Distinct sensitivities of OmpF and PhoE porins to charged modulators

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Abstract The inhibition of the anion-selective PhoE porin by ATP and of the cation-selective OmpF porin by polyamines has been previously documented. In the present study, we have extended the comparison of the inhibitor-porin pairs by investigating the effect of anions (ATP and aspartate) and positively charged polyamines (spermine and cadaverine) on both OmpF and PhoE with the patch-clamp technique, and by comparing directly the gating kinetics of the channels modulated by their respective substrates. The novel findings reported here are (1) that the activity of PhoE is completely unaffected by polyamines, and (2) that the kinetic changes induced by ATP on PhoE or polyamines on OmpF suggest different mechanisms of inhibition. ATP induces a high degree of flickering in the PhoE-mediated current and appears to behave as a blocker of ion flow during its presumed transport through PhoE. Polyamines modulate the kinetics of openings and closings of OmpF, in addition to promoting a blocker-like flickering activity. The strong correlation between sensitivity to inhibitors and ion selectivity suggests that some common molecular determinants are involved in these two properties and is in agreement with the hypothesis that polyamines bind inside the pore of cationic porins.

Key words: Escherichia coli; Porin; Inhibition; Selectivity; Patch-clamp

1. Introduction

The permeability of any biological membrane to hydrophilic solutes is essentially determined by the number and types of membrane-bound transporters and channels. In most cases, the specificity of protein carriers is dictated by the chemical nature or by the charge of their substrates. Not surprisingly, substrate specificity often extends into inhibitor specificity as well. The porins of the outer membrane of Gram-negative bacteria are not an exception, although the extent of specificity varies between the various groups of porins [1,2]. The so-called general diffusion porins select poorly on the basis of chemical structure, although they have a size-exclusion limit and ion preference. Members of this group are the osmotically regulated cation-selective OmpF and OmpC porins, and the PhoE porin.

PhoE is induced by phosphate starvation, and shows some selectivity for anions in general, and phosphate and phosphorylated compounds in particular [3–5]. Phosphorylated molecules, such as ATP, inhibit the flux of small ions through PhoE, observed as a decrease in the amount of total current flowing through a planar lipid bilayer doped with reconstituted PhoE [6,7]. This inhibition is specific for PhoE and is not observed in OmpF, and has been attributed to the presence of a specific lysine residue protruding in the PhoE pore [8,9].

Inhibition of the cation-selective OmpF and OmpC porins by positively charged polyamines, such as spermine and cadaverine, has been reported [10–12]. The investigation of the modulation of site-directed OmpC mutants by polyamines indicates that the interactions occur inside the pore [13]. In light of the apparent correlation that exists between ion selectivity of porins and the charge of their inhibitors, the goal of the present study was to test whether PhoE was sensitive to polyamines and whether OmpF was susceptible to any form of modulation by negatively charged compounds. In addition, by use of the patch-clamp technique, we can obtain detailed information on the open-close kinetics of individual channels in the absence or the presence of inhibitors. By comparing the kinetic patterns of ATP- or spermine-induced modulation of PhoE or OmpF, respectively, we can gain some understanding of the molecular mechanism that underlies the inhibitory effect. This type of investigation yielded extensive information on the modulation of the cationic porins by polyamines [10–12], but has not been performed in the case of PhoE and ATP. The results reported here suggest that the molecular nature of the interactions between porin and inhibitor is different for the two sets: ATP induces a high degree of flickering in the PhoE-mediated current and appears to behave as a blocker of ion flow, while polyamines modulate the kinetics of openings and closings of OmpF.

2. Materials and methods

2.1. Strains, chemicals and media

All strains used were *Escherichia coli* K-12 derivatives. AW738 [14] expresses OmpF only, and CE1265 is a *omp*R *pho*R strain that expresses PhoE only from plasmid pJP29 [15]. Modified Luria-Bertani (MLB) broth was 1% tryptone (Difco), 0.5% NaCl and 0.5% yeast extract. Spermine tetrahydrochloride, ATP (disodium salt) and aspartate (monopotassium salt) were purchased from Sigma. All solutions were adjusted to pH 7.2. Other chemicals were from Sigma or from Fisher.

