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Neisserial porin (PorB) causes rapid calcium influx in target cells and induces apoptosis by the activation of cysteine proteases

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The porin (PorB) of *Neisseria gonorrhoeae* is an intriguing bacterial factor owing to its ability to translocate from the outer bacterial membrane into host cell membranes where it modulates the infection process. Here we report on the induction of programmed cell death after prolonged infection of epithelial cells with pathogenic *Neisseria* species. The underlying mechanism we propose includes translocation of the porin, a transient increase in cytosolic Ca^{2+} and subsequent activation of the Ca^{2+} dependent protease calpain as well as proteases of the caspase family. Blocking the porin channel by ATP eliminates the Ca^{2+} signal and also abolishes its pro-apoptotic function. The neisserial porins share structural and functional homologies with the mitochondrial voltage-dependent anion channels (VDAC). The neisserial porin may be an analogue or precursor of the ancient permeability transition pore, the putative central regulator of apoptosis.

Keywords: apoptosis/calcium/caspase/endosymbiosis/*Neisseria*

Introduction

Apoptosis is a genetically determined form of cell death, characterized by morphological and biochemical features such as nuclear and cytoplasmic condensation, blebbing of the plasma membrane, fragmentation of the nucleus and breakdown of DNA into oligonucleosomes (Kerr *et al*., 1972). In the final stage of apoptosis, cells display special markers on their surface leading to engulfment by phagocytic cells, a process which avoids spillage of intracellular contents and an inflammatory response. Apoptosis plays a central role during development and homeostasis of multicellular organisms.

One of the key signalling pathways to apoptosis starts with ligation of death receptors of the tumour necrosis factor (TNF)/nerve growth factor receptor family (reviewed by Whyte, 1996) which leads to the activation of the main 'executioner' molecules, the caspases (Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997). Caspases comprise a family of cysteine proteases with aspartic acid specificity. All members of this family are synthesized as dormant proenzymes that can be activated by removal of the regulatory prodomain and assembly into the active heteromeric protease. The mechanisms of caspase regulation are currently being actively investigated. One model suggests that cytochrome *c* release from the mitochondria is a prerequisite for subsequent proteolytic activation of caspase 3, a process which is regulated in turn by Bcl-2-like proteins (Golstein 1997; Kluck *et al*., 1997; Li *et al*., 1997; Reed, 1997; Bossy-Wetzel *et al*., 1998; reviewed in Kroemer, 1997a). These molecules have been reported to be localized to mitochondria (Hockenbery *et al*., 1990). The anti-apoptotic family member Bcl-x_L shares structural similarities with the pore-forming domains of bacterial toxins (Muchmore *et al*., 1996) and, like these toxins, is able to insert into synthetic lipid bilayers and to form functional ion channels (Minn *et al*., 1997).

Another protease often activated during apoptosis is the Ca^{2+}-dependent cysteine protease calpain (Sarin *et al*., 1993; Squier *et al*., 1994; Martin and Green, 1995; Nath *et al*., 1996). One of its substrates is reported to be α-fodrin, a cytoskeletal protein that may be involved in the regulation of apoptotic membrane changes such as phosphatidylserine exposure, membrane blebbing and cellular fragmentation into apoptotic bodies, as well as detachment from the surrounding tissue.

The induction of apoptosis in host cells has been described for a variety of bacterial pathogens (Zychlinsky and Sansonetti, 1997). The facultative intracellular pathogens *Salmonella* (L.M.Chen *et al*., 1996; Monack *et al*., 1996) and *Shigella* induce apoptosis in macrophages (Zychlinsky *et al*., 1992, 1994; Y.J.Chen *et al*., 1996b). Another actively investigated bacterial pathogen is *Listeria monocytogenes*, which induces apoptosis in hepatocytes as well as dendritic cells (Guzman *et al*., 1996; Rogers *et al*., 1996). Epithelial pathogen-induced apoptosis has been reported for *Helicobacter pylori* (Chen *et al*., 1997) after infection of human gastric epithelial cell lines.

The facultative, intracellular, human-specific pathogen *Neisseria gonorrhoeae* (*Ngo*) is the etiological agent of the sexually transmitted disease gonorrhea. During the course of infection the pathogen penetrates the mucosa and causes a massive inflammatory response in the subepithelial tissue. *Ngo* can enter epithelial cells as well as professional phagocytes. Several factors play a role in the infection, most notably the pili, which mediate primary adherence (McGee *et al*., 1983; Rudel *et al*., 1992), the Opa-proteins which mediate adhesion and invasion (Makino *et al*., 1991), and the PorB porin. Porins are found in the outer membrane of Gram-negative bacteria and also in mitochondria. In bacteria they function as homotrimers and generate pores allowing the passage of solutes. PorB of pathogenic *Neisseriae* has the unusual feature of translocating from the outer membrane of the bacteria into artificial membranes as well as into target cell membranes (Lynch *et al*., 1984; Blake and Gotschlich,

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1987; Weel and van Putten, 1991). The insertion process leads to the formation of a functional channel which, strikingly, is regulated by the eukaryotic host cell (Rudel *et al*., 1996). Similar to mitochondrial porins, PorB interacts with purine nucleoside triphosphates which down-regulate the pore size and cause a shift in voltage dependence and ion selectivity. The remarkable parallels between PorB and mitochondrial pores led to the hypothesis that early in evolution, intracellular pathogens may have used porin-like channels to induce death of the host when the cytoplasmic level of ATP/GTP was low (Frade and Michaelidis, 1997).

In this study we provide evidence that gonococci cause apoptosis in epithelial and phagocytic cells lines *in vitro*. An intriguing novel mechanism is proposed: translocated neisserial porin induces apoptosis by causing a rapid calcium influx, followed by the activation of the calcium-dependent cysteine protease calpain and the central apoptosis-executing molecules, the caspases.

Results

Infection of the HeLa epithelial cell line with Ngo strains induces apoptosis

Since gonorrhoea is often associated with a strong inflammatory reaction we investigated the potential cytotoxic effects of *Ngo* during *in vitro* infections of HeLa cells, a well established infection model for gonococci. Infection of HeLa cells with invasive or piliated *Ngo* strains at a multiplicity of infection (m.o.i.) of 1 for 15 h resulted in apoptosis detectable by morphological as well as biochemical criteria (Figure 1A–D). The chromosomal DNA of infected cells was fragmented extensively into oligonucleosomes, clearly visible as a characteristic DNA ladder as soon as 12 h after infection (Figure 1A). Neither non-cytotoxic commensal *Neisseria* strains nor an invasive *Escherichia coli* strain expressing the invasin of *Yersinia pseudotuberculosis* induced fragmentation of DNA (data not shown). HeLa cells treated with TNFα in combination with cycloheximide to induce apoptosis exhibited a similar kinetic of chromosomal fragmentation as the infected cells, whereas actinomycin D showed a delayed response. Next, a method that displays fragmented DNA (Figure 1B) was employed, allowing quantification of the cell population. HeLa cells were infected with a piliated (P^+), adherent *Ngo* strain as well as its non-piliated (P^-), non-adherent derivative and a commensal *Neisseria sicca* strain. Cells showing decreased fluorescence after propidium iodide (PI) staining (marked by a gate in Figure 1B) compared to those in G_1 or G_2 phase of the cell cycle are depicted to the left of the diagram. This so-called hypo-diploid part of the population is constituted of apoptotic cells with degraded DNA which incorporate less PI than cells with intact DNA. Infection of HeLa cells with adherent *Ngo* or treatment with TNFα/cycloheximide resulted in an increase in the hypo-diploid population from 15 to 36% and 27%, respectively. Non-adherent (P^-, Opa^-) gonococci and a *N.sicca* strain did not show this effect (21 and 16%, respectively). Apoptotic cells could also clearly be identified by their distinct morphology (Figure 1C). The observed nuclear changes such as chromatin condensation and nuclear fragmentation (data not shown) as well as membrane blebbing, rounding up and detachment from the culture plate are typical apoptotic features. Only cells infected with the strongly binding P^+, Opa^- strain N138 (Figure 1C, panel b) displayed these characteristics. In contrast, cells infected with its P^-, Opa^- variant (panel c) or the commensal *N.sicca* strain (panel d) were indistinguishable from control cells (panel a).

In order to quantify the apoptotic population with an alternative method, the phosphatidylserine exposure was measured by annexin V (FITC) binding and PI counterstaining to distinguish the necrotic cells (Figure 1D). After 15 h, 22% of the cells infected with the P^-, Opa^+ strain N242, and 27% of the cells infected with the P^+, Opa^- strain, N138, stained annexin V-positive, while the non-infected control population showed only 3% spontaneous apoptosis. Infection with a P^-, Opa^- variant of the same strain, which was unable to adhere to or invade epithelial cells, had hardly any effect (6%). In contrast to the adherent *Ngo* strains, a commensal *N.sicca* Opa^+ strain selected for adherence to HeLa cells was unable to induce apoptosis (not shown). The necrotic population remained unchanged in all experiments (~5%). After 60 h of infection with the adherent *Ngo* strains, nearly all the cells were dead but, since the majority of the cells had also incorporated PI at this stage, it was impossible to distinguish apoptosis from secondary necrosis. Adherent *Ngo* strains also induced apoptosis in other epithelial cells, for instance the 293 cell line (data not shown).

