin B6 biosynthesis in *E. coli*. Both proteins are also thought to be involved in resistance against oxidative stress caused by singlet oxygen and in thiamine biosynthesis. It is believed that Pdx1 and Pdx2 form a complex with glutamine-amidotransferase activity in which Pdx2 functions as glutaminase and Pdx1 as acceptor of the ammonia. Here we report the heterologous expression of both His-tagged Pdx1 and Pdx2 in *E. coli* and their purification in high yield by metal affinity chromatography. Interaction of Pdx1 and Pdx2 was studied by size exclusion chromatography and native PAGE. Biochemical characterization of Pdx1 and Pdx2 showed that Pdx2 functions as a glutaminase in the presence of Pdx1. In this project we will further focus on the structure–function relationship between Pdx1 and Pdx2 and investigate the interaction of the two proteins by surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC). Due to the absence of Pdx1 and Pdx2 in humans the elucidation of the function and interaction of Pdx1 and Pdx2 in P. falciparum is of highest interest as both proteins would be excellent targets for the development of new antimalarial drugs.

C6-016P
Structural and functional studies of non-erythroid spectrin fragment at the tetramerization site

L. W.-M. Fung, S. Mehboob and C. Sumandea
Chemistry, University of Illinois at Chicago, Chicago, IL, USA.
E-mail: lfung@uic.edu

We used SpaII-1-149, an aII brain spectrin model peptide similar in sequence to SpaI-1-156, a well characterized model peptide of the aN-terminal region of erythrocyte spectrin, to study its association affinities with a bI-spectrin peptide, SpbI-1898-2083, by isothermal titration calorimetry. We also determined its conformational flexibilities in solution by small angle X-ray scattering (SAXS) methods. The findings were compared with those of SpaI-1-156. Our studies show that the affinity of SpaII-1-149 with SpbI-1898-2083 is much higher than that of SpaI-1-156. Our SAXS findings also indicate a significantly more extended conformation for SpaII-1-149 than for SpaI-1-156. For SpaI-1-156, the SAXS results are consistent with a flexible junction between Helix C' and the triple helical bundle that allows multiple orientations between these two structural elements, in good agreement with our published NMR analysis. The SAXS findings for SpaII-

1-149 support the hypothesis that this junction region is rigid (and probably helical) for aII brain spectrin. Mutations in the partial domain and the junction region in SpaII-1-149 affect its association with SpbII. We used random mutagenesis methods to introduced mutations in the region consisting of residues 1–45 and used the yeast two-hybrid system to study mutational effects on spectrin association to form tetramers. The mutations that affected association were clustered in the region predicted by sequence alignment to be crucial in nonerythroid a-spectrin for tetramerization, a region that spanned Residues 12–36, corresponding to the partial domain Helix C' (Residues 21–45) in erythroid a-spectrin.

C6-017P
Two-photon fluorescence microscopy *in vivo* studies of GFP-yeast ribosomal stalk proteins

A. Garcia-Marcos[1], S. Sanchez[2], P. Parada[1], D. Jameson[3], E. Gratton[2] and J. P. Ballesta[1]
[1]*Centro de Biología Molecular Severo Ochoa, Universidad Autonoma de Madrid, Madrid, Spain,* [2]*Laboratory for Fluorescence Dynamics, Department of Physics, University of Illinois, Urbana, IL, USA,* [3]*Department of Molecular Biology, University of Hawaii, USA. E-mail: alberto.garcia.marcos@gmail.com*

The S. cerevisiae ribosomal stalk, an essential large subunit protuberance, is made of a 34 KD protein, P0, and four 12 KDa acidic proteins, P1α, P1β, P2α and P2β *In vitro* results indicate that in purified ribosomes the four acidic proteins are present as monomers, which can form two preferential associations, P1α-P2β, P1α-P2β. Nevertheless, it has been shown that free P2 proteins can form dimers in solution, and unpublished cross-linking data suggest the existence of P2 dimers in the particles. To test the composition of the stalk inside cells, P0 and the four acidic proteins were GFP tagged, and expressed in yeast strains lacking the corresponding wild type proteins. The four single disrupted strains and all possible double disruptants were prepared. The transformed strains were studied by two-photon fluorescence microscopy and data analyzed using photon-counting-histogram (PCH) method, which allowed estimation of GFP molecules per particle. Using P0-GFP as a reference, the number of tagged acidic proteins associated to ribosomes was estimated. The results show that in cells expressing one tagged acidic protein only one GFP molecule is associated to the ribosomes. When two tagged acidic proteins were introduced simultaneously two GFP molecules were detected, except in the case of the cells expressing P2β and P1α at the same time. The results strongly support that the eukaryotic stalk in wild-type ribosomes is indeed made of protein monomers inside the cell, though when more than one acidic protein are missing two copies of the same protein can bind to the particles.

C6-018P
Construction and characterization of protein rLG, a novel 16.5 kDa hybrid protein with a large binding repertoire for immunoglobulin fragments

S. L. Harrison, N. G. Housden and M. G. Gore
Department of Biomolecular Sciences, School of Biological Sciences, University of Southampton, Southampton, Hampshire, UK. E-mail: slh1@soton.ac.uk

Several proteins isolated from the surface of Gram-positive pathogenic bacteria have been shown to bind immunoglobulin (Ig) in a non-antigenic manner. The most widely studied of these proteins are protein A from *Staphylococcus aureus*, protein G from groups C and G *streptococci*, and protein L from *Peptostreptococcus magnus*. Although very useful reagents these all have limitations to their general applicability.Attempts have previously been made to broaden the binding spectra of individual Ig-binding molecules through the production of multidomain fusion proteins. The binding repertoires of proteins A and G restricts binding of a construct to specific Ig isotypes only. The ability of protein L to uniquely bind the variable domain of κ-chains makes this protein a potential tool for the purification of Ig regardless of isotype. In the present study, a single κ-chain binding domain of protein L was linked to a single Fc-binding domain of protein G to yield the novel, recombinant protein rLG gene. The rLG gene was cloned into the expression vector pKK223-3 allowing it to be over-expressed in *E.coli* JM103 cells. These studies show that despite its small size (Mr = 16.5 kDa), protein rLG exhibits simultaneous binding of ligands to both moieties, thereby making it a more versatile tool for Ig purification. A program of site directed mutagenesis (SDM) has been employed to characterize the binding properties and structural stability of protein rLG by equilibrium and stopped-flow fluorimetry, isothermal titration calorimetry, immuno-diffusion assay, affinity chromatography, circular dichroism and chemical denaturation studies.

C6-019P
Thermodynamics of interactions of natively partially folded EspB from enterohaemorrhagic *E. coli* with human β-catenin tail domain

D. Hamada[1], K. N. Suzuki[1], T. Ikegami[2] and I. Yanagihara[1]
[1]*Department of Developmental Infectious Diseases, Research Institute, Osaka Medical Center for Maternal and Child Health, Izumi, Osaka Japan,* [2]*Laboratory of Structural Proteomics, Institute for Protein Research, Osaka University, Suita, Osaka, Japan. E-mail: daizo@mch.pref.osaka.jp*

Type III secretion system enables several gram-negative bacteria to secrete and inject pathogenicity proteins into the cytosol of eukaryotic host cells. The bacteria contains type III secretion system show a typical filamentous apparatus which is evolutionary related to flagella proteins on the cell surface. In the case of enterohaemorrhagic or enteropathogenic *E. coli* (EHEC or EPEC, respectively), it has been proposed that EspA filaments attach to host cells via EspB/D pore-complexes and that the pore-complex also interacts specifically with the host protein, β-catenin to inhibit F-actin accumulation. These protein complexes regulate effector secretion and delivery into host cells. We have previously shown that EspB is a natively partially folded protein which assumes a partially folded conformation under near physiological conditions. We here analyzed the interaction of EspB and β-catenin tail domain using various methods including the change in fluorescence anisotropy of fluorescein-labeled EspB upon binding to β-catenin tail domain. The binding constant of this interaction is 2.5×10^{-5}/M The free energy of binding calculated from these analysis is –32 kJ/mol at 20ordm;C which include large negative enthalpy (−55 kJ/mol) and entropy (−79 J/mol/K). This result is consistent with the idea that certain conformational restraints are introduced upon binding of natively partially folded EspB to β-catenin. How the interaction of EspB with β-catenin affects other types of interactions in which β-catenin is associated with are discussed further in detail.

