Supporting Online Material (SOM): Measuring Ca^{2+} induced structural changes in lipid monolayers: implications for synaptic vesicle exocytosis

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MATERIALS AND METHODS

Materials

Lipid monolayers were prepared from the lipids dipalmitoyl-sn-glycero-3-phosphatidylcoline (DPPC) and phosphatidylinositol-4,5-bisphosphate (PIP_2). These lipids were purchased as powders from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. Ethylene glycol-bis (2-aminoethyl) -N, N, N', N'-tetraacetic acid (EGTA) was purchased from Fluka (Switzerland). All other chemicals were purchased from Sigma-Aldrich (Germany) and were analytical grade or better.

A stock solution of 1 M $CaCl_2$ was made in HB150 buffer (in mM; 150 KCl, 25 Hepes (pH 7.40 KOH), 1 DTT) using ultrapure water (18 MΩ cm, Millipore, Bedford, MA). $CaCl_2$ solutions of varying concentrations were then prepared by appropriate dilution of the 1 M stock into HB150. The final effective Ca^{2+} concentrations were measured using the fluorescent indicator dyes Fluo-5N and Mag-Fura2 (Molecular Probes, Karlsruhe, Germany), against a standard curve constructed with a Molecular Probes calibration kit. Fluorescence measurements were made at 25°C using a Fluoromax2 spectrophotometer (Jobin Yvon, Bensheim, Germany).

Preparation of lipid monolayers

To prepare lipid monolayers at an air-water interface, we used a custom-made shallow Langmuir trough, equipped with a single movable Teflon barrier, available at ID10B of the European Synchrotron Radiation Facility (ESRF), Grenoble, France. The trough dimensions were 210×170×3 mm^3. For both reflectivity and GIXD measurements, the Langmuir trough was placed on an active anti-vibration table. The trough was also sealed and filled with water-saturated helium vapor. This atmosphere serves not only to reduce evaporation from the subphase and parasitic scattering from the air, but also minimizes radiation damage during the experiments. Each monolayer was exposed multiple times to collect the data at various salt concentrations in the subphase (see below). The special choice of majority lipid dipalmitoyl-sn-glycero-3-phosphatidylcoline (DPPC) in the monolayer follows from the fact that monolayer formation and chain ordering in this lipid is particularly easy, well described, and robust. The ordering in the saturated fatty acids corresponds more to a bilayer's gel phase rather than a bilayer's fluid phase.

Lipid stock solutions were initially prepared in a 20:9:1 (v/v) mixture of chloroform, methanol and water to give a final lipid concentration of 0.2 g/l.

Monolayers were prepared purely from DPPC, or a mixture of DPPC-PIP_2. In the latter case, a constant 5 mol% PIP_2 was substituted for DPPC in the lipid mix, approximating the lower limit of PIP_2 estimated to be present at sites of synaptic vesicle fusion (1, 2). The lipid monolayer was formed by spreading 75 μl of the lipid stock solution onto HB150, using a Hamilton syringe. To ensure complete evaporation of the organic solvent, the system was left undisturbed for 5 min. The lipid monolayer was then compressed or expanded with the Teflon barrier to a final interfacial surface pressure of 30 mN/m, using a barrier velocity of 18 mm/min. Measurements on both the DPPC and DPPC/PIP_2 monolayers were carried out at a temperature of 18°C, which is below the chain-melting temperature of DPPC. $CaCl_2$ and/or synaptic vesicles were subsequently injected into the subphase using a microloader. The 1M $CaCl_2$ stock was added sequentially into the subphase to achieve the desired final Ca^{2+} concentration; in a first step the Ca^{2+} concentration was raised to 1 μM, and for subsequent experiments further Ca^{2+} was added to reach effective concentrations of 10 μM and 30 μM, respectively. These concentrations are known to result in the exocytosis of synaptic vesicles from neurons (3). Synaptic vesicles were routinely used at a final concentration of approximately 1.5 g/l. Figure 1 A of the main manuscript illustrates the experimental systems used.

Grazing incidence x-ray diffraction and x-ray reflectivity measurements

Grazing incidence x-ray diffraction (GIXD) measurements on monolayers were carried out using the undulator beamline ID10B at ESRF. Briefly, a monochromatized x-ray beam was deflected by a downstream mirror to set the incident angle (α_i) onto the interface. This angle was taken to be $0.8\alpha_c$ where α_c is the critical angle for total external reflection at an air-water interface. For the wavelength $\lambda = 1.5$ Å, α_c was 2.68 mrad. To collect the diffracted beam from the sample, a linear position sensitive detector (PSD) (150 mm long Gabriel detector, EMBL, Grenoble, France) was used.