Porin induces apoptosis in epithelial and monocytic cell lines

Since both adherent and invasive strains induced apoptosis to a similar extent in HeLa cells, and adherence alone is not sufficient, we addressed the question whether bacterial factors other than the Opa invasins or pili are responsible for the observed cytotoxic effect. Therefore, purified neisserial factors were tested for their apoptosis-inducing capacity on epithelial cells. The secreted neisserial IgA protease was ineffective in this respect (data not shown). In contrast, incubation of cultured epithelial or monocyte-like cells with purified PorB porins, e.g. $PorB_{IA}$ of *Ngo* strain N242 (Figure 2A–E) or $PorB_{IB}$ of *Ngo* MS11 (not shown), induced apoptosis very efficiently. Cells treated with 7 μg/ml of porin for 15 h showed the DNA fragmentation typical of apoptotic cells, as shown here for U937 cells [Figure 2A, compare lane 4 with lane 2 (control)]. A similar apoptotic effect was achieved by treatment with 10 ng/ml TNFα in combination with 20 μg/ml cycloheximide (Figure 2A, lane 1). Porin at 3.5 μg/ml does not show any effect (Figure 2A, lane 3). Porin-treated cells were assessed further regarding their PI binding capacity (Figure 2B). A dose-dependent increase in the hypo-diploid population was observed after treatment with 4, 7 and 10 μg/ml porin compared with the untreated control. The dose dependence was not linear (15, 21 and 65% apoptotic cells, respectively), indicating that a certain threshold per cell must be exceeded before cells undergo apoptosis. These results were confirmed by microscopy (Figure 2C). The morphology of porin-treated HeLa cells, such as cell shrinkage and membrane blebbing (Figure 2C, phase-contrast, left panel) as well as nuclear fragmentation and chromatin condensation (nuclear staining, right panel) were indistinguishable from apoptotic cells previously observed in infected cultures (Figure 2C,

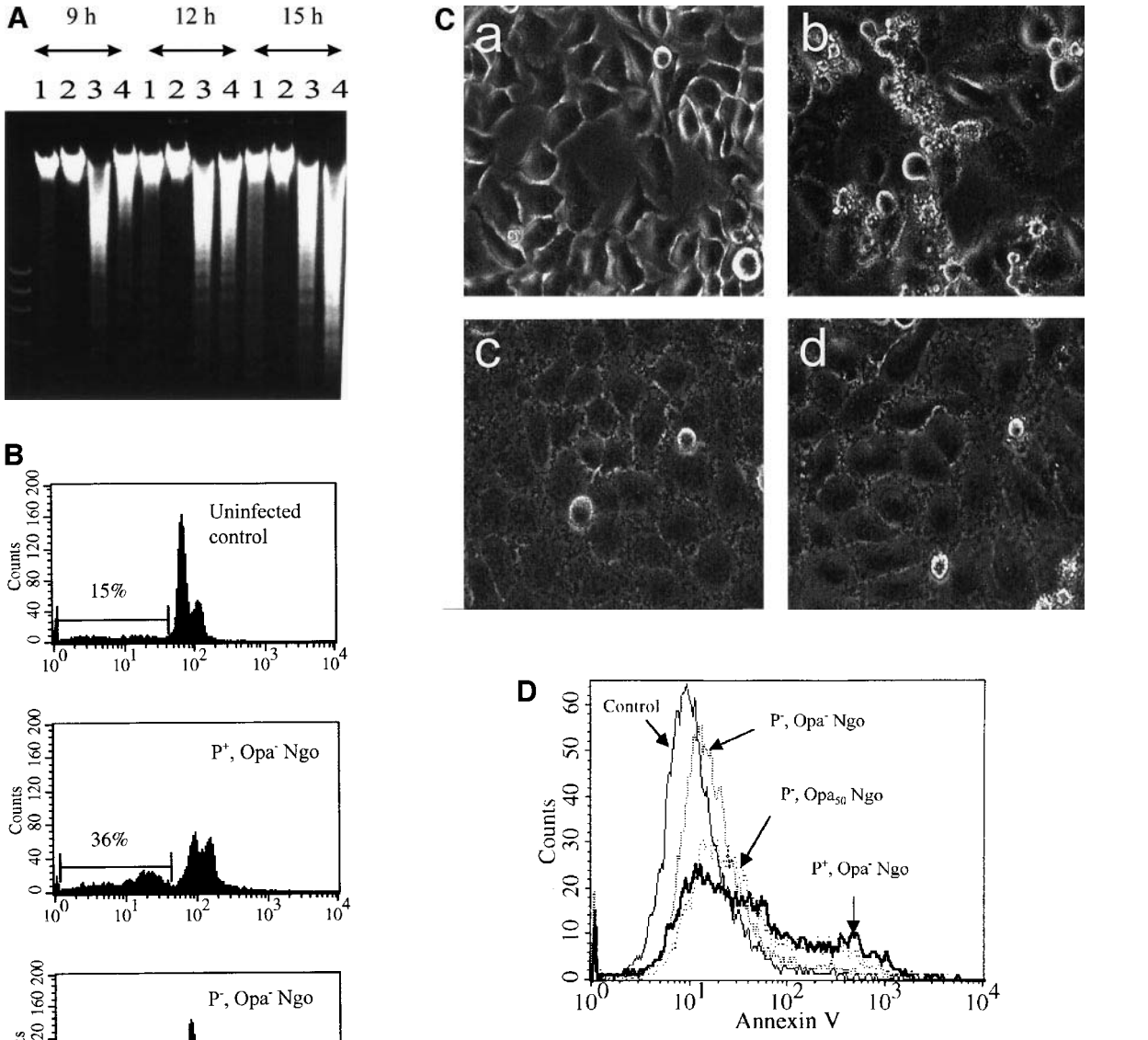

Fig. 1. Prolonged infection of HeLa cells with *Ngo* induces apoptosis. (**A**) Fragmentation of cellular DNA into oligonucleosomes after stimulation of HeLa cells with actinomycin D or TNFα, or infection with gonococcal strain N242 (P^-, Opa^+) for the indicated time points. DNA from 2×10^5 cells was extracted, separated on a 1.8% agarose gel and visualized by ethidium bromide staining. Lane 1, treatment with 1 μg/ml actinomycin D; lane 2, untreated control; lane 3, treatment with 10 ng/ml TNFα and 20 μg/ml cycloheximide; lane 4, infection with strain N242 at an m.o.i. of 10. (**B**) FACS analysis of the DNA content of infected cells. Cells were either infected with the *Ngo* strains of the indicated phenotypes at an m.o.i. of 1, or treated with TNFα and cycloheximide or left untreated. Cells were harvested, permeabilized and stained with PI. 10 000 cells per sample were analysed by FACS and cell counts were plotted against PI fluorescence. The percentage of cells of the hypo-diploid population is indicated in every diagram. (**C**) Morphology of infected HeLa cells. Cells were infected at an m.o.i. of 1 with strain N138 (P^+, Opa^-) (panel b), a P^- variant of N138 (panel c) or *N.sicca*, selected for adherence, (panel d) for 15 h or left untreated (panel a). (**D**) FACS analysis of the annexin V binding properties of infected cell populations. Cells were infected for 15 h with the indicated strains, harvested and stained with annexin V-FITC. After counterstaining with PI which labels the permeable, necrotic cells, 10 000 cells per sample were analysed by flow cytometry. Only the PI-negative cells were included in the histogram. The percentage of apoptotic cells showing strong annexin V binding is 3% in the control, 27% in the population infected with strain N138 (P^+, Opa^-), 6% in the one infected with its P^- variant and 22% in the population infected with strain N242 (P^-, Opa^+).

c and d compared with controls in a and b). Since the porin was purified from *Ngo* in its native trimeric form, it contained lipopolysaccharide (LPS) as well as 0.025% of the detergent LDAO. However, the buffer as well as LPS isolated from gonococcal strain N242 and solubilized in porin purification buffer had no effect on the morphology of the cells (not shown).

A large percentage of the porin-treated cells bound annexin V (Figure 2D). The annexin V-positive cells were not permeable for PI, indicating that the mechanism of