C6-020P
Association of hnRNP S1 proteins with vimentin intermediate filaments in migrating cells

A. Inoue[1], T. Watanabe[2], K. Tominaga[3], K. Tsugawa[4], K. Nishio[5], K. P. Takahashi[6] and K. Kaneda[2]
[1]*Molecular Mechanisms of Biological Regulation, Osaka City University, Graduate School of Medicine, Osaka, Japan,* [2]*Department of Anatomy, Osaka City University, Graduate School of Medicine, Osaka, Japan,* [3]*Department of Third Internal Medicine, Osaka City University, Graduate School of Medicine, Osaka, Japan,* [4]*Department of Environmental Sciences, Osaka Women's University, Sakai, Osaka, Japan,* [5]*Department of Anatomy and Cell Biology, Nagoya University School of Medicine, Nagoya, Aichi, Japan,* [6]*Department of Developmental Anatomy, Osaka Prefecture College of Health Sciences, Habikino, Osaka, Japan,* [7]*Department of Anatomy, Osaka City University, Graduate School of Medicine, Osaka, Japan. E-mail: ainoue@med.osaka-cu.ac.jp*

S1 proteins C2 and D2 are multi-functional hnRNP proteins acting as transcriptional regulators in the nucleus. Besides this, immunofluorescence staining revealed that they occur in the cytoplasm also, often in association with vimentin intermediate filaments (VFs) in various cells in culture. Here, we verified the vimentin-association of S1 proteins by employing vimentin-deficient cells, cross-linking, and immunoprecipitation, and further investigated its biological significance. S1 proteins on VFs, referred to here as S1-fibers, disappeared in highly confluent cells, where cell proliferation and cellular metabolic activity largely decreased due to cell density-dependent arrest. However, the disappearance of S1-fibers was not related to these reduced activities, but to inhibited cell migration. In fact, while undetected in non-migratory tissue cells as well as in confluent cultured cells, S1-fibers were found in all examined migratory cells such as cultured cells in scratch/wound experiment, neutrophils and monocytes from the blood, and fibroblasts engaging in tissue-healing. In addition, S1-fibers re-appeared even in confluent cells when VFs were induced to reorganize with okadaic acid. We interpret these results as suggesting that S1 proteins occur in association with VFs in migratory cells. Possible participation of S1 proteins in the formation/reorganization of VFs is discussed.

C6-021P
Interaction of mitochondrial uncoupling protein UCP2 with spin-labeled fatty acids

P. Jezek[1], N. Raju[2], J. Jezek[1], T. Spacek[1], I. M. Caminiti[2] and W. E. Trommer[2]
[1]*Department75 Membrane Transport Biophysics, Institute of Physiology, Prague, Czech Republic,* [2]*Biochemistry, Department of Chemistry, Technical University of Kaiserslautern, Kaiserslautern, Germany. E-mail: jezek@biomed.cas.cz*

Mitochondrial uncoupling protein UCP2 promotes weak uncoupling attenuating mitochondrial production of reactive oxygen species [1]. Fatty acids (FAs) were found to be essential for UCP2-induced uncoupling [2]. We used EPR spectroscopy with spin-labeled-FAs to investigate the putative FA binding site on UCP2. UCP2 was refolded from inclusion bodies in nonaethylene glycol monodecyl ether (C12E9) micelles. 4-PROXYL-stearic acid bound to UCP2 exhibited a clearly separated h+1I "immobile" peak and highly reduced h+1M "mobile" peak in the EPR spectrum. Competition of 142 μM 4-PROXYL-stearic acid with ω-6 eicosatrienoic acid and octadecane-sulfonate proceeded at micromolar range as indicated by the gradual rise of "mobile" peaks h+1M and h−1M in the low and high field region, respectively. It proves the existence of a common binding site for fatty acids and hydrophobic anions on UCP2. In contrast, 12-hydroxylauric acid, which is unable to activate UCP2, did not competitively replace 4-PROXYL-stearic acid bound to UCP2 at concentrations up to 400 μM. On the contrary, at 0.3 μM ATP the h−1M peak exhibited twice as higher amplitude as in control and the amplitude was again diminishing to the levels equivalent to zero ATP at concentrations above 2.3 μM ATP. At ~30 μM ATP, a charge effect partially released the restricted motion of the 4-PROXYL-stearic acid bound to UCP2. These data provide evidence that the nucleotide binding site resides at a distinct location from the fatty acid binding site.

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C6-022P
Substrate recognition and cleavage in 8-oxoguanine DNA glycosylase catalysis – the dynamics of conformational transitions

V. V. Koval[1,2], N. A. Kuznetsov[1,2], D. O. Zharkov[1,2], G. A. Nevinsky[1,2], K. T. Douglas[3] and O. S. Fedorova[1,2]
[1]*Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Science, Novosibirsk, Russian Federation,* [2]*Novosibirsk State University, Novosibirsk, Russian Federation,* [3]*School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, UK. E-mail: koval@niboch.nsc.ru*

Multiple conformational changes are the general feature of enzymatic mechanisms. Enzyme structure can be pre-formed for substrate recognition or can be optimized for each step in the reaction sequence. In the latter case enzyme plays a dynamic role in the catalytic process by directly coupling conformational changes throughout the macromolecule to the catalytic process. In any event, the conformational adaptability of the macromolecule clearly is essential for enzyme functions. We have studied the dynamics conformational transitions of repair processes catalyzed with 8-oxoguanine DNA glycosylases, *E. coli* Fpg-protein and human hOgg1. These enzymes remove from DNA oxidatively damaged bases, formed due to action of reactive oxygen species. The conformational transitions in 8-oxoguanine DNA glycosylases were studied using stopped-flow technique with tryptophan fluorescence detection. DNA substrates contained oxoG, deoxyribose (AP-site) and tetrahydrofuran (non-cleavable AP-site) residues. In single-turnover conditions multiple transient changes in enzyme fluorescence were observed, indicating sequential conformational transitions in the both protein molecules. The results obtained with stopped-flow experiments were compared to results from gel-electrophoresis analysis. The data provide useful information on the mechanisms of both 8-oxoguanine DNA glycosylases demonstrating the coupling conformational changes with substrate recognition and processing.

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C6-023P
Carbohydrate moiety of glycoproteins – analysis using biosensor BIAcore

B. Krotkiewska[1], M. Buchaniewicz[1], T. Banas[1] and H. Krotkiewski[2]
[1]*Department of Biochemistry, Medical University, Wroclaw, Poland,* [2]*Department of Immunochemistry, Institute of Immunology and Exp. Therapy, Wroclaw, Poland.*
E-mail: krotkiew@iitd.pan.wroc.pl

In attempt to apply a biosensor BIAcore, with surface plasmon resonance (SPR) as a detection method, in analysis of the carbohydrate moiety of glycoproteins we isolated and purified two human glycoproteins: IgG from serum and glycophorin A (GPA) from erythrocyte membranes. IgG was isolated from the sera of healthy individuals and from the sera of patients with rheumatoid arthritis (RA), GPA originated from the blood group A, B and O erythrocytes. In the biosensor methodology, applied here, four lectins were used as the ligands: *Ricinus communis* (RCA-I , reacts with terminal Gal residues), *Griffonia simplicifolia* (GSL-II, reacts with terminal GlcNAc residues), *Triticum vulgaris*(WGA, reacts with GlcNAc and sialic acid residues) and *Sambucus nigra*(SNA-I, reacts with sialic acid linked alpha2–6), and two mentioned above glycoproteins were used as analytes (conc. 2.5 – 7.0 μg/100 μl of a working buffer). It was shown that analyses performed in a biosensor may help to determine some of the structural features of the glycoproteins, i.e. status of sialylation/desialylation (determined for GPA), status of reduction of the disulphide bonds (determined for IgG), presence of terminal galactoses in IgG (comparison of the samples from healthy people and RA patients). During experiments some practical remarks of the biosensor technology were established, which may help in the future work.

