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Modulation of Neisseria Porin (PorB) by Cytosolic ATP/GTP of Target Cells: Parallels between Pathogen Accommodation and Mitochondrial Endosymbiosis

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Summary

PorB of the pathogenic Neisseria species belongs to the large family of pore-forming proteins (porins) produced by gram-negative bacteria. PorB is exceptional in that it is capable of translocating vectorially into membranes of infected target cells and functions in the infection process. Here we report on an unexpected similarity between Neisserial PorB and mitochondrial porins. Both porin classes interact with purine nucleoside triphosphates, which down-regulate pore size and cause a shift in voltage dependence and ion selectivity. Patch-clamp analyses indicate that PorB channel activity is tightly regulated in intact epithelial cells. In light of recent findings on the pivotal role of PorB in virulence and the prevention of phagosome lysosome fusion, these data provide important mechanistic clues on the intracellular pathogen accommodation reminiscent of mitochondrial endosymbiosis.

Introduction

During the course of an infection, the pathogenic Neisseria species, N. gonorrhoeae (Ngo) and N. meningitidis (Nme), closely interact with and enter both human epithelial cells and human professional phagocytes (McGee et al., 1981; Shafer and Rest, 1989; Virji et al., 1992; Kupsch et al., 1993). The internalized bacteria are usually observed in a phagosomal compartment separate from the cytosol. Several factors have been identified that play a role in cellular infection events, most notably the variable opacity (Opa) outer membrane protein adhesins, which are essential for the entry process (Makino et al., 1991). Another group of relatively conserved proteins implicated in the pathogenesis of Neisserial infections is the major outer membrane porins, i.e., P.IA and P.IB of Ngo and class 2 and class 3 of Nme (Tsai et al., 1981; Sandstrom et al., 1984; Blake and Gotschlich, 1987; Weel and van Putten, 1991), here collectively referred to as PorB (Hitchcock, 1989). Different clinical isolates possess structurally variant forms of PorB proteins, upon which serotyping is based. These porins form trimeric β-pleated barrels in the outer membrane reminiscent of other gram-negative bacterial porins (Weiss et al., 1991; Cowan et al., 1992; Weiss and Schulz, 1992). A striking feature of PorB proteins of the pathogenic Neisseria species is their ability to translocate into artificial membranes (Lynch et al., 1984) as well as into target cell membranes (Blake and Gotschlich, 1987; Weel and van Putten, 1991). This property is usually not seen with the porins of commensal Neisseriae and unrelated bacterial pathogens. Translocation occurs vectorially rather than by membrane vesicle fusion, such that the surface-oriented side of the porin in the bacterium contacts the cytosol after translocation into a target cell (Blake and Gotschlich, 1987).

It has been suggested that membrane-inserted PorB operates as a trigger for the uptake of Neisseria into host cells (Blake, 1985; Weel et al., 1991; Weel and van Putten, 1991). An absolute requirement for uptake into epithelial cells is the establishment of multiple zones of very close contact between the bacterial and the cell membrane. PorB protein was shown to bind to, or to be inserted into, the epithelial cell membrane at these close contact sites (Weel et al., 1991; Weel and van Putten, 1991).

While the precise role of PorB in the uptake of Neisseria into epithelial cells needs further analysis, direct evidence exists for the function of PorB on the anti-bactericidal machinery of phagocytes. The transfer of purified PorB porin into polymorphonuclear cell membranes causes a transient change in membrane potential that interferes with cell signaling, and this in turn causes granule exocytosis (Haines et al., 1988, 1991). In the same line of evidence, this laboratory recently showed that in human blood mononuclear cells, the fusion of phagosomes and lysosomes is significantly inhibited upon membrane insertion of PorB, whereas the activity of NADP(H) oxidase is not changed (Lorenzen et al., submitted). This suggests a selective interference of PorB with signaling pathways that activate nonoxidative killing mechansims, which probably account for the major bactericidal activity on gonococci (Shafer and Rest, 1989).

One puzzling question concerning membrane-inserted PorB relates to the expected formation of channels in the host cell membrane. Taking into consideration a pore size of 10 nm or a conductance of 1.5 nS in artificial membranes in 1 M KCl buffer, the membrane

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potential of PorB-bearing target cells is expected to break down. However, experimental data suggest that insertion of PorB into target cells is not toxic (Haines et al., 1988; Lorenzen et al., submitted). This points either to a function of PorB other than channel formation or to the tight regulation of PorB channels. Although ion channels in eukaryotic cells are formed in most cases by amphipathic α helices (Kyte and Doolittle, 1982; Oiki et al., 1988), eukaryotic porins, the so-called voltage-dependent anion channels (VDACs), exhibit significant structural similarities with gram-negative bacterial porins (Benz, 1994a). Interestingly, both porin classes probably form amphipathic β-barrels when inserted into membranes (Kleene et al., 1987; De Pinto et al., 1991; Jeanteur et al., 1991; Weiss et al., 1991; Troll et al., 1992; Cowan et al., 1992; Ha et al., 1993). A unique and therefore name-giving feature of eukaryotic porins (i.e., VDACs) is the relative closing of the pore channel if voltages above 30–40 mV are applied (Schein et al., 1976; Colombini, 1979); porins of gram-negative bacteria do not show this feature. The eukaryotic porins are mainly associated with the mitochondrial membrane and are thought to facilitate diffusion of low molecular weight compounds with the cytosol (Colombini, 1979). However, the human 31HL porin was purified from, and shown to reside in, the plasmalemma of a human lymphocyte cell line (Thinnes et al., 1989; König et al., 1991). A different but highly homologous porin was found to copurify with the plasmalemma-bound central type A γ-aminobutyric acid ($GABA_A$) receptor (Bureau et al., 1992). Additionally, the bovine homolog of the human 31HL was cloned and localized in situ in the plasma membrane of astrocytic cells (Dermietzel et al., 1994). The VDACs correspond to the widely distributed anion channels of large conductance (Benz et al., 1992), which were mainly characterized by patch-clamp techniques (Blatz and Magleby, 1983; Schwarze and Kolb, 1984). It is believed that this channel population is involved in many different cellular functions, including cell volume regulation (Dermietzel et al., 1994), phagocytosis of macrophages (Kolb and Ubl, 1987), formation of cell-to-cell channels mediating the gap-junctional permeability (Kolb and Somogyi, 1991), and modulation of chloride permeability (Bettendorff et al., 1993). One of the characteristics of anion channels of large conductance is that they are in a closed configuration when analyzed under cell-attached conditions and are activated in excised patches after being exposed to the bath solution (Schwarze and Kolb, 1984; Jalonen et al., 1989; Marrero et al., 1991). This indicates that regulatory factors keep the channels closed under cell-attached conditions.