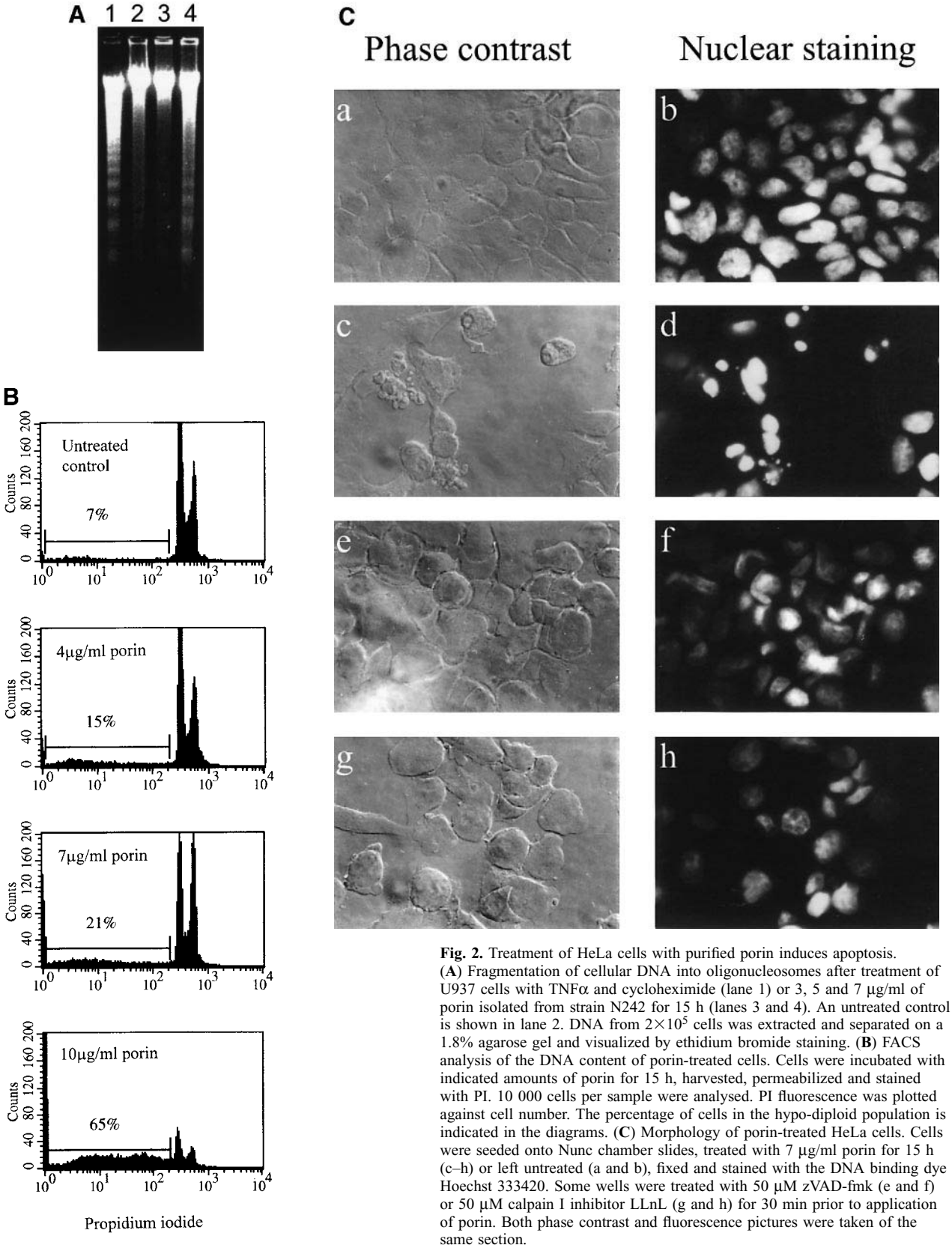

Fig. 2. Treatment of HeLa cells with purified porin induces apoptosis. (**A**) Fragmentation of cellular DNA into oligonucleosomes after treatment of U937 cells with TNFα and cycloheximide (lane 1) or 3, 5 and 7 μg/ml of porin isolated from strain N242 for 15 h (lanes 3 and 4). An untreated control is shown in lane 2. DNA from 2×10^5 cells was extracted and separated on a 1.8% agarose gel and visualized by ethidium bromide staining. (**B**) FACS analysis of the DNA content of porin-treated cells. Cells were incubated with indicated amounts of porin for 15 h, harvested, permeabilized and stained with PI. 10 000 cells per sample were analysed. PI fluorescence was plotted against cell number. The percentage of cells in the hypo-diploid population is indicated in the diagrams. (**C**) Morphology of porin-treated HeLa cells. Cells were seeded onto Nunc chamber slides, treated with 7 μg/ml porin for 15 h (c–h) or left untreated (a and b), fixed and stained with the DNA binding dye Hoechst 333420. Some wells were treated with 50 μM zVAD-fmk (e and f) or 50 μM calpain I inhibitor LLnL (g and h) for 30 min prior to application of porin. Both phase contrast and fluorescence pictures were taken of the same section.

cell death induced by porin is clearly apoptotic and not necrotic. Buffer (data not shown) as well as LPS (Figure 2D) had no effects on the annexin V binding capacity of the cells, even at concentrations 10 times higher than those found in the porin preparations (100 μg/mg porin). Interestingly, recombinant His-tagged porin, produced in *E.coli* and purified from inclusion bodies, induced apoptosis with similar dose-dependence and kinetics as porin purified from *Ngo*, as determined by annexin V (FITC) binding (Figure 2E). This demonstrates clearly that no other neisserial factors beside the porin are necessary for induction of apoptosis. Both preparations were pure as judged by SDS–PAGE analysis (Figure 2E, inset). Whereas porin from *Ngo* was purified in its native trimeric form (lane 2) and disassembled by boiling (lane 3), the recombinant porin was purified as monomers (lane 4). It is conceivable that trimers form immediately after insertion of porin monomers into host cell membranes. Porin also induced apoptosis in Chang conjunctiva cells, 293 cells and in the monocytic cell lines U937 and JOSK-M with a similar kinetics to HeLa cells (data not shown).

Porin induces rapid calcium fluxes in epithelial and monocytic cell lines

A possible mechanism by which the neisserial porin induces apoptosis might depend on its property to form ion-selective channels in the eukaryotic membranes (Rudel *et al*., 1996). Changes in intracellular Ca^{2+} have been reported to occur during the onset of apoptosis (McConkey *et al*., 1988; Jones *et al*., 1989; Martikainen *et al*., 1991). Therefore, we investigated the effect of porin treatment on the cytoplasmic Ca^{2+} levels of the monocytic cell line JOSK-M by FACS analysis. JOSK-M cells grown in medium containing 1 mM Ca^{2+} were loaded with the two calcium-responsive chromophores, Fluo-3 and Fura Red. The fluorescence intensity of Fluo-3 increases after Ca^{2+}-binding, while that of Fura Red decreases. The basal fluorescence level of the indicator-loaded cells was determined for 1 min. After adding the porin, intracellular Ca^{2+} was monitored for another 2 min. The ionophore ionomycin was applied to a separate sample prior to the measurements in order to control the proper loading of the cells.

The level of intracellular Ca^{2+} increased immediately upon addition of porin at concentrations of 5–10 μg/ml and peaked after 1–2 min at 2- to 3-fold (in some experiments up to 4-fold) the basal Ca^{2+} concentration (Figure 3A–D). Application of adequate volumes of detergent-containing buffer, as well as LPS at a concentration of 15 μg/ml, had no effect on the intracellular Ca^{2+} concentration (Figure 3A). Furthermore, a clear dose-dependence could be observed with increasing porin concentrations (Figure 3B). Whereas porin at 4 μg/ml had no effect on intracellular Ca^{2+} levels, a significant increase was recorded with 5 μg/ml which was even exceeded at 7 μg/ml. A similar dose-dependence was observed with assays quantifying apoptosis after porin treatment (Figure 2B). This supports the idea that both processes are linked. The Ca^{2+} measurements by FACS were performed mainly with JOSK-M cells from suspension cultures. However, primary monocytes and granulocytes isolated from fresh blood reacted very similar to JOSK-M cells in the same experiment with regard to the kinetics and dose dependence (data not shown). Adherent cell types like HeLa were not accessible to Ca^{2+} measurements by FACS since removing them from the cell culture plate by trypsination alone resulted in elevated intracellular Ca^{2+} concentrations. However, Fluo-3-loaded adherent HeLa cells monitored by confocal microscopy reacted with an increase in Fluo-3 fluorescence intensity upon addition of porin which was similar to the treatment with ionomycin (data not shown). The elevation of intracellular Ca^{2+} was

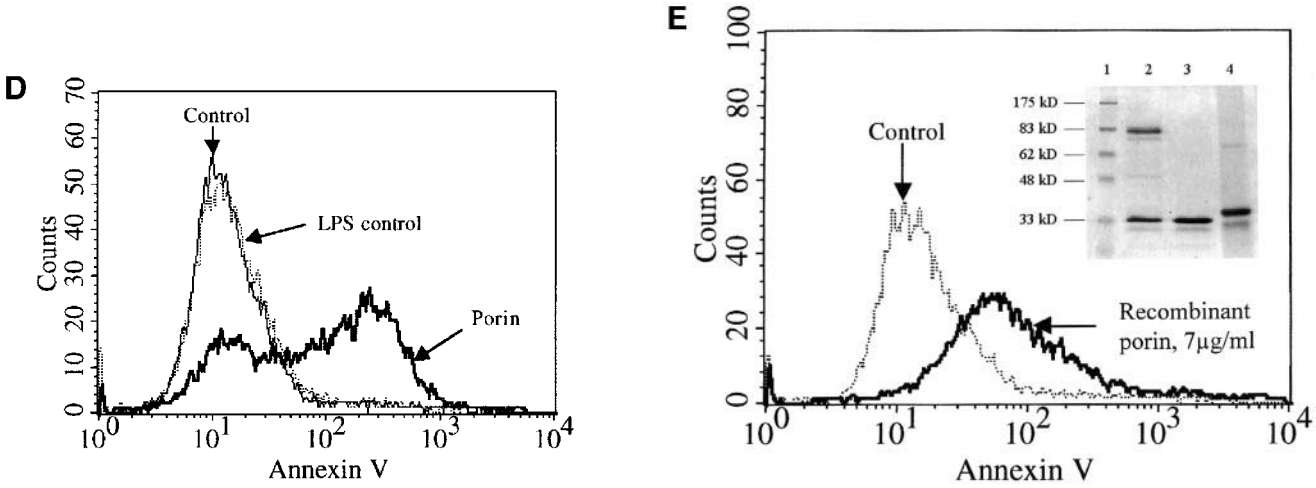

Fig. 2. *Continued.* (**D**) FACS analysis of the annexin V binding properties of a porin-treated population. Cells were incubated with 7 μg/ml porin for 15 h, harvested and stained with annexin V and PI to mark the apoptotic and the permeable, necrotic cells, respectively. 10 000 cells were analysed per sample and annexin V binding was plotted against cell number in a histogram of the PI-negative cells. A control of untreated cells as well as an LPS control is included. The bold line represents the porin treated cells (55% apoptosis), the thin line the untreated control (5% apoptosis) and the dotted thin line the LPS control (5% apoptosis). (**E**) FACS analysis of the annexin V binding properties of cells treated with recombinant His-tagged porin purified from *E.coli*. Cells were incubated with 7 μg/ml porin for 15 h and analysed as described. The dotted thin line represents the untreated control (6% apoptosis), the bold line represents the porin-treated cells (38% apoptosis). A Coomassie Blue-stained gel of purified porins from *Ngo* strain N242 (lanes 2 and 3) and recombinant *E.coli* expressing His-tagged porin (lane 4) is shown as inset. Visible are the monomeric form around 34 kDa and some bands representing oligomeric forms of porin (lane 2). Due to the insertion of six histidine residues, the recombinant porin exhibits a slightly higher molecular weight. Samples where either incubated at 37°C (lane 2) or boiled (lane 3 and 4) prior to electrophoresis. Molecular weight marker (kDa) was applied in lane 1.