2.2. Membrane preparation and electrophysiology

Outer membrane fractions were purified by sucrose gradient centrifugation from bacterial cells grown exponentially in MLB broth, and reconstituted in artificial liposomes [16]. Patch-clamp experiments were performed on blisters induced from the reconstituted liposomes [17]. Since porins strongly associate with each other and with lipopolysaccharides, it is likely that they remain in a protein and lipid environment that is closer to the natural one than if they had been purified. Therefore, we purposely chose not to reconstitute purified porins. We can recognize the activity of OmpF, OmpC and PhoE porins from extensive comparative studies between wild-type strains expressing a single porin type, null mutants, point mutants and chimeras [13,18–20]. Protein:lipid ratios of 1:1600 to 1:1800 (w:w) were typically used yielding seals of ~0.5–1.0 GΩ, due to the presence of multiple open channels in each patch. Experiments were performed on insight-out patches after excision by air exposure. The protein orientation is not random and is identical to the natural one, with the extracellular side

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facing the pipette solution, as previously determined [20,21]. In all figures, the voltages indicated are pipette voltages.

Control experiments were done in symmetric solutions of 150 mM KCl, 5 mM HEPES, 0.1 mM K-EDTA and 0.01 mM $CaCl_2$ at pH 7.2. 15 ml solutions of spermine, cadaverine, aspartate or ATP were prepared in the same buffer (pH adjusted to 7.2) and then applied to the periplasmic side of the patch with bath perfusion on a ~2 min time scale. All solutions were filtered before use through a 0.2 μm filter. Current recordings and analysis were done as described [19,20].

Patches typically contain 20–50 porin subunits in the open state. The current flowing through all the open channels corresponds to a 'baseline' ('BL' in the figures) from which current deflections arise during the transient closures of one or many monomeric pores. For the sake of clarity, we have represented all the traces of the figures in such a way that the closures correspond to upward deflections (even when traces of opposite voltages are compared). The current levels through single monomeric pores are indicated either as tick marks or dotted lines.

3. Results

We have recently characterized the inhibition of OmpC and OmpF porins by natural and synthetic polyamines [10–12]. Here we have extended our studies to the anion-selective porin PhoE and compared the effect of polyanions and polycations on OmpF and PhoE.

When patches of membrane containing many OmpF porins are exposed to bath-applied spermine, an increase in the frequency and duration of closing events is observed. This modification in the gating activity is illustrated in the traces of Fig. 1, where closures are represented by upward deflections. The onset of OmpF inhibition by spermine occurs in the range of a few tens of nanomolar [11]. By contrast, the activity of PhoE is completely unaffected by spermine (Fig. 1D), up to 10 mM, the highest concentration tested. The difference in sensitivity of the two porin types towards spermine indicates that the effects are specific and not artifacts of the experimental procedures. The same results were observed in eight experiments on PhoE obtained from three membrane preparations, and in four experiments on OmpF obtained from two preparations (see also [11]). In addition, six experiments (data not shown) showed that PhoE is also insensitive to cadaverine (up to 300 mM), another inhibitor of OmpF activity [11]. Altogether, these results suggest that a correlation might exist between the selectivity of the pore and the charge of the modulator.

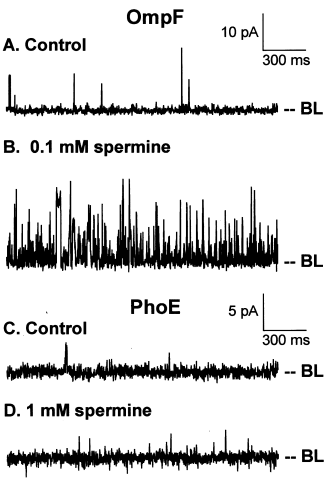

Fig. 1. Representative current traces of OmpF or PhoE activity in the absence (control) or the presence of the bath-applied spermine concentrations indicated. The baseline current level is marked by BL. The pipette voltage is −60 mV.