Here, we report on the noncovalent binding of nucleoside triphosphates to the PorB porins of pathogenic, but not nonpathogenic, Neisseria species. Our data suggest that Neisserial PorB and eukaryotic porins function via similar mechanisms. We show that the channel activity of PorB inserted into target cell membranes is modulated by cellular nucleotide triphosphates, and, in the context of other known biological functions of PorB, this is considered a crucial mechanism of pathogen accommodation inside target cells.

Results

PorB Proteins of Pathogenic, but Not Commensal, Neisseria Species Bind Nucleotides

Concomitant with our studies on the molecular cross-talk between Neisseria and target cells, we covalently labeled whole viable bacteria with radioactive nucleotide triphosphates using the procedure of Peter et al. (1992). By this method, one protein in Ngo strains MS11 and VPI, identified as PorB, reacted strongly with GTP and ATP (Figure 1a). PorB did not covalently react with nucleotides if the chemical cross-linking was omitted, indicating that PorB binds nucleotides in a noncovalent manner (Figure 1a). Covalent labeling of PorB with radioactive ATP or GTP was prevented in the presence of excess nonlabeled ATP or GTP, but less efficiently with ADP, AMP, GDP, GMP, or pyrimidine nucleotides (data not shown). Perforation of the outer bacterial membrane by EDTA treatment did not increase the intensity of the label. This demonstrates a nucleotide-binding site of PorB that is accessible on the surface of intact bacteria.

In meningococcal isolates, which commonly synthesize a PorA porin along with PorB, only PorB was labeled, irrespective of the perforation of the outer membrane (Figure 1b). In commensal Neisseria species, the porins of two N. lactamica isolates bound ATP; however, other commensal Neisseria species tested interacted with neither ATP nor GTP. Likewise, the porins of other gram-negative human pathogenic and nonpathogenic species, including Escherichia coli K12, enteropathogenic E. coli, Salmonella typhi Ty21A, and Pseudomonas aeruginosa, were not labeled either with ATP or GTP (Figure 1a).

To study the interaction of isolated Neisserial porins with radioactive nucleotides and the effect of nucleotide binding on the pore function, we purified several porins to near homogeneity in their native trimeric forms (Figure 1c). Labeling was very efficient with respect to the isolated gonococcal PorB porins regardless of the serotypes, i.e., P.IA and P.IB (Figure 1d), whereas purified OmpC porin of E. coli was not labeled (Figure 1d). The purified PorB porin still contained traces of the PIII protein (Figure 1c), which was shown to associate with PorB (Newhall et al., 1980). However, the PorB porin purified from a *rmp* mutant ($PIII^-$) was labeled with exactly the same efficiency (data not shown). PorB could still be labeled as a monomer obtained after heating the preparation to 65°C for 10 min (Figure 1d), whereas the modification of ϵ-amino groups of lysine residues prevented the nucleotide labeling of the porin, suggesting a lysine residue to be involved in the binding of nucleotides (Figure 1d).

Common Motifs in Nucleotide-Binding Porins

An amino acid alignment of known Neisserial porins reveals a high degree of homology, as published previously (van der Ley et al., 1991; Ward et al., 1992). In such an alignment, we compared the lysine residue–containing regions of the nucleotide-binding porins of Nme (PorB), Ngo (PorB), and N. lactamica (PorB) with the corresponding region in the nonbinding porins of

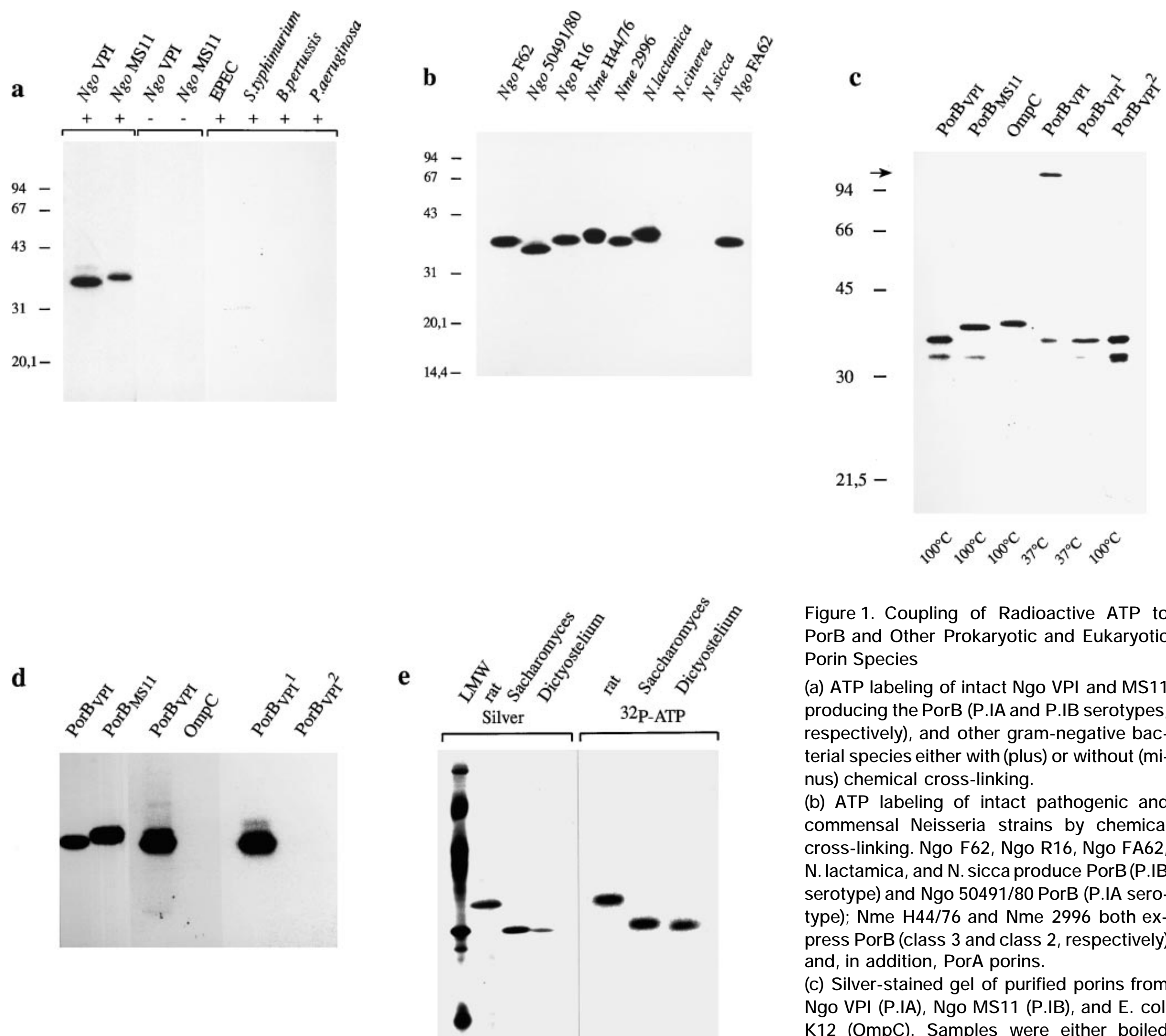

Figure 1. Coupling of Radioactive ATP to PorB and Other Prokaryotic and Eukaryotic Porin Species

(a) ATP labeling of intact Ngo VPI and MS11 producing the PorB (P.IA and P.IB serotypes, respectively), and other gram-negative bacterial species either with (plus) or without (minus) chemical cross-linking.