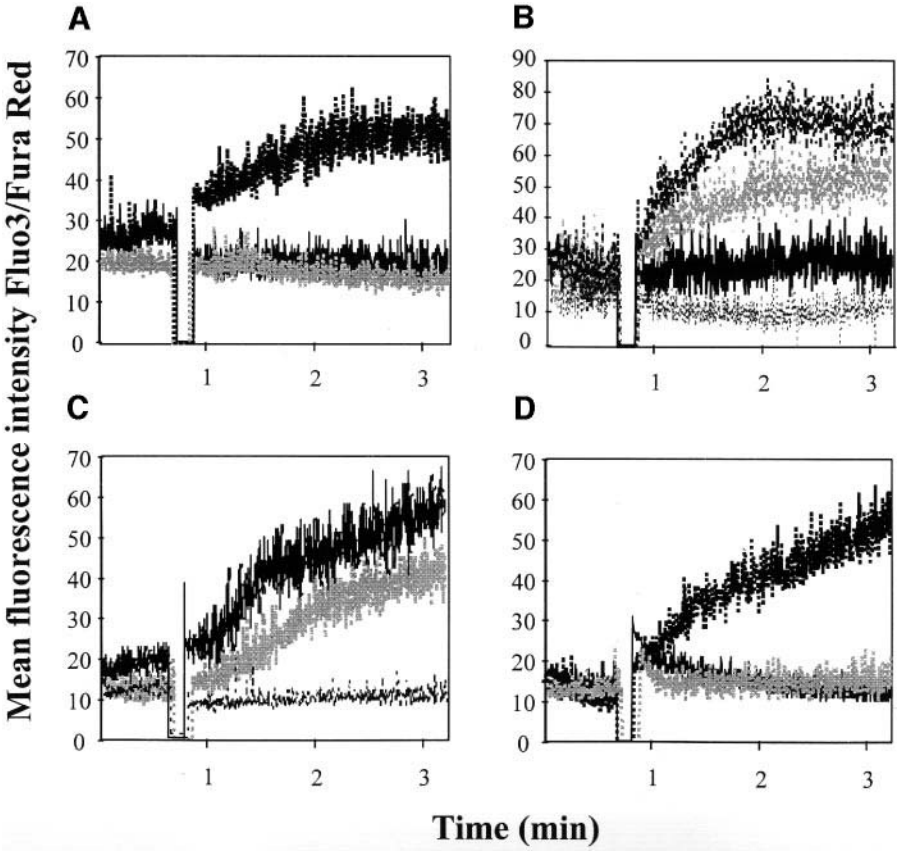

Fig. 3. Porin treatment of JOSK-M cells induces Ca^{2+} influx from the surrounding medium. Cells were loaded with the dyes Fluo-3 and Fura Red at 10 μg/ml final concentration. Fluorescence intensities in channels 1 (515–535 nm) and 3 (665–685 nm) were routinely measured over a period of 3 min. The baseline was determined for 1 min, then an inducer (e.g. porin or LPS) was applied and the measurement continued for another 2 min. The ratio of Fluo3/ Fura Red intensities was formed and mean values of this ratio were calculated for each time point. For presentation the mean fluorescence intensity of Fluo3/Fura Red was plotted against time. (**A**) Addition of 7.5 μg/ml porin-induced a Ca^{2+} influx (black dotted line) while the buffer control (grey dotted line) or 15 μg/ml LPS purified from neisserial strain N242 (black thin line) had no effect. (**B**) Porin induced Ca^{2+} influx is dose-dependent and blocked by EGTA. Measurements were performed in RPMI containing 1 mM Ca^{2+}. Porin was applied at three different concentrations: 4 μg/ml (bold black line), 5 μg/ml (grey dotted line) and 7 μg/ml (black dotted line). Ca^{2+} influx induced by 7 μg/ml is totally abolished in RPMI medium containing 2 mM EGTA (thin black dotted line). (**C**) Porin-induced Ca^{2+} measurements performed in PBS in the absence of Ca^{2+} (black dotted line), in the presence of 3 mM Ca^{2+} (grey dotted line) and 30 mM Ca^{2+} (black plain line). (**D**) The Ca^{2+} measurement was performed with 7 μg/ml porin (black dotted line), 7 μg/ml porin in the presence of 0.1 mM ATP (black plain line) and 0.1 mM ATP as a control (grey dotted line).

only transient and returned to basal levels after 3–5 min. Thus, the neisserial porin evokes transient Ca^{2+} fluxes in both phagocytes and epithelial cells.

Porin triggers the influx of Ca^{2+} from extracellular sources

Cytoplasmic Ca^{2+} originates from two major sources: it is either released from intracellular Ca^{2+} stores like the endoplasmic reticulum (ER), the mitochondria or the nucleus, or enters the cells through Ca^{2+}-specific channels of the plasma membrane from the exterior. Several strategies were used to identify the Ca^{2+} source in the case of porin-induced Ca^{2+} changes. On the one hand, measurements in medium containing 1 mM Ca^{2+} were performed as described above. If the extracellular Ca^{2+} was chelated by the addition of 2 mM EGTA, which reportedly is not cell permeable, the increase in Ca^{2+} was totally abolished (Figure 3B). Additionally, when the measurements were performed in Ca^{2+}-free phosphate-buffered saline (PBS), no increase in cytoplasmic Ca^{2+} was recorded upon treatment of the cells with porin. However, addition of 1–30 mM Ca^{2+} resulted in a concentration-dependent restoring of porin-induced Ca^{2+} fluxes (Figure 3C). Chemicals known to selectively inhibit intracellular Ca^{2+} mobilization (10 μM TMB 8) or receptor-mediated Ca^{2+} entry from the exterior of the cell (10 μM SK&F) had no effect on the time course and the degree of Ca^{2+} influx (not shown). Interestingly, addition of porin preincubated with either 10 mM ATP or GTP (0.1 mM final concentration) resulted in complete inhibition of the Ca^{2+} influx. Also, ATP alone added at the same concentration had no effect (Figure 3D). This effect could not be observed with AMP, which reportedly binds to the porin with much lower affinity (data not shown). These findings suggest that the pore is closed after binding to ATP or GTP, as proposed previously (Rudel *et al.*, 1996), and that the Ca^{2+} influx is regulated by the ATP/GTP level.

Altogether these data support the idea that extracellular Ca^{2+} passes directly through the porin channel. That Ca^{2+} enters the cell via so far undefined, porin-activated Ca^{2+} channels appears less probable.