C6-024P
Characterization of differentiation-inducing epitopes of the cell surface CD44 antigen expressed on human acute myeloid leukemia blasts

N. Lecomte[1], L. Durand[1], A. Roseto[2], J. Kadouche[3], z C. Chomienne[1] and F. Smadja-Joffe[1]
[1]*Institut Universitaire d'Hématologie, Hôpital Saint-Louis, Paris, France,* [2]*Université de Technologie de Compiègne, Compiegne, France,* [3]*Laboratory of Immunology, University of Paris, Paris, France.*
E-mail: fjsmadja@infobiogen.fr, nicolas.lecomte@chu-stlouis.fr

CD44 is a type-1 transmembrane highly glycosylated protein encoded by a single 60 kb gene comprising ten constitutively expressed standard exons encoding the CD44s standard isoform (80–90 kDa) and ten alternatively spliced variant exons expressed in CD44v variant isoforms CD44s and CD44v are strongly expressed on leukemic blasts from patients displaying an Acute Myeloid Leukemia (AML), a heterogeneous clonal malignancy characterized by an excess of immature myeloid cells which are unable to terminally differentiate. We have reported that certain anti-CD44 mAbs (monoclonal antibodies) can trigger terminal differentiation of primary AML blasts [Charrad et al. *Nature Medicine* 1999), raising the possibility to use these mAbs for developing a CD44-targeted differentiation therapy in AML. We presently aimed to characterize, by X-ray crystallography, the epitope(s) mapped by 2 distinct differentiation-inducing anti-CD44 mAbs. To obtain high levels of soluble glycosylated CD44 proteins retaining native conformational properties, we constructed fusion proteins comprising the CD44s extracellular domain plus the human IgG1 Fc fragment. Moreover, we added a polyhistidine tag, which allows to easily purify them by double affinity to Ni^{2+} and G-protein. Eight distinct CD44-Fc constructions have been prepared, transiently expressed in COS-7 cells and the best one, selected because of its higher affinity for the anti-CD44 mAbs and its high expression level, has been transfected into CHO-K1 cells. Using a stable clone, we succeeded to produce large amounts of CD44-Fc, in serum-free culture medium, using CELLine CL1000 bioreactor flasks. The results of crystallographic and Surface Plasmon Resonance (SPR) studies involving CD44-Fc and the differentiation-inducing anti-CD44 mAbs will be presented and discussed.

C6-025P
Characteristics of an immunoglobulin-binding protein based on a single domain of protein G from *Streptococcus* group G

N. M. Muir and M. G. Gore
Department of Biomolecular Sciences, School of Biological Sciences, University of Southampton, Southampton, Hampshire, UK.
E-mail: nmb@soton.ac.uk

Protein G (SpG) is a Type III bacterial Fc receptor found on the surface of or secreted by the cell wall of the β-haemolytic Gram-positive bacterium *Streptococcus* of the C and G strains, and has the ability to bind IgG of different subclasses of most mammalian species. Protein G binds selectively to both the Fc and Fab regions of most mammalian IgGs. NMR and crystallographic studies of others have provided the structure for a single domain of protein G (SpG) in complex with the Fc and Fab of IgG. SpG uses its α-helix and β-strand 3 to bind predominantly to the interface of C_H2-C_H3 heavy chains of IgG-Fc and its β-strand 2 to form an anti-parallel interaction with the last β-strand of the C_H1 domain of IgG-Fab. Specific amino-acid residues that contribute to these binding interactions *via* a network of hydrogen bonds have been identified and studied by a programme of site directed mutagenesis. Unique Trp residues (W48 or W20) have been used to facilitate equilibrium and pre-equilibrium fluorescence studies to observe binding to Fc or Fab and to determine the Kd for the various equilibria. The effect of second substitutions of residues implicated in H-bond formation to either the Fc or Fab IgG fragment have been determined. SpG species which only bind to Fc or Fab have been developed. ITC studies have also been employed to characterize the thermodynamic changes that take place in the reactions.

C6-026P
Study of amino acid complexation with metal by using different methods (Study of thermodynamics between histidine and copper(II))

N. Mortazavi[1], H. Naghibi Beidokhti[1], A. A. Saboury[2] and A. Nasehsadeh[1]
[1]*Physical Chemistry Research, Department of Chemistry, Shahid Bahonar University, Kerman, Iran,* [2]*Biochemistry, Institute of Biochemistry and Biophysics, Tehran University, Tehran, Iran.*
E-mail: niaz58@yahoo.com

Proteins are composed of 20 essential amino acids which have donor atoms suitable for coordination to metal ions. Transferrin is one of the proteins that has an important role in biological systems. Histidine is one of the amino acids which has an important role in metabolism, absorption, repulsion, consumption and reservation of Fe(II) through His-Fe bonding. The excess amount of copper in our body will damage our biological system. In this

work, the thermodynamics of the interaction between Cu(II) in aqueous solution with Histidine, has been investigated by spectrphotometric, potentiometric and Isothermal calorimetric techniques (ITC), at various temperatures (between 5 and 45°C) and two different ionic strengths (I = 0.1 and 0.15 M). Acid dissociation constants of the ligands and the stability constants of Cu(II) chelate compound at various temperatures and ionic strength are reported. The ratio of the metal to ligand was determined by UV-Vis spectrophotometric method in a buffer solution (acetate buffer PH = 6.6) and (max wave length = 620 nm) and ionic strength 0.1 M. Potentiometric titrations of the amino acid in the absence and in the presence of the metal ion were used to determine the thermodynamic parameters at various temperatures and two ionic strengths by using Bjerrum method. The obtained results are compared with the results of the ITC method. ΔH (kJ/mol) is determined and its amount is −92.9 (kJ/mol) and ΔG_o (kJ/mol) = −184 (25 °C). logK1 = 10.03 and logK2 = 8.03 are determined that the results in both methods are similar. These tests and calculations have considered a good coupling with the potentiometric results. This coupling have shown that the methods, tests, resolution, repeatability, accuracy and theoretical prospection have a good precision. The logK1 and logK2 was calculated with the Eureka program. These tests and calculations were considered a good coupling with the potentiometric results. This coupling has shown that the methods, tests, resolution, repeatability, accuracy and theoretical prospection have a good precision. The results have been used to describe the bonding in the Cu-His system.

C6-027P
A force spectroscopic analysis of intracellular antigen-antibody interactions using nanoneedle and AFM

C. Nakamura[1,2], T. Sato[2], I. Obataya[1], N. Nakamura[1,2] and J. Miyake[1,2]
[1]*Research Institute of Cell Engineering, National Institute of Advanced Industrial Science and Technology, Amagasaki, Japan,* [2]*Division of Biotechnology and Life Science, Tokyo University of Agriculture and Engineering, Koganei, Japan.*
E-mail: chikashi-nakamura@aist.go.jp

A development of cell manipulation is one of the most important technologies in the field of life science involving biotechnology, diagnostics and medicine. If very precise method to handle the molecules in a cell body would be developed, we could investigate the cell life deeply. So we are developing a tool for performing low invasive operations on living cells using atomic force microscopy (AFM) and a modified AFM tip. We call the technique, 'Cell Surgery'. The apparatus for the cell surgery is designed to keep cells alive whilst measurement of exerting forces on the AFM tip during cell contact. The AFM tips are sharpened to ultra-thin needles of about 200 nm in diameter using focused ion beam etching. We call the sharpened tip, 'Nanoneedle'. Force-distance curves obtained during insertion process gives information about the nanoneedle position to cell body, contact, penetration and evacuation. The reproducible sudden force relax represents a point of penetration of the nanoneedle through the cell surface. The nanoneedle insertion never kills the cell. Thus, after a nanoneedle insertion the manipulated cell can be used for further analysis or manipulation. This is a novel technology that can be applied for material transfer into cell or intracellular manipulation. When the anti-actin-antibody-immobilized nanoneedle was inserted to a human melanocyte, we could observe a strong interaction from a force curve during a needle evacuation process. The result indicates that intracellular interactions between the cellular actin filaments and the antibodies immobilized on the needle surface can be observed. We expect that this technique can be applied as a novel diagnostics for a living cell.