(b) ATP labeling of intact pathogenic and commensal Neisseria strains by chemical cross-linking. Ngo F62, Ngo R16, Ngo FA62, N. lactamica, and N. sicca produce PorB (P.IB serotype) and Ngo 50491/80 PorB (P.IA serotype); Nme H44/76 and Nme 2996 both express PorB (class 3 and class 2, respectively) and, in addition, PorA porins.

(c) Silver-stained gel of purified porins from Ngo VPI (P.IA), Ngo MS11 (P.IB), and E. coli K12 (OmpC). Samples were either boiled (100°C) or incubated at 37°C (37°C) prior to electrophoresis. Boiling the native porins causes conversion of the trimer (indicated by arrow) into the denatured monomeric form. In lane $PorB_{VPI}{}^1$, the native porin was heated to 65°C for 10 min at a concentration of 50 μg/ml to obtain soluble monomers. In lane $PorB_{VPI}{}^2$, the native porin was acetylated. The PIII protein present in some gonococcal PorB preparations is represented by the lower protein band.

(d) ATP labeling of different purified PorB and OmpC porins by chemical cross-linking. Note that both trimeric and monomeric, but not acetylated, PorB are capable of binding labeled nucleotides. VPI^1 and VPI^2 lanes are as in (c).

(e) Mitochondrial porins from rat liver, D. discoideum, and S. cerevisiae. At left, silver-stained porin preparations; at right, the corresponding ATP-labeled porins using the same chemical cross-linking procedure as for labeling Neisserial porins.

Nme (PorA) and N. sicca. Three lysine residues were identified in the putative membrane-spanning regions 2, 3, and 4 of nucleotide-binding porins that were absent in the nonbinding Neisserial porins. To strengthen the assumption that the identified regions are indeed involved in nucleotide binding, we analyzed additional Neisseria strains for nucleotide binding and subjected the *por* genes of these strains to sequence analysis. The result was an absolute correlation between the presence of lysine residues at positions 59, 73, and 101 and the nucleotide binding (Figure 2C). Thus, the putative nucleotide-binding motifs are within the predicted porin channel.

Nucleotide Binding of Mitochondrial Porins

Common to all three lysine residues supposedly involved in nucleotide binding are glycines at the relative position −2. Moreover, Lys-73 and Lys-101 are part of a GLK tripeptide that has been suggested to be a motif common to all eukaryotic porins (Troll et al., 1992; Fischer et al., 1994). One of the GLKs of Neisserial porins exhibits homology in addition to the GLK of eukaryotic porins (Figure 2A). The other GLK motif of Neisserial porin shows homology to a GTP-binding region of the T cell receptor ζ subunit (TCRζ) (Figure 2B). An interesting connection between the latter homology and nucleotide binding is that this particular lysine residue (Figure 2B)

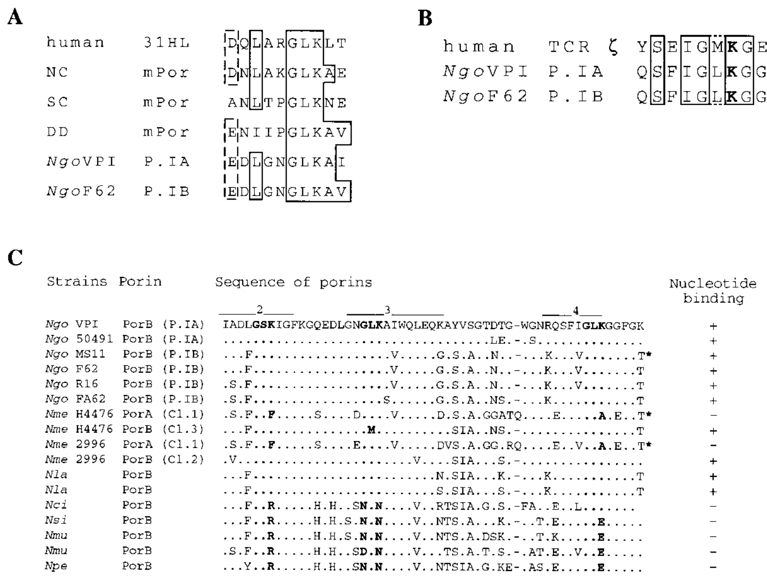

Figure 2. Sequence Alignment of Putative ATP/GTP-Binding Motifs in Neisserial PorB and Other ATP- or GTP-Binding Proteins

(A) Comparison with eukaryotic porins. GLK motives and adjacent amino acids of human 31HL porin (human 31HL) and the mitochondrial porins of Neurospora crassa (NC), S. cerevisiae (SC), and D. discoideum (DD) (Troll et al., 1992; Fischer et al., 1994) were aligned to the first GLK of PorB of Ngo strains VPI (P.IA) and R16 (P.IB).

(B) Comparison of the second GLK motif of PorB with a known GTP-binding site in the TCRζ subunit (Peter et al., 1992). The lysine (bold K) was shown to be the relevant amino acid for cross-linking GTP to the TCRζ subunit. Identical amino acids are indicated by continuous lines and similar amino acids by broken lines.

(C) Comparison of several porins of pathogenic or commensal Neisserial origin. Nucleotide binding was tested with whole intact bacteria. The generated PorB sequences begin at position 33 (isoleucine) with reference to the MS11 porin sequence and include the putative nucleotide-binding region. Note that the presence of GSK, G(L/M)K, and GLK strictly correlates with the nucleotide binding of the porin. The amino acid sequences were obtained by direct sequencing of PCR-amplified *por* genes or (asterisks) were derived from the EMBL data base with the following accession numbers: Nme 2996 class 1 porin, X60105; Nme H44/76 class 1 porin, X52995; Ngo MS11 P.IB, M21289.

was identified as the site of covalent cross-linking between GTP and TCRζ (Peter et al., 1992) by the same method used here for cross-linking nucleotides to Neisserial porins.