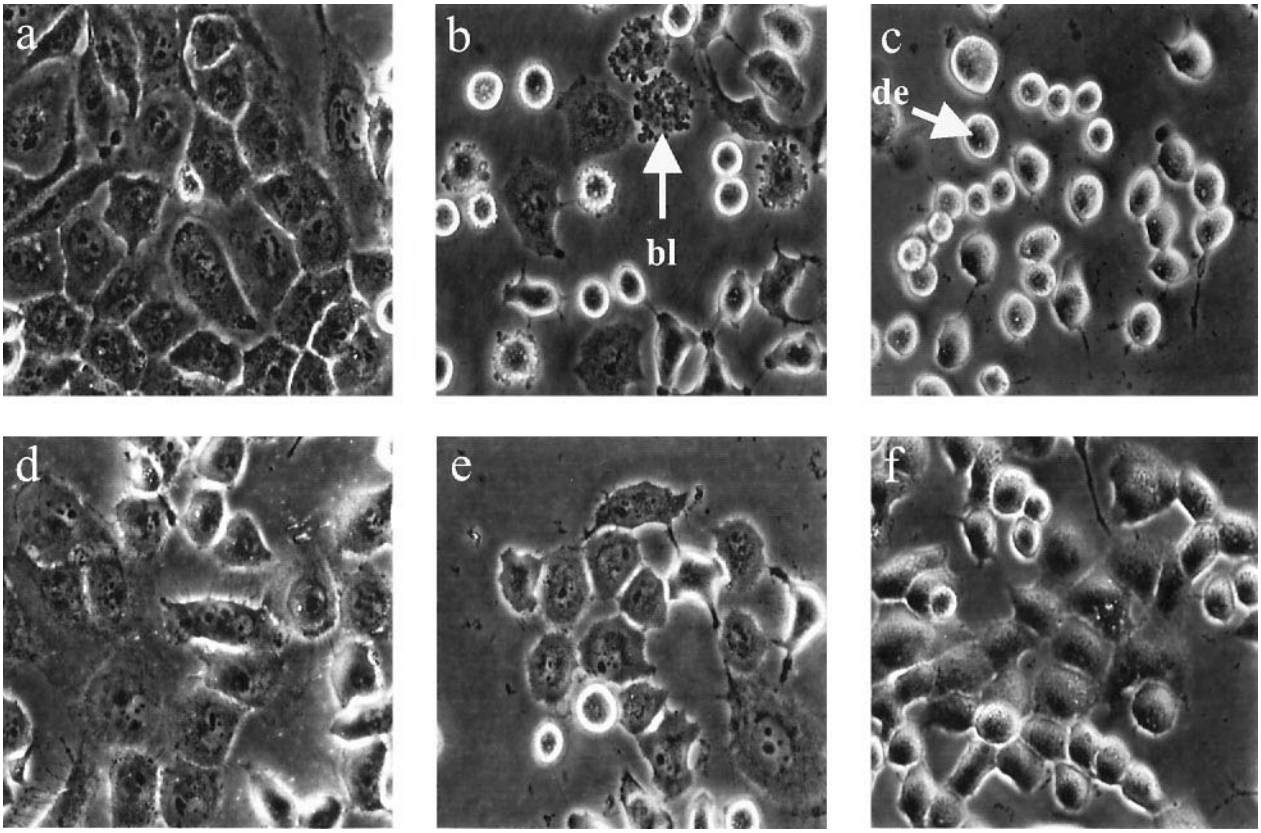

Fig. 4. Early features of apoptosis are inhibited by pretreatment of porin with ATP. Purified porin was preincubated with 10 mM ATP for 20 min at 37°C before application to HeLa cells at a concentration of 7 μg/ml, resulting in a final ATP concentration of 20 μM. Non-fixed cells were photographed after 30 and 90 min, respectively. Cells in (**a**) were left untreated, cells in (**b**) and (**c**) were treated with porin for 30 and 90 min. (**d**) shows a control treated with 20 μM ATP for 30 min; the cells in (**e**) and (**f**) were treated with preincubated porin for 30 and 90 min. bl, cell showing extensive blebbing; de, cell detached from culture plate.

Inhibition of porin-induced apoptosis by ATP

The first striking morphological features reminiscent of apoptosis appeared as early as 30 min after application of porin to HeLa cells (Figure 4b). Cells showed extensive blebbing of the plasma membrane followed by rounding up, shrinkage and detachment from the culture vessel. After 60–90 min, blebbing gradually declined: the majority of the cells now detached (Figure 4c) and blebbing only occurred in a small population. At these early stages, the cells showed neither annexin V-binding nor DNA fragmentation. Since blocking pore channel formation with ATP completely prevented the rapid Ca^{2+} signal elicited by porin (Figure 3D), we asked whether ATP-treated porin still induces apoptosis. As previously demonstrated, preincubation of porin with 10 mM ATP closes the channel (Rudel *et al*., 1996). The above described early signs of apoptosis did not appear after incubation of porin with 10 mM ATP for 20 min at 37°C prior to application (Figure 4e and f). The resulting final concentration of 20 μM ATP alone had no effect on the cells (Figure 4d). These results provide evidence that both processes, Ca^{2+} influx and apoptosis, are mechanistically linked. Later biochemical features of porin-treated cells such as annexin V-binding do not reveal a clear difference with the ATP-bound form. This discrepancy between early and later characteristics can most likely be attributed to the fact that the non-covalently bound ATP readily dissociates from the porin.

Caspases and the calcium-dependent protease, calpain, are activated during porin-induced apoptosis

Cysteine proteases of the caspase family and the Ca^{2+}-dependent protease, calpain, play a critical role in the execution of apoptosis. In order to dissect which types of proteases are involved in porin-induced apoptosis, cell lysates of porin or TNFα-/cycloheximide-treated HeLa cells were subjected to immunoblotting. Poly-ADP ribose polymerase (PARP) and α-fodrin were monitored as specific substrates for each class of proteases.

α-fodrin 150 and 120 kDa signature fragments were detected in porin and TNFα-/cycloheximide-treated cells (Figure 5A). While the generation of the 120 kDa fragment was inhibited by the specific caspase inhibitor zVAD-fmk at 50 μM (Villa *et al*., 1997), the calpain inhibitor LLnL at 50 μM (Nath *et al*., 1996) blocked the formation of the 150 kDa fragment. This very specific inhibition pattern indicates that α-fodrin is cleaved by both types of proteases at different sites, suggesting that both caspases and calpain are activated during porin-induced apoptosis in HeLa cells. Calpain activation may be a consequence of the transient Ca^{2+} increase measured shortly after porin application. In the case of PARP, the 85 kDa fragment was detected in TNFα-/cycloheximide-treated, as well as in porin-induced apoptotic cells (Figure 5B). This cleavage was completely inhibited by zVAD-fmk, whereas LLnL was ineffective, as expected. Similar observations were made for p21-

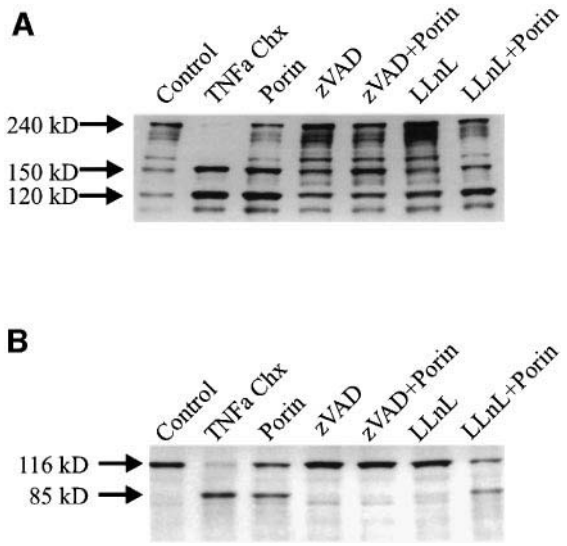

Fig. 5. Cleavage of α-fodrin and PARP during porin-induced apoptosis and inhibition of cleavage by caspase and calpain inhibitors. HeLa cells were treated as indicated, harvested and lysed by boiling for 10 min in lysis buffer. The proteins were separated on a 6% (α-fodrin) or 10% (PARP) SDS–polyacrylamide gel. α-fodrin and PARP were detected by ECL using specific monoclonal antibodies and horseradish peroxidase coupled secondary antibodies. (**A**) The full-length 240 kDa form of α-fodrin is cleaved into a 120 and 150 kDa fragment after treatment with porin or TNFα, respectively. The caspase inhibitor zVAD-fmk inhibits the formation of the 120 kDa fragment while the calpain inhibitor I (LLnL) prevents the formation of the 150 kDa fragment. (**B**) PARP cleavage results in a breakdown product of 85 kDa which is blocked by zVAD-fmk but not by LLnL.

activated kinase 2 (PAK2) (not shown), another caspase 3 substrate involved in apoptotic body formation (Rudel and Bokoch, 1997).

Porin- and Neisseria-induced apoptosis are blocked by the same protease inhibitors

The observation that caspases and the Ca^{2+}-dependent protease calpain are activated in porin-treated HeLa cells prompted us to investigate whether inhibitors of these proteases also interfere with apoptotic events further downstream of protease substrate cleavage. Pre-treatment of HeLa cells with 50 μM zVAD-fmk or 50 μM LLnL abolished the typical apoptotic phenotype seen upon incubation with porin (Figure 2C). Fragmented nuclei, condensed chromatin or membrane blebbing, characteristic of porin-treated cells, were no longer detectable. It should, however, be noted that especially the LLnL treated cells showed a moderately altered appearance as a result of prolonged treatment with this compound. The same inhibitors significantly reduced annexin V binding of porin-treated cells (Figure 6A, right panels). zVAD-fmk or LLnL reduced the population of annexin V positive cells by more than 50% while the caspase 1 and 4 inhibitor zYVAD-cmk had only a minor effect (Figure 6B). The specific caspase 3 inhibitor DEVD-fmk blocked annexin V staining more effectively than zVAD-fmk. Interestingly, the combined pretreatment of cells with inhibitors of caspases and calpain had an additive effect (Figure 6B): this indicates that both calpain and caspase activation is necessary for porin induced apoptosis and that these proteases act independently.

Strikingly, apoptosis induced after infection of HeLa cells with *Ngo* showed an inhibition pattern very similar to porin induced apoptosis (Figure 6A, left panels). Both inhibitors significantly reduced the number of annexin V-positive cells in the population. These similarities between *Ngo*- and porin-induced apoptosis provide strong support for the function of porin as the responsible bacterial factor.

A different inhibition pattern was obtained in studies with TNFα/cycloheximide treated HeLa cells where annexin V binding was totally abolished by zVAD-fmk but not by LLnL treatment (Figure 6C). In these cells, zYVAD-cmk reduced annexin V binding by 50% (Figure 6C). Thus, calpain activity seems to be critical in porin-, but not TNFα-induced apoptosis of HeLa cells.