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C6-028P
Understanding interaction properties in β-lactoglobulin in solution

M. G. Ortore[1,2], F. Carsughi[2], P. Mariani[2], R. Sinibaldi[2], F. Spinozzi[2] and G. Onori[1]
[1]*Dipartimento di Fisica, Università degli Studi di Perugia, Perugia, Italy,* [2]*Dipartimento di Scienze Applicate ai Sistemi Complessi, Università Politecnica delle Marche, Ancona, Italy.*
E-mail: mg.ortore@alisf1.univpm.it

The application of high pressure to a protein solution perturbs the structure of the protein and its interactions with solvent in a controlled way. We are studying the effects of increasing pressures on β-lactoglobuline (BLG), a protein belonging to the lipocaline family whose function *in vivo* is still unclear. Small Angle Neutron and X-Ray Scattering (SANS and SAXS) experiments have been performed to evaluate both structural modification and protein–protein effective interactions in different experimental conditions. In fact, we used buffers with different percentages of ethylene-glycol (EG: 0–50% w/w), since EG modifies solvent's permittivity in a controlled and continuous way. In particular, it is known that at acidic pH (a condition similar to the physiological one) the quaternary dimeric structure of BLG is modified, with a partial dissociation into two monomers still showing a fairly ordered structure. SAS results indicated that compression induces dissociation of oligomers and that the presence of ethylene-glycol modifies this behaviour moving the transition to higher pressures. Since the form factors of both the species are known, we derived the protein–protein effective interactions. Moreover, we compared the trend of dissociation free energy versus pressure with the one expected considering a model potential. The results showed that a simple interaction model that accounts for the excluded-volume effects and a screened Coulomb repulsion is not able to reproduce experimental data. The contribution of non-polar interactions and desolvation effects reveals to be the key to give a more realistic interaction model. Furthermore, the combination of SANS and SAXS techniques provide information about modifications of the hydration shell during pressure treatment.

C6-029P
The autocatalytic reaction of hydrogenase enzyme

J. Ösz, G. Bodó, R. M. M. Branca and C. Bagyinka
Department of Biophysics, Biological Research Center, Szeged, Hungary. E-mail: oszj@brc.hu

Hydrogenases are metalloenzymes that catalyze the reaction $H_2 \rightarrow H^+ + 2e^-$. Though the enzymatic activity of hydrogenase is determined routinely, a number of contradictory results have been published and the activity of this class of enzymes has not yet been thoroughly explained. We examined the special spatial distribution of the hydrogenase uptake reaction, the dependence of front velocity on the concentration of hydrogenase and electron acceptors, the kinetic characteristics of the reaction such as lag phase, enzyme concentration dependence, and also the enzyme activation. We have made some autocatalytic and non-autocatalytic model calculations as well. Our results showed that the hydrogenase reaction can only be explained by assuming an autocatalytic step in the reaction cycle. The activation of hydrogenase is not necessarily autocatalytic it is rather a twofold process. The first step is the removal of the oxygen, the second one

is the redistribution of the enzyme forms in the enzyme cycle producing available amount of hydrogen bound enzyme form which can autocatalytically interact with another enzyme form which does not bind hydrogen.

C6-030P
Oligomerization confers hydrophobicity and fusogenic activity to a domain of VSV (Vesicular Stomatitis Virus) -G protein.

F. Carneiro[1], A. Da Poian[1], G. Vandenbussche[2] and **J.-.M. Ruysschaert**[2]
[1]Department Bioquimica Medica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [2]Centre of Structural Biology and Bioinformatics, Université Libre de Bruxelles, Brussels, Belgium. E-mail: jmruyss@ulb.ac.be

VSV (Vesicular Stomatitis Virus) peptide (VTPHHVLVDEYTGEWVDSQF) is as efficient as the whole virus in catalyzing membrane fusion at pH 6.0. 20 amino acid long VSV peptide contains four acidic amino acid residues and two histidines [1]. We found that liposomes fusion was highly increased at concentration above 2 μg/ml. Interestingly, hydrophobicity (detected by ANS binding) increased drastically at the peptide concentration required for fusion. Peptide labelling with DCCP (dicyclohexylcarbodiimide) of negatively charge amino acids and of histidines with diethylpyrocarbonate inhibited both fusion and hydrophobicity. In most cases, the oligomerization of fusion peptides is driven by changes in their secondary structure. Here, there is no change in secondary structure (IR spectroscopy) and oligomerization is triggered by the protonation of His residues that interact electrostatically with negatively charge residues. Such an oligomerization reorients hydrophilic residues and creates a hydrophobic envelope. This organization explains the capacity of this peptide to induce cell fusion and to generate syncytia formation.

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C7 – Membrane proteins - membrane traffic

C7-001
New insights into the role of lipid rafts in GPCR signal transduction using Plasmon Waveguide Resonance (PWR) spectroscopy

V. J. Hruby[1], I. D. Alves[1], Z. Salamon[1] and G. Tollin[1]
[1]Laboratory of Victor J. Hruby, Department of Chemistry, University of Arizona, Tucson, AZ, USA, [2]Laboratory of Gordon Tollin, Department of Biochemistry and Molecular Biophysics, University of Arizona, Tucson, AZ, USA. E-mail: hruby@u.arizona.edu

Plasmon Waveguide Resonance (PWR) spectroscopy is a powerful new biophysical method which allows for the first time the use of p and s polarized light to examine structural changes, kinetics and thermodynamics of anisotropic biological systems and processes such as those in proteolipid membrane environments. We have used this method to probe the mechanisms of G-protein coupled receptor (GPCR) signal transduction, and have obtained new insights into the specific signaling pathways upon interaction of agonists and antagonists with GPCRs. We have extended these studies to examine the effects of lipid microdomains (rafts) on the binding, signaling and transduction pathways. In particular, we have examined the microdomains that result from a 1:1 mixture of palmitoyloleoylphosphatidylcholine (POPC) and sphingomyelin (SM), and their influence on the partitioning and functioning of the human delta opioid receptor (hDOR) into these microdomains and the influence of ligands on the process. We have directly observed in real time the formation of two lipid bilayer microdomains, and the preferred segregation of the delta opioid receptor (hDOR) into SM lipid rafts when the agonist ligand was bound, but not for the unoccupied receptor, which preferentially incorporated into the POPC-rich domain. Furthermore, we can demonstrate directly that, G-proteins bind much more strongly to the hDOR receptor in the lipid raft (SM-rich) environment than in the fluid non-raft (POPC-rich) domain of the lipid bilayer. The implications of these findings for novel design of drugs, and drug screening will be emphasized.