Recent work suggests that eukaryotic porins associate with, and allow the transport of, nucleotides such as ATP and ADP (Benz et al., 1988; Flörke et al., 1994). To assess whether eukaryotic porins interact with ATP by a mechanism similar to Neisserial PorB, we labeled a few purified eukaryotic porins available to us with radioactive ATP, again using the conditions for the labeling of the Neisserial porins. As shown in Figure 1e, the purified porins from rat, Saccharomyces cerevisiae, and Dictyostelium discoideum could indeed be covalently labeled with ATP.

Effect of Nucleotides on PorB Conductance

The addition of small amounts of the purified Ngo VPI PorB to the aqueous solution on one or both sides of an artificial lipid bilayer membrane allowed the resolution of step increases in conductance; each step corresponded to the incorporation of one channel-forming unit into the membrane (Figure 3A), similar to the steps observed when other gram-negative bacterial porins were used (Benz et al., 1978; Benz, 1994b). Most of the steps had a single channel conductance of 1.5 nS in 1 M KCl, but we also observed steps corresponding to a single channel conductance of about 3 nS, which are probably caused by the simultaneous insertion of two trimers (see also histogram of Figure 4). Under the low voltage conditions of Figure 3A, all the steps were directed upward, indicating the pore was in an open state. At higher transmembrane potentials, starting with 40 to 50 mV, the channels closed (see below) in a fashion similar to that described previously for another gonococcal PorB (P.IB) (Mauro et al., 1988).

To test whether the binding of ATP or GTP has any influence on the channel function of PorB, we performed conductance measurements in the presence of nucleotides. PorB was added to black lipid bilayer membranes at a concentration of ~500 ng/ml. At 30 min after the addition of the protein, the rate of conductance increase had slowed down considerably. At this timepoint, small amounts of concentrated GTP solutions were added to the aqueous phase on both sides of the membrane, with stirring to allow equilibration. In these experiments, we observed a strong decrease followed by an increase of the membrane conductance (data not shown). While the immediate response to the addition of GTP can be explained as a partial or complete blockage of the PorB channels, the following increase of the membrane conductance was probably due to an enhanced insertional activity of membrane-attached PorB in the presence of GTP, rather than to the catalytic decay of GTP. Owing to this effect, the determination of the half-saturation constant of GTP binding in multichannel experiments was difficult to achieve. We therefore measured the GTP-induced block of conductivity in single channel experiments. Figure 3B shows a single channel record at a GTP concentration of 3.8 mM. Under these conditions, PorB exhibited a single channel conductance of 0.55 nS per trimer insertion into the membrane (see the histogram of Figure 4). Experiments with other concentrations of GTP suggested a dose-dependent decrease of the single channel conductance, with a limiting conductance of 0.4 nS at very high GTP concentration. The half-saturation constant was approximately 0.5 mM under these conditions. ATP had a similar effect on the conductance of the PorB channel (data not shown).

The ion selectivity of the PorB channel was measured in zero-current membrane potential measurements in the presence of KCl gradients. After the incorporation of a large number of channels in membranes bathed in 50 mM KCl, 10-fold salt gradients were established by

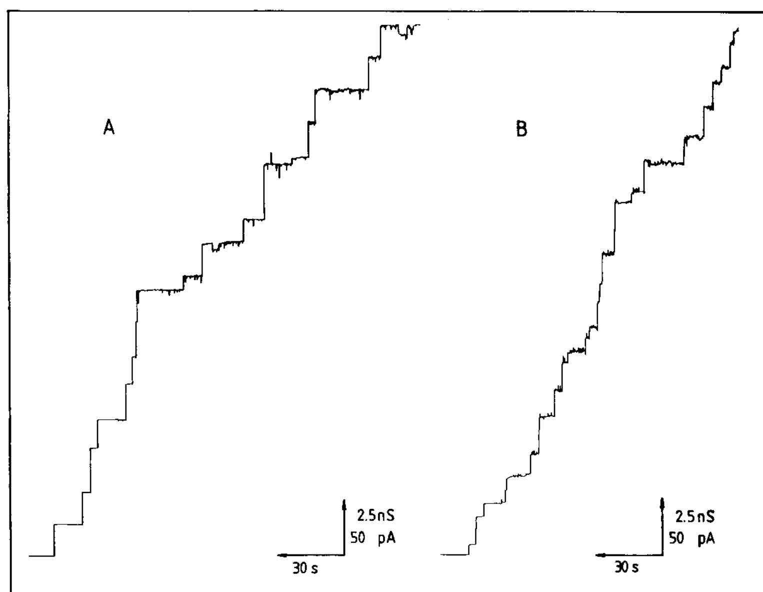

Figure 3. Single Channel Recordings of Diphytanoyl Phosphatidylcholine/n-Decane Membranes in the Presence of PorB (P.IB) of Ngo MS11

The membrane current was measured with a pair of calomel electrodes (with salt bridges) switched in series with a voltage source and a current amplifier (Keithley 427). The amplified signal was monitored with a storage oscilloscope and recorded with a strip chart recorder at a bandwidth of 300 Hz. Curve A, aqueous phase containing 1 M KCl and 10 ng/ml PorB; curve B, aqueous phase containing 1 M KCl, 3.8 mM GTP, and 10 ng/ml PorB. The applied voltage was 20 mV; temperature was 20°C.

the addition of small amounts of concentrated KCl solution to one side of the membrane. In all cases, the more diluted side of the membrane became slightly positive (12 mV at pH 7), an indication of the preferential movement of cations through the PorB channel (ratio of the permeabilities $P_K/P_{Cl} = 1.8$). The addition of increasing concentrations of GTP to both sides of the membrane had a substantial influence on the membrane potential and on ion selectivity. The zero-current membrane potential was 38 mV at a 10-fold KCl gradient and a GTP concentration of 2 mM, which corresponded to a ratio of the permeabilities P_K/P_{Cl} of about 8, according to the Goldman–Hodgkin–Katz equation.

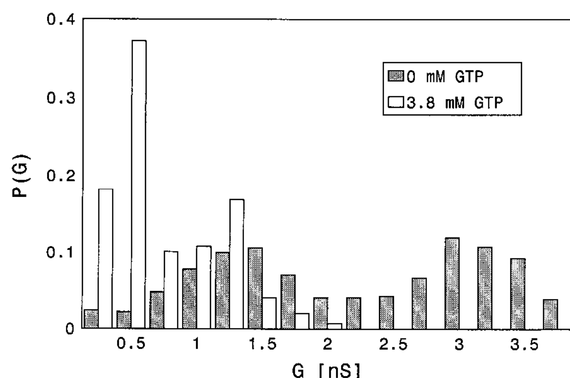

Figure 4. Single Channel Distribution of PorB with and without GTP

Histogram of the probability of the occurrence of certain conductivity units observed with membranes formed of diphytanoyl phosphatidylcholine/n-decane membranes in the presence of 10 ng/ml PorB (P.IB) of Ngo MS11. The applied membrane potential was 20 mV; temperature was 20°C. Stippled bars indicate that the aqueous phase contained 1 M KCl. The average single channel conductance was 1.5 nS for 225 single channel events (left-hand maximum) and 3.1 nS for 232 events (right-hand maximum). The data were collected from ten different membranes. Open bars indicate that the aqueous phase contained 1 M KCl and 3.8 mM GTP. The average single channel conductance was 0.5 nS for 185 single channel events (left-hand maximum) and 1.1 nS for 113 events (right-hand maximum). The data were collected from eight different membranes.