Isogenic gonococci expressing commensal or mutated porins differ in their ability to induce apoptosis

In order to investigate further the role of porin in *Neisseria*-induced apoptosis, we tested several isogenic gonococcal strains producing recombinant porins, i.e. *Ngo* MS11 synthesizing $PorB_{IA}$ of strain VP1 (MS11 $PorB_{IA}$), MS11 $PorB_{IB}$ synthesizing the authentic $PorB_{IB}$, MS11 $PorB_{Nla}$ synthesizing a *Neisseria lactamica* PorB, and MS11 $PorB_{\Delta loop1}$ synthesizing a mutant MS11 PorB with a deletion in loop 1 (Bauer *et al*., 1999). All strains contained a single copy of the respective *porB* gene integrated in the chromosome and expression of the gene was driven by the genuine *porB* promoter of strain MS11. In order to derive piliated versions of these isogenic strains the $pilE_{F3}$ gene contained on plasmid pEMK40 was introduced via conjugation (Kupsch *et al*., 1996). The resulting strains thus produced defined porins and, in addition, pili that allowed adherence to epithelial cells (Rudel *et al*., 1992). HeLa cells were infected with these strains for 15 h and apoptosis was quantified with the annexin V binding assay. As shown in Figure 7, the MS11 strain producing wild-type $PorB_{IA}$ (set as 100%) induced apoptosis more efficiently than the isogenic MS11 $PorB_{IB}$ (59%), MS11 $PorB_{Nla}$ (60%) and MS11 $PorB_{\Delta loop1}$ (55%) strains. In conclusion, this result provides strong evidence for a direct role of porin in *Neisseria*-induced apoptosis.

Discussion

Neisseria gonorrhoeae infection *in vivo* as well as in various model systems is associated with a strong inflammatory response and the destruction of the epithelial cell layer (McGee *et al*., 1981). One model closely resembling natural sites of gonococcal infection is provided by human distal urethers from kidney donors, which display a native complex epithelium susceptible to infection. Electron microscopic studies revealed that infection of this tissue with piliated gonococci results in massive cell lysis, exfoliation of infected as well as non-infected cells and subsequent thinning of the mucosa (Mosleh *et al*., 1997). Similar observations were made with the cornea organ culture model (Tjia *et al*., 1988) and the Chang conjunctiva cell line (Virji and Everson, 1981). In both systems, adherence via pili or Opa proteins was required to produce the cytotoxic effects. Here we show that pathogenic *Ngo* strains induce cell death in epithelial cells reminiscent of apoptosis. This is in contrast to many other bacterial

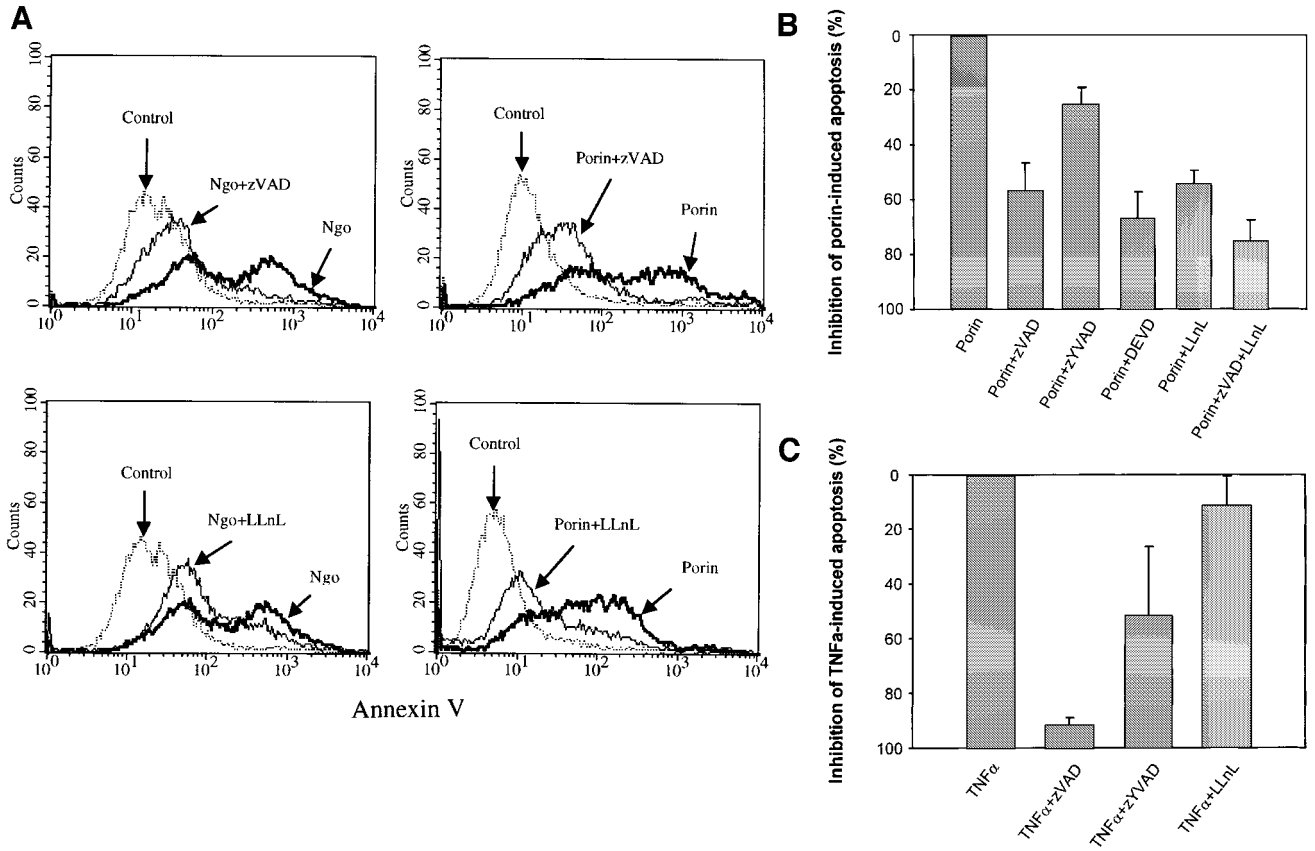

Fig. 6. Caspase and calpain I inhibitors prevent apoptosis induced by both porin treatment and *Ngo* infection of HeLa cells. Cells were pretreated with the respective inhibitors at a final concentration of 50 μM for 30 min, then infected with *Ngo* (N138) or treated with porin for 15 h and the annexin V binding assay was performed. (**A**) Infection with *Ngo* (left panels): Annexin V-positive cells are reduced from 49 to 14% by the caspase inhibitor zVAD-fmk (upper left panel), and from 49 to 28% by LLnL (lower left panel). Treatment with porin (right panels): Annexin V-positive cells are reduced from 48 to 16% by zVAD-fmk (upper right panel), and from 55 to 26% by LLnL (lower right panel). Spontaneous apoptosis in control samples was around 2–5%. The diagram in (**B**) shows the mean inhibition and standard deviations derived from at least three and up to six separate experiments. The population of apoptotic annexin V positive cells in the porin treated sample is set as 100% and inhibition is calculated in percent of the respective porin control. Caspase inhibitors DEVD-fmk and zYVAD-cmk as well as a combination of zVAD-fmk and LLnL were employed. The diagram in (**C**) shows the mean inhibition and standard deviations in TNFα-/cycloheximide-treated HeLa cells with selected inhibitors.

pathogens that have been described to induce apoptosis predominantly in phagocytes, such as *Yersinia* (Monack *et al*., 1997; Ruckdeschel *et al*., 1997), *Legionella* (Müller *et al*., 1996), *Shigella* (Zychlinsky *et al*., 1992) and *Salmonella* species (Chen,L.M. *et al*., 1996; Monack *et al*., 1996). In the case of *Ngo*, apoptosis may constitute a mechanism to disrupt the mucosal lining and to allow access of the bacteria to deeper tissues.

Ngo-infected cells did not express significant amounts of CD95 (Apo-1/Fas) nor Fas-ligand (data not shown). Also, an inhibitory anti-TNFα antibody was unable to block pathogen-induced apoptosis (data not shown). This excludes a primary role of these well-known receptor-mediated pathways in *Ngo*-induced apoptosis. Furthermore, a dominant effect of bacterial LPS (e.g. acting via the CD14 receptor) appears unlikely since epithelial cells do not express CD14, and because all experiments were carried out in the absence of serum and thus in the absence of soluble CD14. This assumption was confirmed here in several experiments involving LPS controls, suggesting a mechanism different from that described by Dunn *et al*. (1995) for endothelial cells.

Besides LPS, we assessed several proteinacious factors purified from *Ngo* for their ability to induce apoptosis in host cells. The only prominent effect was elicited by the neisserial porin which rapidly induced apoptotic phenotypes in target cells indistinguishable from those generated by the pathogen. Native and recombinant porin purified from *Ngo* and *E.coli*, respectively, elicited identical effects with respect to induction of Ca^{2+} fluxes (not shown) and all apoptotic phenotypes, thus excluding other neisserial factors. Furthermore, the unusual dependence of *Neisseria*- and porin-induced apoptosis on both calpain and caspases points to the same mechanism of apoptosis initiation.