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C7-002
Transport, assembly and turnover of wild-type and mutant connexins linked to human disease

D. Laird, T. Thomas, E. McLachlan, J. Simek, A. Maher, C. Lounsbury, G. I. Veitch, H. Wang, J. Manias, J. Riley, R. Bhalla and Q. Shao
Department of Anatomy and Cell Biology, University of Western Ontario, London, Ontario, Canada. E-mail: Dale.Laird@fmd.uwo.ca

Gap junctions have a ubiquitous distribution in mammalian tissues and these specialized intercellular channels are essential for normal cell function and survival. It has been shown that loss-of-function mutations of specific gap junction proteins (connexins) are linked to diseases affecting the eye, ear, skin, heart, bone and nerves. In the current study we are examining connexin mutations linked to inherited syndromic deafness, various skin diseases and oculodentodigital dysplasia (ODDD). ODDD is an autosomal-dominant human disorder linked to Cx43 mutations where patients display symptoms of congenital craniofacial and limb deformities suggestive of defective osteoblast function. We are examining the consequences of Cx43 mutations on transport, assembly, stability and function, which can potentially result in either gain or loss of channel function. In parallel studies we have also expressed diseased-linked Cx26 mutants and examined their transport, assembly and function in mammalian cells that include organotypic keratinocyte cultures. While the Cx43 mutants G21R, G138R, and G60S mutants readily traffic to the cell surface, these mutants showed complete loss-of-function, not unlike the Cx26 G59A mutant. However, the loss-of-function Cx26 D66H mutant was not stabilized at the cell surface but was localized to the trans Golgi network. Importantly, when any of these mutants were co-expressed with their wild-type connexin counterpart, they dominantly inhibited gap junction function and trans-dominantly inhibited the function of other connexin family members. Collectively, these studies suggest that patients suffering from either Cx43-linked ODDD or Cx26-linked skin diseases express connexin mutants that greatly inhibit overall gap junctional intercellular communication.

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C7-003
The scaffolding protein Shank and associated glutamate receptors induce formation and maturation of dendritic spines

G. Roussignol[1], F. Ango[1], S. Romorini[2], J. C. Tu[3], C. Sala[2], P. Worley[3], J. Bockaert[1] and **L. Fagni**[1]
[1]IGF, CNRS-INSERM-UM1,2, Montpellier, France, [2]Cell Mol Pharmol, CNR-Inst. Neurosci., Milan, Italy, [3]John Hopkins, Medical School, Baltimore, MD, USA.
E-mail: lfagni@ccipe.cnrs.fr

Formation of a dendritic spine begins with apparition of a filopodia that maturates into a spine displaying neck and head. The spine head supports a protein network, the so-called postsynaptic density that organizes glutamate receptors at the postsynaptic membrane. Shank is a large scaffolding protein that is part of this network and which links the ionotropic and metabotropic glutamate receptor complexes together, as well as to intracellular signaling pathways and actin filaments. We studied the role of Shank and glutamate receptors in dendritic spine formation. Knock down of Shank with siRNA lowered the density of dendritic spines in spiny hippocampal neurons. In aspiny cerebellar granular cells, over-expression of Shank induced formation of dendritic spines that co-localized with axon terminals, increased the number of endogenous functional glutamate receptors and increased spontaneous synaptic activity. Shank mutations that altered interactions with glutamate receptor complexes reduced functional expression of these receptors, as well as spine maturation, including formation of functional synaptic contacts. Treatments with glutamate receptor agonists partially rescued these effects, whereas glutamate receptor antagonists impaired maturation of the Shank-induced spines. These results show that Shank can trigger formation of spines, whereas glutamate receptor activation controls maturation of these spines.

C7-004
SNAREs can promote complete fusion and hemifusion as alternative outcomes

C. G. Giraudo, C. Hu, T. J. Melia and **J. E. Rothman**
Department of Physiology and Cellular Biophysics, Columbia University, College of Physicians and Surgeons, New York, NY, USA.
E-mail: jr2269@columbia.edu

Cognate v- and t-SNARE on vesicles and target membrane pair to form the core machinery for intracellular membrane fusion, and the compartmental specificity of intracellular membrane fusion can be recapitulated from the pattern of fusion by isolated SNARE proteins. Here we expand upon a cell fusion assay in which ''flipped''-SNAREs are ectopically expressed on the cell surface [Hu et al., 2003] to monitor single fusion events between cells. Using a range of extracellular and intracellular membrane markers, content markers, and protein constructs, we find that, in addition to complete fusion, SNAREs also promote hemifusion as an alternative outcome. About 65% of events resulted in full fusion; the remaining 35% in hemifuion; of whose about 2/3 were permanent and approximately 1/3 were reversible. We imagine that this relative close balance among outcomes could be tipped by binding of regulatory proteins to the SNAREs allowing for dynamic physiologic regulation between full fusion and reversible ''kiss and run''-like events.

C7-005
The SNARE complex protein syntaxin13 and the PDZ-domain protein β2-syntrophin interact with the ATP binding cassette transporter A1 (ABCA1) and regulate its function

C. Rudolph, A. Sigruener, S. Maa Bared, C. Buechler and G. Schmitz
Institute of Clinical Chemistry and Laboratory Medicine, University of Regensburg, Regensburg, Germany.
E-mail: christian.rudolph@klinik.uni-regensburg.de

ABCA1 facilitates the release of cholesterol and choline-phospholipids to apoA-I particles and may be involved in SNARE complex mediated vesicular fusion. Syntaxins as SNARE complex constituents interact with members of the ABC-transporter family. Immunoprecipitation revealed a direct association of syntaxin13 (STX13) with ABCA1 and both were identified together with flotillin-1 in detergent resistant microdomains and the phagosomal compartment in macrophages. Knock down of STX13 by siRNA decreased lipid efflux and ABCA1 protein levels. In addition, phagocytosis of latex beads by fibroblasts with mutated ABCA1 and in ABCA1-siRNA treated macrophages was enhanced and the recombinant expression of functional ABCA1 normalized phagocytic uptake.Yeast Two-Hybrid screening with the ABCA1 C-terminus revealed five PDZ-proteins, namely GIPC, MAGI-3, Syntenin, Scribble, and TIP-1. These proteins and the recently described β2-syntrophin interact with the C-terminal peptide of ABCA1 in a PDZ specific manner not affecting ABCA1 function in non-polarized cells. Therefore, the association of ABCA1 with PDZ-proteins may not modulate the extent of lipid efflux in this context. This hypothesis was further supported by the analysis of lipids, apoA-I and HDL-cholesterol in fasting plasma of β2-syntrophin deficient mice revealing no significant difference in these animals. Recombinant expression of the β2-syntrophin PDZ-domain reduced basolateral cholinephospholipid efflux and induced apical efflux in Caco-2 cells. In conclusion STX13 is involved in SNARE complex fusion events of ABCA1 containing vesicles with the plasma membrane and the identified PDZ-proteins may play a role in trafficking of ABCA1 to the basolateral membrane of polarized cells.

C7-006
Regulation of mitochondrial function by membrane fusion, fission and biogenesis

G. Szabadkai, K. Bianchi, D. De Stefani, A. M. Simoni and R. Rizzuro
Department of Experimental and Diagnostic Medicine, University of Ferrara, Ferrara, Italy. E-mail: szg@unife.it

Mitochondrial morphology is regulated by multiple mechanisms. The recently described family of mitochondria associated ''large GTPase'' dynamin-like proteins synchronize fusion and fission events of mitochondrial membranes. Drp-1, a member of this family, localizes to mitochondrial fission points and promotes mitochondrial division, while mitofusins (Mfn-1 and 2) drive the fusion of individual mitochondria. On the other hand, the nuclear transcriptional coactivator PGC-1α is responsible to induce an increase in the mitochondrial network volume as a result of increased mitochondrial biogenesis and changes of its proteome. By modifying the expression and function of Drp-1, Mfn-1,2 and PGC-1α, we induced morphological changes of the mitochondrial network and examined its effect on its functional state. For this purpose we applied 3D reconstitution microscopy for morphological imaging and mitochondrially targeted Ca^{2+} sensitive luminescence and fluorescent protein probes for imaging the

spatio-temporal dynamics of mitochondrial [Ca^{2+}]. Our results demonstrate that the regulated process of mitochondrial biogenesis and fusion and fission controls the spatiotemporal properties of mitochondrial Ca^{2+} responses, and thus physiological and pathological consequences of cellular $CaCa^{2+}$ signals.