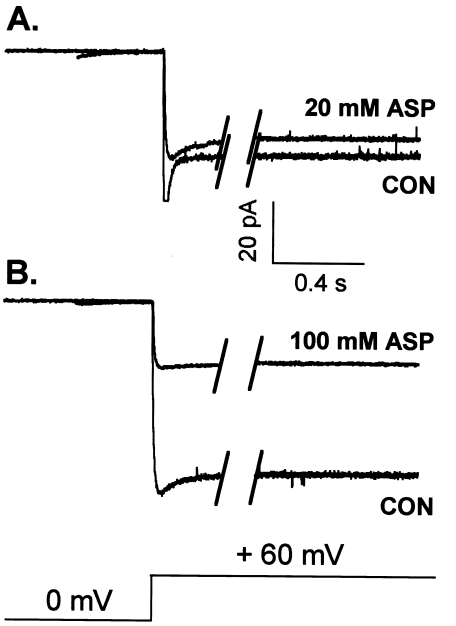

Fig. 2. Inhibition of macroscopic current through PhoE-containing patches in the presence of the bath-applied aspartate (ASP) concentrations indicated. Traces in the absence (CON) and the presence of aspartate have been overlaid for the sake of comparison. Capacitative currents have not been subtracted and are seen as a slow relaxation of current following the voltage step. Some variability exists from patch to patch depending on the patch area and the extent of capacitance cancellation. The voltage protocol is shown below panel B.

In order to test whether PhoE can be inhibited by negatively charged compounds, we applied aspartate to patches containing PhoE only. We did not observe any increase in gating activity, but only a decrease in the macroscopic current flowing through the patch. Fig. 2 shows traces obtained from two PhoE patches before (CON) and after the perfusion of 20 or 100 mM aspartate in the bath. For the sake of comparison, the control and modulated traces are overlaid. The downward deflection observed when the pipette potential is switched from 0 to +60 mV represents the total amount of current flowing through the patch. This macroscopic current (called seal current in our previous publications) is largely due to ions passing through many open porin channels (the true seal current of porin-free patches is ~2 pA at 60 mV). The decrease in macroscopic current in the presence of aspartate is not observed on patches made of pure lipids only. Since the current through single pores is not affected (25 and 27 pA in the absence and the presence of 100 mM aspartate, respectively), the decrease in macroscopic current is indicative that a number of porins have been inhibited. For these experiments, 18% and 63% decreases were observed in the presence of 20 mM and 100 mM aspartate, respectively. The effect of this compound on PhoE is similar to that of putrescine on OmpF [11]: both induce a macroscopic current decrease but no modulation of the gating kinetics. Millimolar concentrations of either compound are also required for inhibition. It is possible that, as suggested for putrescine [11], a mild increase in closing