A two dimensional (2D) crystalline arrangement of lipid molecules in the monolayer plane can be calculated from the observed d-spacings using the horizontal components q_{xy}^{hk} of the momentum transfer vector. The primitive unit cell parameters a and b provide the area per chain (A_I). Further, the projected area of the chains gives a measure of the area in an untilted phase and is obtained by $A_{II} = A_I cos(\tau)$, where τ is a tilt in the hydrocarbon chain from the layer normal. It can be calculated from the peak positions of q_{xy}^{hk} and the vertical component q_z^{hk}. In the case of an orthorhombic

lattice one non-degenerate peak (q^n) and one 2-fold degenerate peak (q^d) should be registered. In the present study, the non-degenerate peak $q^{(02)}$ and the degenerate peak $q^{(11,1\bar{1})}$ are observed (see below). If none of the lipid molecules are tilted, the vertical component (q_z^{hk}) of both these two peaks will vanish. In contrast, a nearest-neighbor tilt will lead to $q_z^n = 0$ and $q_z^d > 0$, whereas a next-nearest-neighbor tilt produces $q_z^n > 0$ and $q_z^d > 0$. In this study, a single peak in the vertical component of the momentum transfer vector was observed indicating a nearest neighbor tilt in the lipid chain. We calculated the corresponding tilt angle (τ) from the expression (4, 5):

$$tan(\tau) = \frac{q_z^d}{\sqrt{(q_{xy}^d)^2 - (q_{xy}^n/2)^2}} \tag{1}$$

The shape of each Bragg peak or Bragg rod was quantified by least-square fits to a Lorentzian (6). The 2D crystalline domain size (L) was determined using the Scherrer formula, $L = \frac{2\pi \times 0.9}{FWHM}$ with $FWHM = \sqrt{w_{fit}^2 - \Delta_q^2}$ where w_{fit} is the full width at the half-maximum (FWHM) of the peak obtained from the fit, and Δ_q is the known resolution of the instrument (7, 8). The derived length provides the average size of a perfect crystal in the direction of the corresponding momentum transfer vector.

X-ray reflectivity experiments were performed at the same beamline using the same incident wave length. The beam size was 1×0.5 mm^2. The basic physical and chemical properties of the samples were similar to the GIXD measurements with a surface pressure of 30 mN/m. Once again, the scattered beam originating from the interface was collected on a PSD and measured as a function of the incident angle α_i assuming a specular recording configuration, where the exit angle $\alpha_f = \alpha_i$ and the horizontal scattering angle was $2\theta = 0$. Under these conditions, only the q_z component of the momentum transfer vector $\vec{q}$ was obtained where the z-axis is parallel to the sample normal.

The x-ray reflectivity from an interface between two media is characterized by the electron density profile $\rho(z)$ and is given by the master formula for semi-kinematical reflectivity theory (9):

$$R(q_z) = R_F(q_z) \mid \int \frac{df(z)}{dz} e^{-iq_z z} dz|^2 \tag{2}$$

where $f(z) = \rho(z)/\triangle\rho_{12}$ with $\triangle\rho_{12}$ being the density contrast between the media. The Fresnel reflectivity $R_F(q_z)$ from an ideal interface is expressed as $|(q_z - q_z^{'})/(q_z + q_z^{'})|$ with ${q_z^{'}}^2 = q_z^2 - q_c^2$. In this study, the critical momentum

transfer, q_c, is related to the critical angle, α_c. Data were analyzed using a standard box model, where each box is defined by both its electron density (height) and thickness (width) (10, 11). Both the height and width of the boxes were taken as free parameters, while the interface between the slabs was smeared with a finite value of surface roughness (maximum value $\sim$ 4 Å). Data fits were performed using a deterministic algorithm called the 'simplex method' in MATLAB.

Preparation of synaptic vesicles

Synaptic vesicles were purified from rat brain as described in Takamori et al. (13), through differential centrifugation, sucrose density centrifugation and size-exclusion chromatography. After chromatography an additional centrifugation step was introduced to allow buffer exchange and synaptic vesicle concentration. Vesicles were subsequently resuspended in HB150 and immediately snap-frozen for transportation to the synchrotron. Importantly, samples prepared and stored using this procedure remain functionally intact, as judged by the capacity of vesicles to acidify and transport neurotransmitter (12), and fuse. The weight of the vesicle population was obtained by measuring the protein mass using a modified Lowry assay (14) and assuming a constant 10:5:2 ratio of proteins, phospholipids and cholesterol (13). The final mass concentrations of the vesicle were in the range of 4.5-5.4 g/l. No aggregation was observable either by electron microscopy or dynamic light scattering (not shown).

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