C7-007P
Dissecting the functional roles of the *E. coli* signal recognition particle in membrane protein biogenesis

E. Bochkareva and E. Bibi
Department of Biological Chemistry, Weiznann Institute of Science, Rehovot, Israel. E-mail: elena.bochkareva@weiznann.ac.il

The bacterial signal recognition particle (SRP) system plays an important role in biogenesis of membrane proteins. The *E. coli* SRP system includes two proteins Ffh and FtsY that are homologues of the eukaryotic SRP54 and the SRP-receptor a-subunit, respectively [1]. Despite the notion that both proteins function in the same pathway, it has been observed that their genetic depletion might confer different effects upon biogenesis of membrane proteins [2]. In order to examine this difference methodically, we investigated and compared the expression, localization, and assembly of several membrane proteins in isogenic and non-isogenic *E. coli* strains depleted of FtsY or Ffh. The results showed that in cells depleted of FtsY, the level of expression of polytopic membrane proteins is markedly reduced, although no increased mRNA degradation or post-translational proteolysis was observed. In contrast, membrane proteins are expressed and localized normally in Ffh-depleted cells, but their membrane topology and activity are disrupted [3]. A possible role for SRP in the inhibition of membrane protein expression in FtsY-depleted cells was examined using an *in vitro* translation system. The results showed that SRP added in excess to the system specifically inhibits synthesis of membrane proteins. Collectively, the results raise the possibility that SRP plays a dual role in membrane protein biogenesis in *E. coli*: (i) it is required for proper assembly of membrane proteins and (ii) it is involved in a quality control mechanism that prevents membrane protein synthesis in the cytoplasm [3].

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C7-008P
Proteins that modulate the heteromeric amino acid transporters: identification and functional characterization

P. Bartoccioni, A. Zorzano, M. Palacín and J. Chillarón
Laboratory of Molecular Patology, Department of Biochemistry and Molecular Biology, University of Barcelona, Barcelona, Spain. E-mail: chiarabartox@yahoo.com

Six families of plasma membrane amino acid transporters have been described in mammals.These heteromeric amino acid transporters (HATs) are composed of a heavy subunit (HSHAT; the two members known to date are rBAT and 4F2hc) and the corresponding light subunit (LSHAT), linked by a disulfide bridge. The heavy subunit rBAT and 4F2hc consist of 685 and 529 amino acids, respectively with an identity of 30.They are type II membrane N-glycoproteins with a single transmembrane domain, an intracellular N-terminal domain, and an extracellular C-terminal domain that shows significant homology with bacterial α-glucosidases. rBAT is mainly expressed in the brush border of epithelial cells of the kidney proximal tubule and of the small intestine. In contrast, 4F2hc is ubiquitous and basolateral in epithelial cells. Seven light chains (LAT1, LAT2, y+LAT1, y+LAT2, ascAT1, xCT and b0+AT) have been identified. These lights chains have 12 transmembrane domains with intracellular N and C-termini. LSHATs are not N-glycosylated and are highly hydrophobic. Our aim is to identify and characterize proteins that interact with the heteromeric amino acids transporters. We will use, rBAT, 4F2hc, y+LAT1 and b0+AT as our model proteins for the following reasons: these subunits are involved in the human aminoacidurias cystinuria and lysinuria and some interacting proteins could be involved in: anchoring of transporters to different domains of the plasma membrane of polarized cells; polarized transport to basolateral, or apical membrane domains; folding; transport activity and regulation. We will use at least two independent strategies to identify the eventual interactions.(i) GST-pull down. The N-terminal cytoplasmic domain of the light subunit y+LAT1 were fused C-terminally to GST coding region and over-expressed in *E. coli*. Fusion protein were purified from bacterial lysates by affinity chromatography and detected by Comassie. Our fusion protein were incubated with kidney tissue which may contain interacting proteins. The proteins that could interact will be detected by silver stain or coomassie stain on SDS-PAGE gels. This assay show the presence of two bands of 96 and 92 Kda corresponding to metalloproteases meprin A and B. Meprin may play a role in the pathophysiology of acute renal failure. The redistribution of this metalloendopeptidase to the basolateral membrane domain during acute renal failure could be results in degradation of extracellular matrix and damage to adjacent peritubular structures. (ii) Two-hybrid system The cytoplasmic domains NH2 of light subunit y+LAT1 and of the heavy subunit 4F2 were our bait. The cDNA prey derived from kidney libraries. We used the Matchmaker Gal4 Two-Hybrid system 3 of Clontech. In the first assay with 4F2 we obtained 18 positives clones but unfortunately none could be confirmed by the usual tests of two hybrid. In the second assay with y+Lat1 we obtained 25 positives clones. At the moment two of these were confirmed like positives and they are the protein Pias1 (E3-Sumo ligase) and the protein Ubc9 (E2-Sumo conjugating enzyme). There are some membrane transporters (GLUT1 and GLUT4) that are modified by sumoylation and we observed that the N-terminal domain of Y+LAT1 have a sumoylation motif Ψ-KxE/D. Actually we will confirm these interaction by GST-pull down assay.

C7-009P
The influence of Pex8p on formation of the importomer within peroxisomal matrix protein import

K. Emmrich and W.-H. Kunau
Abteilung Zellbiochemie, Institut für Physiologische Chemie, Ruhr-Universität Bochum, Bochum, Germany. E-mail: kerstin.emmrich@rub.de

Peroxisomes are ubiquitous organelles that fulfill a large variety of metabolic functions. The organelles consist of a proteinaceous matrix enclosed by a single membrane. Peroxisomes transport folded and even oligomeric proteins (synthesized in the cytosol) across their membrane. Along with approximately 12 membrane-bound peroxins two cycling cytosolic receptors, Pex5p and Pex7p, are involved in the import process. Recently the organization of components of the peroxisomal import machinery in two major subcomplexes has been defined [Agne et al., 2003]. These

are the docking subcomplex containing of Pex14p, Pex17p and a fraction of Pex13p, and the RING finger subcomplex comprising Pex2p, Pex10p and Pex12p. Furthermore Pex8p, a very low abundant peroxin, located at the inner face of the peroxisomal membrane, was demonstrated to mediate the association of these two core complexes into a larger import complex named the importomer. To further study the function of Pex8p within the formation of this importomer we carried out an extensive mutational analysis. Strains expressing C-terminal truncations of Pex8p fused to the IgG binding domain derived from *Staphylococcus aureus* proteinA (ProtA) were constructed. Furthermore we created a collection of point mutations within PEX8. To determine the biological functionality of the modified proteins we investigated strains expressing these proteins concerning their ability to grow on medium containing oleic acid as sole carbon source. Additionally we analyzed peroxisome morphology using electron microscopy and investigated the functional import of peroxisomal matrix proteins by fluorescence microscopy. When using Pex8p-ProtA as bait to isolate protein complexes from detergent extracts in wild type cells the components of the importomer Pex14p, Pex17p, Pex13p, Pex10p, Pex12p as well as the receptor Pex5p were coisolated. As in Δpex8 cells there is no peroxisomal import as well as no formation of the importomer it was concluded that the association of the two core complexes via Pex8p is essential for protein import into the peroxisome. Here we present a modified form of Pex8p that has high influence on formation as well as on composition of the importomer.