Voltage-Dependent Gating of PorB and the Role of GTP

As stated above, the PorB channels started to close at voltages of between 40 and 50 mV. The steady-state conductance displayed a bell-shaped curve as a function of the applied voltage, as shown in Figure 5. PorB was added to the *trans* side of a lipid bilayer membrane. After reconstitution of ~1150 channels into the membrane, positive and negative voltages were applied with respect to the *cis* side. Similar bell-shaped curves have been described for the voltage dependence of mitochondrial porins, also known as VDACs (see Figure 5C; Schein et al., 1976). Surprisingly, nucleotides increased the voltage dependence of PorB, and the channel closed at much lower voltages. Symmetrical addition led to a symmetrical change, whereas the asymmetrical addition of GTP resulted in an asymmetrical shift of the steady-state conductance (Figures 5A and 5B). As depicted in Figure 5A, 2.9 mM GTP was added to the *cis* side, and voltages of opposite polarity were applied to the same membrane. Small negative voltages closed the channels, while the response for positive voltages was similar to that without GTP. Remarkably, the shift of the voltage dependence correlated with the side to which GTP was added (compare Figures 5A and 5B) and not with the side to which the porin was added. The response toward the voltage was always such that the channels closed in the presence of GTP when the sign of the voltage was negative on the side of the addition of GTP. In contrast, other bacterial porins are not normally voltage dependent, particularly not in the voltage range that is of interest here. Figure 5C shows the voltage dependence of the OmpC porin of E. coli. Up to 100 mV, we did not observe any closure of the pores in response to the applied transmembrane potential. This is in sharp contrast to the results obtained with the mitochondrial porin from S. cerevisiae (Figure 5C).

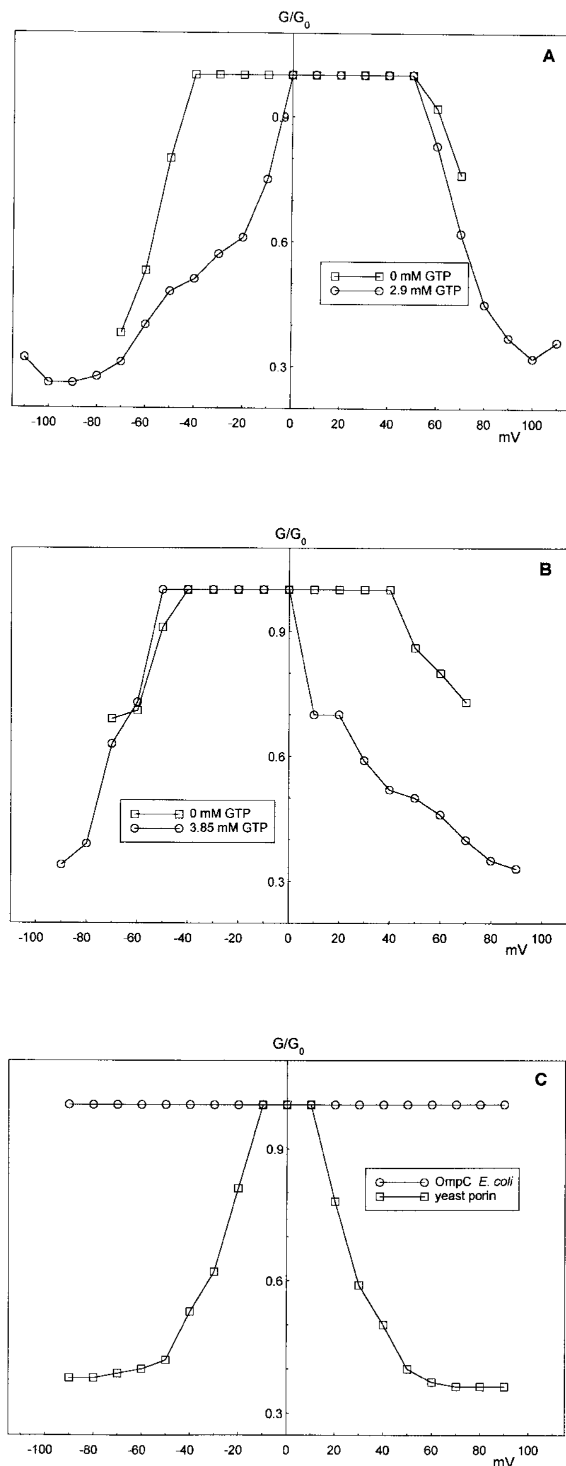

Figure 5. Influence of GTP on the Voltage Dependence of Ngo VPI PorB

(A) Ratio of the conductance G and the starting conductance G_0 (V_m = 10 mV), as a function of V_m. The conductance was calculated as the ratio of current divided by the voltage. The membrane current was measured using a pair of calomel electrodes (with salt bridges) switched in series with a voltage source and an electrometer; temperature was 20°C. The membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 1 M KCl and 100 ng/ml PorB added to the *trans* side of the membrane (squares) or, in addition, 2.9 mM GTP added to the *cis* side (circles).

(B) The same conditions as in (A), except that 3.85 mM GTP was added to the *trans* side (circles).

(C) The aqueous phase contained 1 M KCl and 50 ng/ml OmpC of E. coli or 100 ng/ml mitochondrial porin from S. cerevisiae.

PorB Inserted into Epithelial Cell Membranes

The data derived from the black lipid experiments indicate a conspicuous modulation of PorB channel activity in response to the applied voltage or the presence of ATP/GTP. However, the combination of both, i.e., increasing voltage in the presence of nucleotides on the side of negative voltage, caused the most prominent closing of the pore channel. These observations led us to assume a situation in living cells that favors maximal pore closing, consistent with the high nucleotide concentrations in the cytosol and the negative membrane potential that exists at the cytosolic side of the plasma membrane. To investigate the effect of purified PorB on native cell membranes, we performed patch-clamp experiments in the "on cell" mode and in the "excised" mode. The experiments were carried out on cultured Chang conjunctiva epithelial cells, a suitable infection model which was previously used to demonstrate the association of PorB with target cell membranes (Weel and van Putten, 1991; Weel et al., 1991). In a total of 32 experiments, channels of large unit conductance could not be observed in the on-cell mode, either in control experiments or after addition of variable amounts of PorB to the pipette filling solution. In the excised mode and with control pipette filling solution, channels of large unit conductance were also absent after repetitive application of step depolarizing voltage pulses of −100 mV to the pipette interior for up to 3 min. This last procedure was used to exclude the presence of silent anion channels in the membrane patch, as it is known that in excised membrane patches various anion channel populations can be activated by step voltage gradients, preferentially by depolarization (Schwarze and Kolb, 1984; Li et al., 1989; Bettendorff et al., 1993).