Additional evidence in support of this result is provided by experiments with isogenic *Ngo* strains producing variant or mutant porins: P^+ strains producing wild-type $PorB_{IA}$ induced apoptosis more efficiently than strains producing wild-type $PorB_{IB}$, mutant *Ngo* porin, or porin of a commensal *N.lactamica* strain. This observation coincides very well with earlier results, according to which $PorB_{IA}$ strains show an up to 10 times higher efficiency of porin translocation into lipid bilayers (Lynch *et al.*, 1984). Strains expressing $PorB_{IA}$ are commonly associated with the more virulent disseminated form of gonorrhoea, while $PorB_{IB}$-bearing strains predominantly cause localized infections (Morello and Bohnhoff, 1989). Recently, van Putten *et al*. (1998) demonstrated that gonococcal invasion

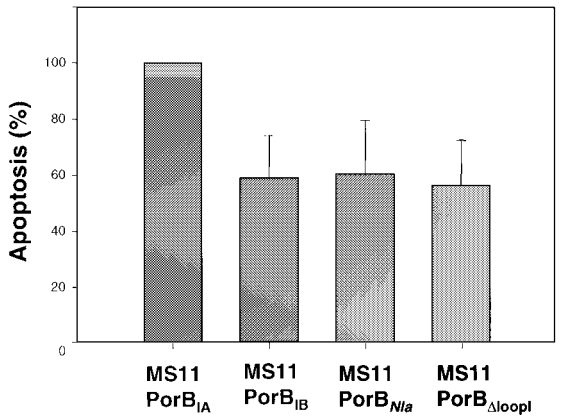

Fig. 7. Isogenic strains expressing different recombinant porins vary in their ability to induce apoptosis. Cells were infected for 15 h with the indicated strains, harvested and stained with annexin V-FITC. Strain MS11 PorB$_{IA}$ induced apoptosis most efficiently and was set as 100%. The results of at least six independent experiments are shown for each strain. Statistical analysis revealed that all values are significant ($p < 0.05$ for MS11 PorB$_{IB}$ and $p < 0.001$ for MS11 PorB$_{Nla}$ and MS11 PorBΔ_{loop1}).

into Chang epithelial cells under low-phosphate conditions is restricted to PorB$_{IA}$-expressing strains.

Since non-adherent *Ngo* mutants are unable to induce apoptosis, close contact to the target cell membrane seems to be important for the induction of apoptosis. This is consistent with the notion that close contact between pathogen and host cell is necessary for the transfer of porin molecules from the outer membrane to the target cell membrane (Weel and van Putten, 1991).

To assess whether the channel function of the porin has any implication in porin-induced cell death, intracellular Ca^{2+} measurements were performed in different cell types ranging from HeLa to purified monocytes and PMNs. All these cells reacted to porin treatment with a rapid and transient increase in cytosolic Ca^{2+} which originated from extracellular sources rather than from the ER, nucleus or mitochondria. Other pore-forming factors have been described which also act to induce apoptosis (reviewed by Chen and Zychlinsky, 1994). An example is the *Staphylococcus aureus* α-toxin which induces apoptosis in human peripheral T-lymphocytes by forming small ion channels (Jonas *et al*., 1994). Interestingly, α-toxin integrates spontaneously into cell membranes and, in analogy to the Gram-negative porins, the transmembrane domain is also formed by anti-parallel β-barrels (Song *et al*., 1996).

Consistent with our earlier report on the regulation of the channel by purin nucleotides (Rudel *et al*., 1996), preincubation of the porin with ATP or GTP totally abolished its ability to induce this immediate Ca^{2+} influx, while AMP did not. Strikingly, ATP pretreatment also prevented the typical early features of porin-induced apoptosis in HeLa cells, such as membrane blebbing, shrinkage, rounding up and detachment from the culture plate. Furthermore, porin-induced Ca^{2+} influx and apoptosis exhibited identical dose-dependence. These observations point to a mechanistical connection between Ca^{2+} influx and the onset of apoptosis.

Many reports have suggested that elevated concentrations of intracellular free Ca^{2+} correlate with apoptosis (McConkey *et al*., 1988; Jones *et al*., 1989; Martikainen *et al*., 1991). For instance, Yoshino *et al*. (1996) reported that treatment of the promyelotic cell line HL-60 with the mycotoxin T-2 results in a transient increase of intracellular Ca^{2+} lasting 3–5 min, followed by typical features of apoptosis. Probably as a consequence of the porin-induced Ca^{2+} fluxes, the Ca^{2+}-dependent protease calpain is activated, which then results in the cleavage of the calpain substrate α-fodrin into a 150 kDa fragment in the absence of caspase activity. Calpain has been shown to play a role in several forms of apoptotic cell death (Squier *et al*., 1994; Chard *et al*., 1995; Cryns *et al*., 1996). Calpain peptide inhibitors reportedly inhibit nuclear events linked to these forms of apoptosis. However, while the roles of intracellular Ca^{2+} fluxes and calpain activation in apoptosis is still a matter of debate (Anagli *et al*., 1993; Yoshino *et al*., 1996), the contribution of caspases and the effects of caspase inhibitors on almost all forms of apoptosis are well described (for reviews, see Nicholson and Thornberry, 1997; Villa *et al*., 1997). It is not surprising, therefore, that the typical caspase substrate PARP is cleaved in the course of porin-induced apoptosis and that broad-spectrum caspase inhibitors and those specific for caspase 3 also inhibit the morphological and biochemical features associated with this form of cell death. Whereas caspase 1 has been reported to be involved in apoptosis of macrophages induced by *Shigella flexneri* (Y.J.Chen *et al*., 1996), the caspase 1 specific inhibitor zYVAD-cmk was ineffective in blocking porin-induced apoptosis. Interestingly, treatment of cells with a combination of inhibitors for caspases and calpain elicited an additive inhibitory effect, suggesting that both types of proteases belong to distinct rather than the same signalling pathway. The concerted action of both protease families has earlier been demonstrated for neuronal apoptosis (Nath *et al*., 1996).

The effects of the purified porin alone were more drastic than those generated by infection with whole bacteria, suggesting that the pathogen also elicits antagonistic effects. Naumann *et al*. (1997) have shown that infection of HeLa and other epithelial cell lines with adherent and invasive gonococcal strains leads to a very immediate activation of the early response transcription factor NF-κB. A protective role in TNFα-induced cell death has clearly been attributed to this factor (Beg and Baltimore, 1996; Liu *et al*., 1996; Van Antwerp *et al*., 1996). One can speculate that one part of the population reacts to the neisserial infection by activating NF-κB and is thus protected against apoptosis. Recent experiments in our laboratory indicate that kinases of the JNK pathway, i.e. PAK and c-Jun N-terminal kinase (JNK) are activated in the course of gonococcal infections, probably via the acidic sphingomyelinase (Grassmé *et al*., 1997; Hauck *et al*., 1998; Naumann *et al*., 1998). Although the role of JNK in signalling to apoptosis is still controversial, several recent reports indicate that the JNK pathway indeed plays a role in apoptotic signalling (Xia *et al*., 1995; Verheij *et al*., 1996; Bossy-Wetzel *et al*., 1997; Chen *et al*., 1998). Considering the other data acquired so far on the issue of *Ngo*-induced signalling it is reasonable to conclude that more than one pathway is triggered by bacterial infection (Dehio *et al*., 1998), resulting in a more complex and totally different picture from the one seen, e.g. after *Shigella* infection of macrophages (Zychlinsky *et al*., 1992, 1994). It is a matter of speculation whether translocation of

the porin and the subsequent cytotoxic effects are late events in the course of infection that are preceded by a prolonged phase of survival of both host and parasite.

The striking structural and functional homology between eukaryotic voltage-dependent anion channels (VDACs) and porins from pathogenic *Neisseria* points to a common origin (Rudel *et al*., 1996). Therefore, the intracellular accommodation of *Neisseria* inside tight vacuolar membranes may resemble the mechanism of mitochondrial endosymbiosis (Taylor, 1979, 1987). One feature of apoptotic cells is the so called permeability transition (PT) of mitochondria regulated by a multi-protein complex also containing mitochondrial porin (VDAC) (Zoratti and Szabo, 1995; Beutner *et al*., 1996; Kroemer, 1997b). Several homologues of other factors of the PT complex are found in the bacterial kingdom (Kroemer, 1997b). This was the basis for the recently raised hypothesis that apoptosis has evolved together with the ingestion of aerobic bacteria (the precursor of mitochondria) into primitive anaerobic cells (Frade and Michaelidis, 1997; Kroemer, 1997b). Conceivably, the translocation of porin from the outer bacterial membrane to the host vacuolar membrane, later constituting the outer mitochondrial membrane, has led to the formation of the initial PT pore. Via the ATP sensor function of the porin, the invader could induce death of its host when the nucleotide level was low (and the nutritional situation of the symbiont critical) and the pore, therefore, in the open state. During the co-evolution, the gene encoding the bacterial porin may have become integrated into the nuclear genome. Our observation that PorB indeed induces apoptosis strengthens this speculative scenario by proposing a candidate for the putative origin of the central part of the PT complex, the pore.

Materials and methods

Human cell culture and infection

Epithelial cells (the human cervix carcinoma cell line HeLa) were grown in RPMI 1640 (Life Technology, Eggenheim) supplemented with 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (FCS) in a humidified, 5% CO_2 atmosphere. The monocyte-like cell lines U937 and JOSK-M were grown in suspension in supplemented RPMI. All cell types were routinely passaged every 2–3 days. The cells were seeded in 24-well plates 24 h prior to infection or porin application. Directly before infection, they were washed several times and further incubated in RPMI without FCS. For infection experiments, bacteria were centrifuged onto the cells for 5 min at 500 r.p.m. For inhibition experiments, the cells were preincubated with caspase inhibitors (Bachem) and Calpain inhibitor I (Amersham) for 30 min if not indicated otherwise.