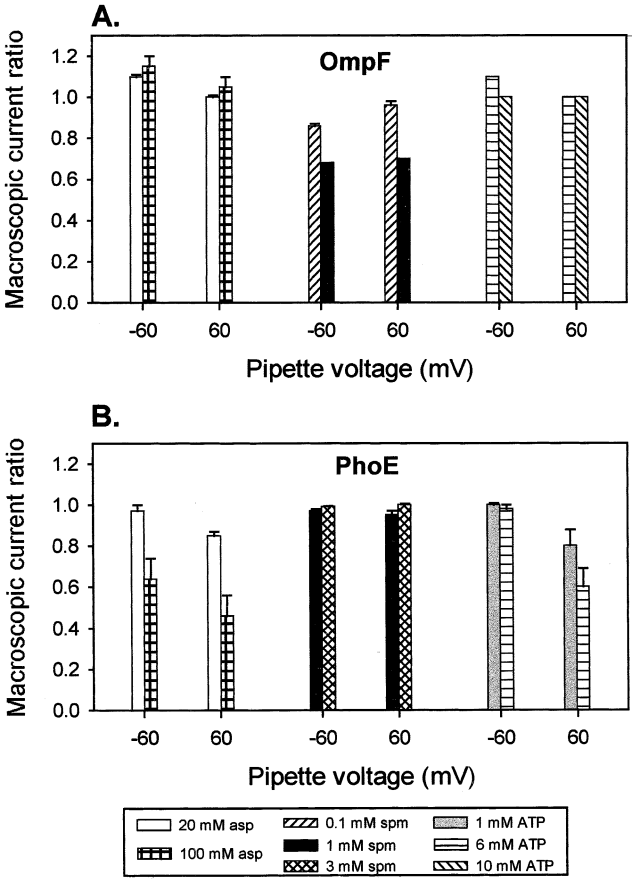

Fig. 3. Macroscopic current ratios obtained with OmpF and PhoE at the voltages indicated, in the presence of the bath-applied compounds at the concentrations indicated. The y axis is calculated as the ratio of macroscopic current in the presence of the compounds to that in their absence. The slight increase in OmpF macroscopic current observed with aspartate (especially at 100 mM) is likely to reflect some deterioration of the seal due to the osmotic imbalance between bath and pipette solutions.

kinetics might have been observed if the number of open porins in the patch had not changed after aspartate perfusion. Alternatively, both aspartate and putrescine, which are fairly small molecules, might affect their respective porin targets differently than the longer modulators, such as spermine and ATP (see below).

Fig. 3 documents that such macroscopic current decrease was not induced by aspartate on OmpF-containing patches. Panels A and B of Fig. 3 compare the ratio of macroscopic current in the presence of a compound to that in its absence, for OmpF and PhoE respectively. Negatively charged molecules, such as aspartate and ATP, induce a macroscopic current reduction in PhoE but not OmpF. Positively charged molecules, such as spermine, decrease the macroscopic current through a patch of OmpF porins, but not of PhoE porins. In addition, in the cases where porin inhibition is observed, a concentration and voltage dependence is manifested, as seen for all polyamines acting on OmpC and OmpF [11]. The macroscopic current reduction induced on PhoE by bath-applied anions is more pronounced at a pipette potential of +60 mV than at −60 mV. The converse is true for the inhibition of macroscopic current induced by spermine on OmpF. It is noteworthy, however, that high concentrations of modulators can override an unfavorable voltage to some extent.

Taken together these results suggest that the charged modulators are attracted to the porin mouth by pipette potentials of the opposite sign, and that the specific charge configuration within the pore – that also influences selectivity – might participate in the modulatory effect. Similar observations had been previously reported for the specific inhibition by ATP and other phosphorylated compounds of PhoE porin [6]. In their study, Dargent and colleagues [6] investigated PhoE in planar lipid bilayers and focused their investigation to the effect of ATP on the overall ionic current flowing through the bilayer (akin to the macroscopic current data reported above). In order to compare the mechanism of action of polyamines on the cation-selective porins with that of ATP on PhoE, an investigation of the gating kinetics is required. Such a detailed analysis of the kinetics of single PhoE channels was not previously provided.

Fig. 4 compares the kinetic signatures of OmpF channels modulated by spermine at −60 mV with those of PhoE channels modulated by ATP at +60 mV. Both patches contained about the same number of open pores (∼40 as determined from the macroscopic current and the single monomer conductance), the single monomer currents of OmpF and PhoE porins are similar (1.8 and 1.7 pA, respectively, for these experiments), and the extent of modulation is identical as determined by the ratio of macroscopic current in the presence to that in the absence of modulator (∼86%). The two traces, however, look very different, suggesting that both modulators might act differently on their respective targets. We have argued that the natural polyamines exert an allosteric effect on OmpF and OmpC, because they appear to stabilize closed states rather than only blocking the pore [10,11]. Thus, polyamines induce the closures of many monomers at the same time, often for prolonged periods (our interpretation of cooperative gating of porins in the absence or the presence of modulators has been discussed previously; see [11,13,20,22], and also Section 4). Examples of such long closures are shown