Results obtained in the presence of PorB differed dramatically. If the pipette filling solution contained PorB, a channel of large unit conductance became spontaneously activated after transition to the excised-patch configuration in 6 of a total of 34 experiments. A representative record of a channel current versus pipette voltage relationship is shown in Figure 6. The figure shows the simultaneous presence of three conducting channels of one population. From the linear current–voltage relationship, a single channel conductance of 490 pS can be derived. This value was obtained at an electrolyte concentration of about 150 mM and would fit to the single channel conductance of 1.5 nS observed for bilayer membranes at 1 M electrolyte solution, since a saturation behavior of the single channel conductance as a function of electrolyte concentration has been reported (Schwarze and Kolb, 1984). Furthermore, the voltage dependence of the corresponding single channel open probability can be read from Figure 6. The open probability is high for pipette voltages close to zero and declines about symmetrically for increasing voltages of both polarities, indicating a bell-shaped distribution of

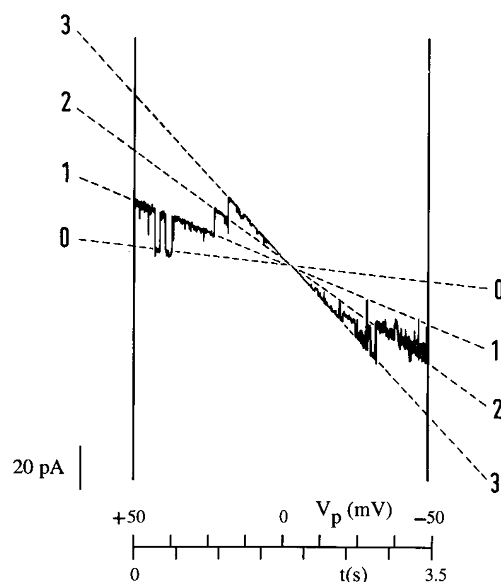

Figure 6. Current Fluctuations in the Inside-Out Configuration Evoked by PorB in the Pipette Filling Solution

Single channel currents versus pipette potential V_p. V_p was continuously decreased from 50 mV to −50 mV within 3.5 s, as indicated. Up to three channels of one population are simultaneously open. The broken lines denoted 1, 2, and 3 indicate the current obtained by superimposition of up to three single channel currents in the channel-open state. For the corresponding mean single channel conductance, 490 pS is derived. The broken line denoted 0 indicates the basal current of the membrane patch, which corresponds to conductance of 180 pS. The transition to the inside-out configuration was performed about 5 min after seal formation. About 1 min thereafter, the presented single channel current fluctuation became visible. The bath and pipette solution was high NaCl electrolyte. In addition, the pipette filling solution contained purified PorB, as described in Experimental Procedures.

the open probability versus voltage. Such behavior observed with PorB has previously been considered a fingerprint of VDAC channels (Bettendorff et al., 1993).

The PorB-induced channel could be blocked in the excised-patch configuration by addition of 5 mM ATP to the bath solution. In the continuous presence of ATP, the channel remained closed for more than 30 min (Figure 7). After washing out the ATP, the channel became activated again in one of these experiments.

Discussion

The porins of gram-negative bacteria form channels in the otherwise impermeable outer membrane and thereby facilitate the diffusion of solutes into the periplasmatic space and subsequent uptake into the cytosol via specific inner membrane transport complexes. The picture of porins as unspecific diffusion pores has been revised in the last years since a new class of porins with specific ligand-binding sites was characterized (Luckey and Nikaido, 1980; Hancock et al., 1982, 1992; Maier et al., 1988; Trias et al., 1988; Trias and Nikaido, 1990). A common property of the specific porins is that, owing to a specific substrate-binding site, diffusion of the substrates or ions is saturable. The specific porins build one part of a nutrient transport system, facilitating uptake under substrate-limiting conditions. It is interesting in this regard that the genetic expression of all known specific porins is induced under substrate limitation (Krieger-Brauer and Braun, 1980; Schwartz, 1987; Siehnel et al., 1988). However, the Neisserial PorB porins represent constitutively expressed, major outer membrane proteins.

Here we report on an unusual ligand-binding property of the PorB porins, i.e., the binding of nucleoside triphosphates. In contrast with the substrates of other specific porins, such as nitrogen, carbon, or phosphate, nucleotides play no role as nutrients in an extracellular environment, which is generally low in this compound. Nevertheless, it cannot be excluded that nucleotides represent an energy source of the pathogenic Neisseria species while they reside in an intracellular environment. Likewise are the intracellular Chlamydia known to utilize ATP from the cytosol of their infected cells (Moulder, 1991). The finding that only pathogenic Neisseria species produce porins capable of interacting with nucleotides suggests a role for nucleotide binding in pathogenesis. Porins of the pathogenic, but not of the nonpathogenic, Neisseria species were found to translocate from the outer membrane to the target cell membrane (Lynch et al., 1984; Blake and Gotschlich, 1987). Thus, the ability to interact with ATP appears to correlate with the translocation property of the Neisserial porins. We do not know yet whether the binding of nucleotides is a prerequisite for the translocation, as it was previously shown to be for the import of soluble mitochondrial porins into the outer membrane of mitochondria (Kleene et al., 1987; Ha et al., 1993). The translocation mechanism may be different for the Neisserial porin, which has to flip between two membranes in an environment at low concentrations of nucleotides. However, platelets and endothelial cells have been shown to secrete ATP (Meyers et al., 1982; Pearson and Gordon, 1979). Furthermore, attaching Neisseriae might well be able to induce or enhance an ATP release from target cells, thereby triggering the transfer of PorB molecules by a mechanism similar to the import of mitochondrial porin into the mitochondrial membranes.