Bacteria and plasmids

Several different strains have been used for infection. N242 is invasive (*Ngo* strain VP1: $PorB_{IA}$; P^-; $Opa_{27;27,5;28;29;30}$; LPS type L1), whereas N138 is a non-invasive P^+ (Opa^-, $PilE_{F3}$) derivative of *Ngo* strain MS11. A spontaneous P^- variant of N138 (N898) was selected by colony morphological criteria. Adherent, Opa^+ (N899) and non-adherent (N79) *N.sicca* strains were used as non-pathogenic control strains. H1422, an *E.coli* derivative, which contains the *inv* gene of *Y.pseudotuberculosis* cloned in pACYC184, has been described (Grassmé *et al*., 1997). The MS11 strains expressing variant and recombinant porins have been described previously (Bauer *et al*., 1999). Expression of isotypic F3 pili in these strains was achieved by introducing pEMK40, which contains a $pilE_{F3}$ gene in Hermes-8 under the control of an IPTG-inducible Ptrc promoter (Kupsch *et al*., 1996).

Porin purification

An overnight culture of *Ngo* strain VP1 or MS11 was harvested, washed and resuspended in buffer A (10 mM HEPES pH 7.6, 10 mM $MgCl_2$). Bacteria were disrupted by passing twice through a French Press in the presence of RNase (Boehringer Mannheim) and DNase (Benzonase, Merck). Debris was removed by centrifugation at 7000 *g*. The crude membranes were pelleted by a further centrifugation at 45 000 *g*. To remove the inner membrane, the pellet was extracted twice with a teflon homogenizer in buffer B [0.5% *N*-lauroylsarcosine (Sigma), 20 mM Tris–HCl pH 7.6] for 30 min at 37°C. Porin was extracted with buffer C [2% LDAO (Fluka), 20 mM Tris–HCl, 500 mM NaCl, 20 mM $MgCl_2$ pH 7.3] for 30 min at 37°C. Supernatants were subjected to gel filtration (Superdex 200, Pharmacia) in buffer D (0.025% LDAO, 20 mM Tris–HCl, 150 mM NaCl, 20 mM $MgCl_2$ pH 7.8). Porin fractions were pooled and dialysed (Slide-A-Lyzer, Pierce) against buffer E (0.025% LDAO, 20 mM Tris–HCl, 50 mM NaCl, 2 mM $MgCl_2$ pH 8.25). For further purification, the samples were loaded on a Resource Q (Pharmacia) and washed with 10 column volumes of this buffer. The native trimeric porin complex was eluted in a linear gradient ranging from 50 to 800 mM NaCl in buffer E. Samples were dialysed against buffer D and stored at 4°C. Recombinant His-tagged porin expressed in *E.coli* (F.J.Bauer, T.Rudel and T.F.Meyer, unpublished) was purified from inclusion bodies as described (Quiagen Handbook, 1996) and refolded in LDAO containing buffer.

Purification of neisserial LPS

LPS was purified according to the method of Galanos *et al*. (1969). Briefly, the washed and dried bacterial pellet was homogenized and extracted twice with a mixture of phenol, chloroform and petroleum ether (in a volume ratio of 2:5:8, respectively). From the recovered supernatant, petroleum ether and chloroform were removed with a rotary evaporator. The gonococcal LPS was precipitated by the dropwise addition of bidistilled water. The precipitate was washed with phenol (80%). Traces of phenol could be removed by several washes with ether. After evaporation of the ether, the LPS was stored at –20°C.

Extraction of cellular DNA and gel electrophoresis

Cells (2×10^5 per sample) were trypsinized, washed with PBS, lysed for 1 h at 50°C in lysis buffer (0.1% Triton X-100, 5 mM Tris, 0.5 mM EDTA, pH 7.2) supplemented with proteinase K (5 mg/ml) and digested for 1 h with RNase A (0.5 mg/ml). After two phenol and one chloroform/isoamylalcohol (24:1) extraction, the DNA was precipitated with ethanol, dried, resolved in TE (10 mM Tris, 1 mM EDTA pH 8), separated on a 1.8% agarose gel and visualized by ethidium bromide staining under ultra-violet light.

Assessment of chromatin condensation and membrane blebbing

Cells were seeded in chamber slides (Nunc) and infected or treated with porin as described above. At the indicated time points the medium was removed, cells were washed twice, fixed in 3% paraformaldehyde for 20 min, washed again and stained with Hoechst 333420 (10 μg/ml, Sigma) for 10 min in the dark. After another two washes, the slides were covered with moviol and viewed with an Axiovert fluorescence microscope (Zeiss) at 450–490 nm.

Quantification of phosphatidylserine exposure

The flipping of phosphatidylserine from the inner to the outer leaflet of the plasma membrane is assessed by *in vitro* binding of the serum factor annexin V, which is coupled to FITC for detection by flow cytometry (Vermes *et al*., 1995). Cells were seeded in 24-well plates and infected or treated with porin as described. They were then harvested by trypsination, washed twice in PBS and suspended in 100 μl binding buffer (10 mM HEPES–NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$ pH 7.4). Annexin V–FITC (Bender Med Systems) was added and cells were incubated for 15 min in the dark. After one wash, cells were resuspended in 200 μl binding buffer and counterstained with 1 μg/ml PI for determination of permeable (necrotic) cells. Ten-thousand cells per sample were analysed with a Becton Dickinson FACS Calibur equipped with a 15 mW, 488 nm air cooled argon laser using Cell Quest software. All PI-positive cells were excluded from the analysis. Histograms showing the FITC fluorescence intensity in 1024 channels revealed the amount of annexin-positive cells that were determined as apoptotic.

Quantification of hypodiploid cells in the population

Since the fragmented DNA of apoptotic cells incorporates less PI than the intact DNA of healthy cells, the DNA content as determined on a single-cell level by flow cytometry after DNA staining is another method which allows accurate quantification of the percentage of apoptotic (hypodiploid) cells in a population (Nicoletti *et al*., 1991). Cells (2×10^5) were trypsinized and washed twice in PBS before being resuspended in 300 µl PBS containing 5% FCS. The cells were permeabilized by addition of 750 µl ice-cold ethanol and incubation at 4°C for 10 min. After one more wash in PBS, they were resuspended in 300 µl PBS containing 50 µg/ml PI (Sigma) and 0.5 mg/ml RNase A, and incubated in the dark for 20 min. After another two washes, the DNA content of 10 000 cells per sample was assessed on a flow cytometer by plotting PI fluorescence intensity in a histogram plot.

Preparation of cell lysates for immunoblotting

Cells were trypsinated and washed twice with cold PBS at 4°C. Cell pellets containing 2×10^5 cells were resuspended in 50 µl lysis buffer (62 mM Tris–HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol pH 6.8) and heated in a boiling water bath for 10 min. Cell debris was removed by centrifugation for 5 min at 13 000 r.p.m. at 4°C. Lysates were stored at –70°C until further analysis.

Electrophoresis and immunoblotting of proteins

Proteins were separated under reducing conditions for 1 h at 160 V in 6% (for α-fodrin blots) and 10% (for PARP blots) SDS–polyacrylamide gels and then blotted overnight at 100 mA onto PVDF membranes (Millipore). Membranes were blocked for 1 h in 3% bovine serum albumin in Tris-buffered saline (TBS)/0.1% Tween for α-fodrin blots or 5% blocking agent (Amersham) for PARP blots, and then incubated for 1 h with anti-α fodrin (Chemicon) or anti PARP antibody (PharMingen) in TBS/0.1% Tween 20. After washing the blots three times for 10 min in TBS/0.1% Tween, they were incubated for another hour with peroxidase-coupled secondary antibody, and bound antibody was detected by enhanced chemiluminescence (Amersham).

Measurement of intracellular calcium

The changes in intracellular calcium levels resulting from porin treatment were measured by flow cytometry with the help of a combination of two fluorescent dyes, each of which react differently to calcium binding: Fluo-3 shows increased fluorescence in channel 1 (515–535 nm) after calcium binding, whereas Fura Red fluorescence intensity in channel 3 (665–685 nm) decreases with calcium binding. Cells were washed twice in RPMI. After being suspended in 500 µl RPMI (at a density of 2×10^6 cells per ml) the cells were loaded with Fluo-3 and Fura Red (Molecular Probes) simultaneously at a final concentration of 10 µg/ml. Probenecid (4 mM; Amersham) was added to avoid compartentation of the dyes. After 30 min of incubation at 30°C, the loaded cells were washed twice before measurement with a flow cytometer using Cell Quest software. The ionophore ionomycin was applied to one sample at 2 µg/ml before each experiment to check for correct loading of the cells and thus served as a positive control. The basal fluorescence of both dyes was usually determined for 1 min prior to application of the stimulus and the measurement was continued for 2 min thereafter. Fluorescence intensities in channels 1 and 3 were plotted against time (3 min for each measurement). The quotient of Fluo3/Fura Red intensities was formed and mean values of this quotient were calculated for each time point with the help of special calcium software suited for this purpose (FCS Assistant v 1.3.1a beta) to ensure better survey and facilitate semi-quantification. For presentation, the mean fluorescence intensity of Fluo3/Fura Red was plotted against time.

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