C7-010P
Post-translational modifications of the mitochondrial outer membrane proteins: carnitine palmitoyltransferase-I, long-chain acyl-CoA synthetase, and voltage dependent anion channel

C. Hoppel[1], J. Kerner[2] and A. M. Distler[2]
[1]*Pharmacology / Medicine, Case Western Reserve University School of Medicine, Cleveland, OH, USA,* [2]*Nutrition, Case Western Reserve University School of Medicine, Cleveland, OH, USA.*
E-mail: charles.hoppel@case.edu

Long-chain fatty acids require both activation and transport into the mitochondrial matrix before β-oxidation can occur. Carnitine palmitoyltransferase-I (CPT-I), long-chain acyl-CoA synthetase (LCAS), and voltage dependent anion channel (VDAC), localized in the mitochondrial outer membrane play a critical role in this transport process and are the focus of our study. To achieve our goals, we developed a method for the analysis of hydrophobic membrane proteins. The method is based on the isolation of the resident membranes, isolation of the proteins by gel eletrophoresis, electroelution of the proteins, and enzymatic digestion of the proteins by both trypsin and proteinase K. The resulting peptide mixtures from the digestions were analyzed by MALDI-TOF and HPLC ESI-MS/MS. With this method, we have achieved 75 to 99% sequence coverage for the above mentioned transmembrane proteins. This methodology provides a targeted approach for examining membrane proteins in detail including post-translational modifications and identification of less abundant isoforms. In summary using our method: (a) we documented several phosphorylation sites on CPT-I, VDAC-1, and LCAS-2 using peptide mass fingerprinting and ESI-MS/MS, (b) on the CPT-I protein we identified a nitrated tyrosine in position 589, (c) we provided evidence for acetylation of the N-terminal alanine in VDAC-1, and (d) we found that in the mature rat liver CPT-I the cDNA deduced N-terminal methionine is removed and alanine is acetylated. Studies regarding the functional/regulatory impact of these post-translational modifications are currently being addressed.

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C7-011P
Protein translocation to the intermembrane space of mitochondria

N. Mesecke and J. M. Herrmann
Institute of Physiological Chemistry, University of Munich, Munich, Germany.
E-mail: nikola.mesecke@bio.med.uni-muenchen.de

Proteins located in the intermembrane space (IMS) of mitochondria perform a large number of crucial functions in the eukaryotic cell, e.g. in protein import, oxidative phosphorylation, metal ion transport and apoptotic signalling. In contrast to most mitochondrial precursors, IMS proteins do not contain presequences and are imported in an ATP and membrane potential-independent process. Many IMS proteins are of low molecular mass, contain highly conserved cysteine residues and coordinate metal ions. Studies on one of these proteins, Tim13, showed that this IMS protein diffuses rather randomly in and out of the IMS. Unidirectionality of the translocation reaction is conferred by a protein folding event in the IMS triggered by zinc binding [Lutz Neupert and Herrmann, *Embo* **22**: 4400f]. Whereas several reports now support this "folding trap" mechanism, components of this translocation pathway were not known so far. We have now identified the first component of this translocation pathway. The protein encoded by the yeast reading frame YKL195w is essential for cell viability. It exposes a highly conserved domain in the IMS that is able to bind zinc and copper ions, which directly binds to newly imported IMS proteins. In cells lacking Mia40 the endogenous levels of Tim13 and other metal-binding IMS proteins are strongly reduced due to the impaired import of these proteins. We conclude that Mia40 is the first component of a specific translocation pathway used by small metal-binding proteins of the mitochondrial IMS.

C7-012P
Mechanism of translocation of PAR-2 from the plasma membrane to the nuclear membrane

S. Nim[1,2], T. Zhu[2], A. Vazquez-Tello[2], N. Heveker[3] and S. Chemtob[1,2]
[1]*Department of Pharmacology, University of Montreal, Montreal, QC, Canada,* [2]*Laboratory of Dr Sylvain Chemtob, Department of Pharmacology, Ste-Justine Hospital, Montreal, QC, Canada,* [3]*Laboratory of Dr Nikolaus Heveker, Department of Biochemistry, Ste-Justine Hospital, Montreal, QC, Canada.*
E-mail: nim_satra@hotmail.com

The PARs are activated by the cleavage of a part of the N-terminal domain of the receptor and this cleavage generates a tethered ligand at the end of the N-terminal domain that binds to the extracellular domains of the receptor. We noted recently that the proteinase activated receptor 2 (PAR-2) can be translocated to the nuclear membrane after stimulation with trypsin or its synthetic ligand SLIGRL. The functionality of PAR-2 at the nucleus has been confirmed using calcium mobility and gene expression assays. The molecular domains responsible for translocation of the receptor to the nuclear envelope are still unknown. It has been suggested that the intracellular loops and the C-terminal domain of some GPCRs are implicated in internalization, and we proceeded to investigate this possibility. We found that truncation of the C-terminus at the position S363 prevented mobiliza-

tion of the receptor to the nuclear membrane. On the other hand, mutation of the beta-arrestin interacting domain (mutation of ST363/366AA) did not affect translocation to nucleus. While chimeric substitution of the third intracellular loop of PAR-2 with that of beta-3 adrenoceptor (which normally does not acutely internalize) abrogated translocation of the receptor to the nucleus upon stimulation. These results unveil a previously undescribed translocation of GPCRs to the nucleus, and identify critical domains required for this purpose; essentially, the C-terminus and third intracellular loop (possibly through direct or indirect interactions) are important for PAR-2 translocation to the nucleus.

C7-013P
Fusogenic activity of conformationally flexible *de novo* designed transmembrane peptides

M. W. Hofmann[1], **B. C. Poschner**[1], K. Weise[2], J. Ollesch[3], P. Agrawal[4], W. Stelzer[1], F. Hulsbergen[4], H. de Groot[4], K. Gerwert[3], J. Reed[2] and D. Langosch[1]
[1]Lehrstuhl Chemie der Biopolymere, Technische Universität München, Freising, Germany, [2]German Cancer Research Center, Heidelberg, Germany, [3]Lehrstuhl für Biophysik, Ruhr-Universität Bochum, Bochum, Germany, [4]Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands. E-mail: langosch@lrz.tum.de

Fusion of biological membranes is mediated by distinct integral membrane proteins, e.g. SNAREs and viral fusion proteins. Previous work has indicated that their transmembrane segments play an important role in fusion. Further, peptide mimics of the transmembrane part can drive the fusion of liposomes and evidence had been obtained that fusogenicity depends on their conformational flexibility. In previous experiments, the fusogenicity of synthetic polypeptides mimicking the transmembrane segments of SNARE-proteins or viral fusion proteins have been shown to depend on their structural flexibility [1, 2]. To test this hypothesis, we present a series of non-natural transmembrane segments that was designed *de novo* based on the structural properties of hydrophobic residues. A well-established lipid mixing fluorescence assay was used to measure fusion of liposomal bilayers. We find that the fusogenicity of the peptides depends on the ratio of α-helix-promoting leucine and β-sheet-promoting valine residues and is enhanced by helix-destabilizing proline and glycine residues within their hydrophobic cores [3]. The fusogenic ability of an alternating leucine/valine sequence is comparable to the transmembrane segment of the SNARE synaptobrevin and depends on the length of the peptide, as a core peptide sequence of 16 residues shows an optimum in fusion rate. Furthermore, we investigated the effects of small molecules, which have been proposed to modify the secondary structure of a peptide by stabilizing sheet conformation. Assuming that sheet-promoting molecules are able to enhance flexibility and thus fusogenicity of helical peptides, they might allow further insights into structure-function relationships of transmembrane segments and their ability to support membrane fusion. Based on our results we propose that structural flexibility of these transmembrane segments is a prerequisite of fusogenicity.