Insertion of PorB into the cytoplasmic membrane causes apparently no toxic effect to the target cells (Blake and Gotschlich, 1987; Weel and van Putten, 1991; Lorenzen et al., submitted), although toxicity might be expected considering the high pore conductance measured for PorB in black lipid membranes. Therefore, unless PorB activity is regulated, the insertion would be expected to cause a breakdown of the membrane potential. Here we provide evidence that membrane-inserted PorB porin is tightly regulated by the target cell. Surprisingly, the single channel properties of the bacterial PorB were very similar to the eukaryotic VDACs described for a variety of different cell types (Blatz and Magleby, 1983; Schwarze and Kolb, 1984; Gray et al., 1984; Nelson et al., 1984; Kolb et al., 1985; Bettendorff et al., 1993). Like other VDACs, PorB channels remain closed in cell-attached membrane patches and become activated in excised patches in a manner similar to those previously described (Schwarze and Kolb, 1984; Kolb et al., submitted). This mode-dependent open probability

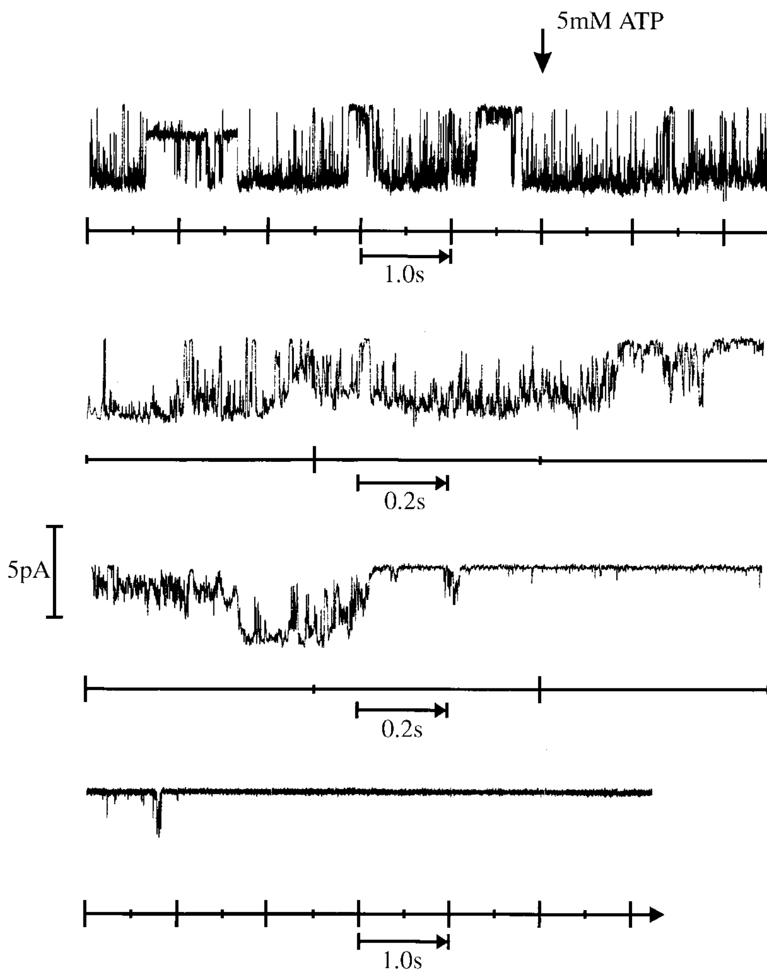

Figure 7. Current Fluctuations in the Inside-Out Configuration Evoked by PorB in the Pipette Filling Solution

Continuous single channel current record prior to and after exposure of the inside-out membrane area to the bath containing 5 mM ATP (see arrow). V_p was set to −10 mV. Channel openings are drawn downward. Improved resolution of the time scale is seen in the two center traces, which are expanded by a factor of 4. The pipette filling solution contained purified PorB (see Experimental Procedures).

suggests the involvement of cellular factors in the regulation of the high conductance pore channels.

Likewise, one member of the VDACs, the 31HL porin, identified in the plasma membrane of a lymphoblastoid cell line (Kayser et al., 1989) and also found in several other human transformed and primary cells (König et al., 1991), has a conductance of ∼2.4 nS in black lipid membranes. The voltage-dependent in vitro activity of this porin always exceeds the corresponding in situ pore activity, suggesting a superimposed regulation of porin conductance in situ (Benz et al., 1992). The mechanism of 31HL porin regulation is still unclear; however, consistent with our observations on mitochondrial porins, the 31HL porin interacts with ATP (Flörke et al., 1994), and hence ATP might also be involved in 31HL porin regulation.

ATP is the likely regulator of cell membrane–inserted PorB porin, since ATP is present in the cytosol of target cells at 5 to 15 mM concentrations. This would allow the modulation of the PorB channel, which has a K_m for these nucleotides of about 0.5 mM. However, the channel does not close completely in artificial lipid bilayers even in the presence of excess nucleotides. Additional mechanisms must be postulated that keep the porin channel closed in epithelial cells. The pronounced voltage-dependent gating of PorB, which is increased if nucleotides are present at the side of the applied negative potential, could be the key regulation mechanism. In this sense, nucleotides act as modulators of the voltage dependence. Only recently, such modulators of VDACs have been characterized (Benz et al., 1988; Holden and Colombini, 1993; Zizi et al., 1994) and found to share a common feature: like ATP in the case of PorB, the VDAC modulators increase the voltage dependence of the particular porin only if present at the site of the negative membrane potential. Activation of membrane-inserted PorB channels and VDACs might therefore involve membrane potential changes, thus causing the dissociation of modulators and subsequent opening of the porin channel.

The biological properties of PorB porins of pathogenic Neisseria (Haines et al., 1988, 1991) together with the influence of nucleotides on PorB physiology described here provide mechanistic clues to how pathogens manage to survive in phagosomal compartments. Purified PorB prevents degranulation of activated phagocytic cells without inhibiting the generation of superoxide anions by the NADP(H) oxidase, indicating the interference of inserted PorB with vesicular transport (Haines et al., 1988, 1991). Changes in membrane potential, a rapid depolarization followed by hyperpolarization, have been described as an early event of stimulus-induced

activation of neutrophils (Korchak and Weissmann, 1978; Whitin et al., 1980). Haines et al. (1988) have demonstrated that neutrophils respond entirely differently upon stimulation if PorB is present as compared with the control without PorB: instead of the rapid depolarization, a transient hyperpolarization can be observed. In light of the data presented here, it is quite possible that PorB channels counterregulate early events of neutrophil activation by changing, e.g., the ion composition of the cell. A similar mechanism probably underlies a survival strategy of Mycobacterium avium, which is found in phagosomes lacking intact H^+-ATPases (Sturgill-Koszycki et al., 1994). Because H^+-ATPases are contained in vesicles and are released and activated upon fusion with the phagosome, M. avium might express a factor operating in a manner similar to PorB in preventing the phagosomal maturation.