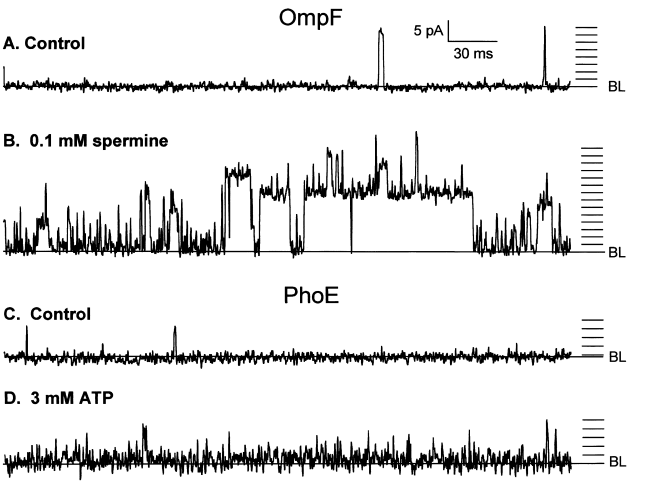

Fig. 4. Expanded traces of OmpF and PhoE activity in the absence (control) or the presence of their respective modulators as indicated. The favored current level corresponding to a large number of open pores is indicated as the baseline (BL). Upward deflections are closures. Ticks mark the single-pore current amplitudes (1.7 pA for PhoE; 1.8 pA for OmpF). The potentials were −60 mV for the OmpF traces and +60 mV for the PhoE traces.

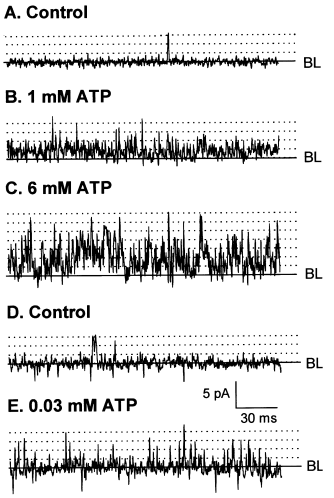

Fig. 5. Concentration dependence of the ATP-induced kinetic effect on PhoE activity. Traces were obtained from two separate patches in the absence (control) and the presence of bath-applied ATP at the concentrations indicated. A horizontal line through the trace marks the estimated position of the baseline current level (BL). Dotted lines indicate the single-pore current amplitudes (for this experiment, 1.5 pA and integer multiples). The pipette potential was +60 mV.

in the trace of Fig. 4B. On the other hand, the inhibition of PhoE by ATP is reflected by a high degree of flickering of the current trace between levels corresponding to closures of one or a few monomers. This kinetic signature is typical of an open-channel blocker [23]. This type of activity leads to a greatly thickened baseline. At concentrations of 10 or 30 mM, the thick baseline occasionally shifts to a lower current value, due to the prolonged closures of many channels, but these events are relatively rare. We have not been able to test higher concentrations because of patch instability.

We typically quantify the effect of modulators by comparing the total number of closures per unit time in control and modulated conditions [11,12]. This type of analysis requires that we set the baseline level of current from which the closures originate. Although we have marked this approximate location of the baseline in the figures, we do not feel that we can set this level with the accuracy needed for a quantitative analysis of the ATP block. The reason is the high level of flickering activity in the presence of ATP that yields to extremely short dwell times at the baseline level. For the same reason, we have not attempted to measure the single channel conductance, although visual inspection of the traces suggests that the same single channel current can be used to fit multiple current levels in both the presence or the absence of ATP. Hence, the tick marks or current levels lines displayed in Figs. 4 and 5 are identical for the control and modulated traces.

The ATP-induced flickering activity is both voltage- and concentration-dependent. As for the inhibition of macroscopic current, the effect is observed at positive pipette potentials only, i.e. voltages that are positive on the side of the membrane opposite to the side of ATP application. As we have seen for polyamines, this type of voltage dependence appears to stem from the attraction of the modulators inside the pore, and argues that the site of interaction is within the pore. This 'electrophoretic' interpretation had already been proposed by Dargent and coworkers [6].