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C7-014P
In *Haloferax volcanii*, membrane protein biogenesis and protein synthesis rates are affected by decreased ribosomal-binding to the translocon

G. Ring and J. Eichler
Laboratory of Prof. Jerry Eichler, Department of Life Sciences, Ben-Gurion University, Beer-Sheva, Israel. E-mail: ringg@bgumail.bgu.ac.il

In *Haloferax volcanii*, ribosomes are found in the cytoplasm and membrane-bound at similar levels. Transformation of *H. volcanii* to express chimeras of translocon components SecY/E fused to a cellulose-binding domain substantially decreased ribosomal membrane binding, likely due to steric hinderance by the cellulose-binding domain. Treatment with puromycin, with or without low salt washes previously shown to prevent *in vitro* haloarchaeal ribosomal binding, did not release translocon-bound ribosomes, indicating that release is not directly related to the ribosomal translation status. Release was, however, achieved during cell starvation or stationary growth, pointing at regulated ribosomal release in *H. volcanii*. Decreased ribosomal binding selectively affected membrane protein levels, suggesting that membrane insertion occurs co-translationally in Archaea. In the presence of chimera-incorporating sterically-hindered translocons, the reduced ability of ribosomes to bind modulated protein synthesis rates over time, suggesting that transformed cells manage to compensate for the reduction in ribosome binding. Possible strategies for this compensation include a shift to a post-translational mode of membrane protein insertion or maintained ribosomal membrane binding.

C7-015P
Calcium-driven structural changes in toposome, a protein which mediates membrane–membrane interactions in the developing embryo

J. J. Robinson and M. Hayley
Department of Biochemistry, Memorial University, St John's, NL Canada. E-mail: johnro@mun.ca

The sea urchin embryo has long been used as a model system to study the regulatory mechanisms that control developmental processes. In this report we present the results of an analysis of calcium binding to toposome, a protein extrinsically associated with the yolk granule. This membrane-bounded organelle is prominent in the cytoplasm of the sea urchin egg and embryo and has been implicated as a source of membrane for repair of lesions in the plasma membrane. Earlier studies have suggested that toposome possesses a calcium-dependent, membrane aggregating/fusion activity. Using circular dichroism measurements, calcium-dependent secondary structural changes were detected with an apparent Kd (calcium) of 23 μM. In parallel experiments, calcium-dependent toposome binding to liposomes occurred with an apparent Kd (calcium) of 25 μM. In contrast, tertiary structural changes occurred with an apparent Kd (calcium) of 240 μM. Interestingly, the calcium concentration-dependence of toposome- driven liposome aggregation paralleled the effect of this cation on tertiary structure. Equilibrium dialysis measurements revealed that toposome binds up to 600 moles of calcium/mole of protein with an intrinsic Kd of 240 μM. These data suggest the following two-step

model describing the role of calcium-dependent structural changes in the biological activity of toposome. At low concentrations of calcium, toposome undergoes a secondary structural change, which facilitates protein binding to membranes. As increasing amounts of calcium bind, toposome undergoes a change in tertiary structure, which endows this protein with the capacity to drive membrane–membrane interactions. These interactions are required for a number of biological processes during development.

C7-016P
Biophysical investigations of synaptotagmin-SNARE interaction

A. Radhakrishnan, D. Fasshauer, K. Wiederhold and R. Jahn
Department of Neurobiology, Max Planck Institute for Biophysical Chemistry, Göttingen, Lower Saxony Germany.
E-mail: anandkpr77@yahoo.com

The SNARE complex consisting of Syntaxin 1A, SNAP-25 and Synaptobrevin 2 drives membrane fusion by forming a helical bundle. The formation of this helical complex brings two opposing membranes in close proximity eventually resulting in membrane fusion and exocytosis. The regulation of this process by other factors is essential to ensure efficient exocytosis. At the presynaptic terminal, synaptic vesicle exocytosis is dependent on a highly regulated stimulus, calcium. Synaptotagmin 1 is believed to be the calcium sensor on the synaptic vesicle. However, very little is known about how it transmits the calcium signal onto the SNARE fusion machinery. To answer these questions, a number of single cysteine surface positions of the four-helix bundle were coupled to the environmental dye IAANS. Only a few positions located in the middle of the four-helix bundle, showed clear, stoichiometric changes in fluorescence intensity upon addition of Synaptotagmin. These changes were strongest in the presence of Ca^{2+}, suggesting a direct Ca^{2+}-dependent 1:1 interaction of Synaptotagmin with the surface of the SNARE bundle. These findings were supported by anisotropy measurements using the fluorescent dye Oregon Green™ (OG) and by isothermal titration calorimetry. In addition, we constructed several single cysteine mutants of Synaptotagmin. Preliminary results using OG™ labeled Synaptotagmin single cysteine mutants and Texas Red™ labeled liposomes, have showed these mutants to interact with the lipid membrane in a calcium dependent manner.

C7-017P
Transport and reduction of dehydroascorbate in plant mitochondria

A. Szarka[1,2], N. Horemans[3], G. Bánhegyi[2], M. Mayer[1] and H. Asard[3,4]
[1]*Department of Biochemistry and Food Technology, Budapest University of Technology and Economics, Budapest, Hungary,* [2]*Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University Budapest, Budapest, Hungary,* [3]*Department of Biology, University of Antwerp, Antwerp, Belgium,* [4]*Department of Biochemistry, University of Nebraska, Lincoln, NE, USA. E-mail: szarka@mail.bme.hu*

Ascorbate, dehydroascorbate and glucose transport was investigated in plant mitochondria and mitoplasts prepared from cultured BY2 tobacco cells. Using a rapid filtration method with radio-labeled ligands, we observed a specific glucose and dehydroascorbate transport, which was temperature and time dependent and saturable. Inhibition of mitochondrial respiration by KCN and the uncoupler 2,4-dinitrophenol did not influence the transport of the investigated compounds. Dehydroascorbate transport was inhibited by glucose and genistein, while glucose uptake was decreased upon 3-O-methyl-glucose, D-mannose, cytochalasin B or genistein addition. On the other hand, a low affinity low capacity ascorbate transport was found. Oxidizing agents (potassium ferricyanide or ascorbate oxidase) increased ascorbate uptake. On the base of these facts we expected a mitochondrial ascorbate recycling machinery. The addition of dehydroascorbate to freshly purified BY2 mitochondria resulted in measurable amount of ascorbate both in the incubation media and inside the mitochondrial matrix.The results demonstrate the presence of dehydroascorbate and glucose transport in mitochondria and suggest a mitochondrial ascorbate recycling system in plant cells.

C7-018P
DMPK splice isoforms associate with the endoplasmic reticulum or mitochondrial outer membrane via unique C-terminal anchors

R. E. van Herpen, R. J. Oude Ophuis, M. Wijers, J. Fransen, B. Wieringa and **D. G. Wansink**
Department of Cell Biology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. E-mail: r.wansink@ncmls.ru.nl

Myotonic dystrophy protein kinase (DMPK) is a Ser/Thr-type protein kinase with unknown function and a member of the Rho kinase family. It was originally identified as the product of the gene mutated by CTG triplet repeat expansion in patients with myotonic dystrophy type 1 (DM1). Constitutive and regulated alternative splicing of DMPK transcripts is conserved between man and mouse and results in multiple protein isoforms carrying distinct C-termini. We set out to investigate how structural subdomains determine biochemical and cell biological properties of individual DMPK splice isoforms. By expressing single DMPKs in various cell types, including Neuro2A, C2C12 and DMPK-/- myoblasts, we demonstrate that unique sequence arrangements in DMPK C-terminal tails control the specificity of anchoring into intracellular membranes. Mouse isoforms DMPK A and C were found to associate specifically with either membranes of the endoplasmic reticulum (ER) or the mitochondrial outer membrane, respectively. Unexpectedly, however, the corresponding human DMPK A and C proteins localized both to mitochondria. Membrane association of DMPK isoforms was resistant to alkaline conditions and mutagenesis analysis showed that proper anchoring was critically dependent on basic residues – different between mouse and man – flanking putative transmembrane domains, demonstrating that DMPK C-termini form unique tail anchors. This identifies DMPK as the first kinase in the class of tail-anchored proteins. Preliminary results suggest a role for DMPK in ER or mitochondrial distribution and dynamics, or apoptosis. We are currently investigating these observations in more detail and will present our latest findings.