Here we demonstrate the binding of ATP and GTP to both eukaryotic and Neisserial porins and that this interaction probably occurs via the same mechanism. In light of the apparent structural and functional similarities between Neisserial and eukaryotic porins, it is tempting to speculate on a similar regulation of Neisserial porin after transfer to the target cell membranes. The Neisserial porin is thus an intriguing example of the molecular cross-talk that takes place between bacterial pathogens and host cells. Moreover, the structural and functional similarities between eukaryotic and Neisserial porins provide an interesting perspective on the evolutionary origin of mitochondria. Considering the fact that pathogenic Neisseriae are ingested by host cells and subsequently maintained viable in tight vacuolar membranes (McGee et al., 1981; Shafer and Rest, 1989; Virji et al., 1992; Kupsch et al., 1993) with an inserted ATP-regulated pore, these pathogens constitute a tantalizing model for comparing the molecular and cellular mechanisms underlying organelle endosymbiosis and pathogen accommodation in mammalian cells.

Experimental Procedures

Bacterial Strains and Cell Lines

Ngo strains MS11-N139 (Meyer et al., 1984) and VPI-N131 (van Putten, 1993) have been described previously. Ngo strains FA62-N115 and R16-N126 were obtained from Dr. E. C. Gotschlich (Rockefeller University, New York, NY), Ngo strain FA62-N437 was from Dr. V. Clark (University of Rochester, Rochester, NY), and Nme strains H44/76-N687 and 2996-N688 and Ngo strain 50491/80-N689 were from the Department for Medical Microbiology (University of Amsterdam). N. lactamica, N. cinerea, N. sicca, N. mucosa ssp. mucosa, and N. perflava were from Dr. U. Berger, provided via the Reference Laboratory for Bacterial Menigitidis (Amsterdam). The Chang conjunctiva cell line was derived from Flow Laboratories, Irvine (Ayshire, United Kingdom). The *rmp* ($PIII^-$) mutant (N357) was constructed by allelic replacement using pTF33 (*rmp::cat*; T. Fujiwara, unpublished data).

Nucleotide Binding Experiments

For labeling intact bacteria, 3×10^8 cells were suspended in 100 μl of phosphate-buffered saline (PBS) containing 10 mM $MgCl_2$ and incubated for 10 min at room temperature in the presence of 5 μCi of [α-^{32}P]ATP (3000 Ci/mmol; Amersham). Aliquots were either treated with 1 mM $NaIO_4$, 20 mM $NaCNBH_3$, and 20 mM $NaBH_4$ to link nucleotides covalently to proteins as described previously (Peter et al., 1992) or were incubated in PBS under the same conditions. The labeled bacteria were washed twice with PBS, lysed in loading buffer, and separated over 12.5% SDS–polyacrylamide gels, and the dried gels were then exposed for 24 hr to a Fuji RC film. For labeling purified porins, 50 ng of protein was reacted with 5 μCi of [α-^{32}P]ATP (3000 Ci/mmol; Amersham). After the cross-link reaction, porins were precipitated with acetone, washed with 70% acetone, and analyzed accordingly. The native PorB protein was acetylated according to the method described by Tokunaga et al. (1981). Therefore, aliquots of 1% acetic acid anhydride were added slowly to 100 μg fractions of PorB protein, which was solubilized in 200 μl of buffer B (20 mM MES, 0.08% LDAO, 100 mM LiCl) at pH 6.0, 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 8.0) to a final concentration of 0.2%.

Purification of Porins

Ngo strain VPI (PI.A) was cultured overnight in liquid medium, harvested, washed, and resuspended in buffer A (10 mM HEPES, 10 mM $MgCl_2$, 0.03% NaN_3) at pH 7.6. Crude membranes were obtained by lysing the bacteria in the presence of RNase and DNase by glass beads in a Vibrogen cell disrupter and pelleting the lysate at 110,000 $\times$ g for 60 min at 4°C. The membranes were extracted twice with 20 mM MES, 10 mM $MgCl_2$, 2% Triton X-100, 0.02% NaN_3 (pH 6.0) to remove the inner membranes. The remaining outer membranes were collected by centrifugation at 48,000 $\times$ g and washed with buffer A before outer membrane proteins were solubilized with 20 mM MES, 10 mM $MgCl_2$, 10% glycerol, 2% LDAO, 100 mM $NaCl_2$ (pH 6.0). After an additional centrifugation step, supernatants were applied to a cation exchange column (CM–Sepharose), washed with 4 vol of buffer B (20 mM MES, 0.08% LDAO, 100 mM LiCl) at pH 6.0, and eluted with a salt gradient from 100–800 mM LiCl in buffer B. OmpC of E. coli K12 and mitochondrial porin from S. cerevisiae were isolated and purified as previously described in full detail (Benz et al., 1985; Ludwig et al., 1988).

DNA Sequence Determination

Neisseria strains were grown on CG agar plates overnight. Chromosomal DNA was purified by the procedure described by Boom et al. (1990). The PCR reaction to amplify *por* genes was performed with primer pair TR39 (TGTAAAACGACGGCCAGTACTAGTCCAAAA AAGGAATACAGC) and TR46 (CAGGAAACAGCTATGACCAAGCTTT GCAGATTAGAATTTG) with chromosomal DNA templates. The primers contain M13 forward or reverse binding sequences, which allowed direct sequencing of amplified *por* genes according to a previously described method (Rudel et al., 1992).

Lipid Bilayer Experiments

The methods used for lipid bilayer experiments have been described previously in detail (Benz et al., 1978). Membranes were formed from a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids) in n-decane. Zero-current membrane potentials were measured with a Keithley 610C electrometer 5–10 min after a 10-fold salt gradient was established across the membranes (Benz et al., 1979).

Patch-Clamp Experiments

Chang human conjunctiva epithelial cells were seeded on coverslips and incubated in RPMI 1640 tissue culture medium (GIBCO) supplemented with 5% fetal calf serum at 37°C, 5% CO_2. On the day of the experiment, the culture medium was replaced by a high NaCl solution containing 142 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 18 mM glucose, and 20 mM HEPES at pH 7.4 (310 mOsm). The cells were used thereafter within 3–4 hr. Patch-clamp experiments were carried out as described previously (Hamill et al., 1981) with an EPC-7 amplifier (List Electronic). The seal resistance was in the range of 10 to 90 GΩ. The pipette medium contained high NaCl medium. A stock solution of solubilized and purified PorB in buffer B (see above) was used in experiments with PorB-containing pipette filling solutions. Aliquots of the stock solution were diluted 1:100 with high NaCl solution to a final porin concentration of 5 μg/ml. To improve the seal formation in the presence of PorB, we filled the outermost tip of the pipette with pure high NaCl solution and then back-filled the pipette with the PorB-containing solution. The indicated potential is the pipette potential (V_p) referred to a bath potential of 0 mV. Positive current deflections correspond to an anion flux from the

cytoplasmic side to the pipette interior. Experiments were performed at 20°C–22°C.

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GenBank Accession Number

The accession number of the sequence of N. gonorrhoeae VPI *porB* reported in this paper is Z69259.