An enhancement of the flickering activity occurs at higher ATP concentrations (Fig. 5). This observation supports the interpretation that the change in kinetic signature is truly due to ATP, and not the reflection of a non-specific effect. The traces of Fig. 5B,C show that as the ATP concentration increases, the current oscillates between a great multiplicity of levels corresponding to the block of multiple monomeric pores. Again, in contrast with the concentration dependence of the polyamine inhibition of OmpF [11], the effect of greater ATP concentrations is not to prolong the non-conducting states, but to induce the flicker of an increasing number of pores. As illustrated in Fig. 5E, a mild effect is produced by submillimolar concentrations: 0.03 mM ATP appears to be the threshold concentration for inducing flicker spikes, but does not produce any reduction of macroscopic current. We have found that concentrations of at least 1 mM were required to induce macroscopic current decrease, in agreement with the $\sim$2 mM apparent dissociation constant previously reported from a similar kind of measurement [6]. Thus, the measurement of macroscopic current decrease by itself can be misleading regarding the ability of a specific ATP concentration to produce inhibition. It is also possible that, as suggested for polyamines and cationic porins, the reduction in macroscopic current and the modification of channel kinetics arise from binding of the modulators to two different classes of sites [11,13].

4. Discussion

The inhibition of PhoE by anions and polyanions has been documented previously [6,7], as well as the modulation of the cation-selective porins OmpC and OmpF by positively charged polyamines [10–12]. In the present study, we have extended the comparison of the modulator-porin pairs by investigating the effect of anions and polyamines on both OmpF and PhoE, and by comparing directly the gating kinetics of the channels modulated by their respective substrates. The novel findings reported here are (1) that the activity of PhoE is completely unaffected by spermine and cadaverine, and (2) that the kinetic changes induced by ATP on PhoE or polyamines on OmpF suggest different mechanisms of inhibition.

Although the first observation is a negative result, it has some important implications on the modulation of cationic porins by polyamines from both biochemical and physiological standpoints. At the level of protein chemistry, it implies that the effect involves specific interactions between polyamines and their porin targets. The correlation that exists between the sensitivity of porins to charged modulators and their intrinsic ion selectivity suggests that these two processes share similar molecular determinants, and hence that the modulator binding site resides within the pore. From the physiological standpoint, the distinct sensitivities of porins to charged modulators suggests that the impact of natural or synthetic inhibitors of porin will be considerably influenced by the protein makeup of the outer membrane. It appears that polyamines may play some role as endogenous modulators of outer membrane permeability [22,24,25]. Together with the opposite voltage dependence displayed by OmpF and PhoE [20] and the well-known environmental control of porin genes

[1], the distinct sensitivities of porin channels towards polyamines and other compounds extend the possibility for a fine-tuning of outer membrane permeability in a variety of environmental conditions.

The lack of a spermine effect on PhoE fits well with the postulate that the presence of positively charged residues lining the constriction zone of the pore (the 'hot spot' postulated by Bauer and colleagues [7]) plays the dual role of repelling cations and attracting anions. The crystal structures of OmpF and PhoE have revealed two prime candidates for such a role: K18 of PhoE substituted for glycine in OmpF, and K125 substituted for G131 in OmpF [9]. Both protrude towards the lumen of the channel and are accessible to modulators applied from the periplasmic side, as in our experiments. The contribution of each residue to the repelling effect towards polyamine needs to be assessed, and preliminary data indicate that K18, along with K125, may be important in this respect (Samartzidou and Delcour, in preparation).

Our finding that the macroscopic current through patches containing multiple PhoE pores is affected by ATP and aspartate is in agreement with previous reports that there does not appear to be a specificity for polyphosphates, but rather a general anion selectivity [4,7]. However, we must keep in mind that the two compounds affect PhoE differently, since aspartate does not promote kinetic effects in addition to macroscopic current reduction, while ATP does; at this point, it is hard to distinguish whether this difference is due to the fact that ATP is a more charged and longer molecule, or whether the types of interaction with PhoE are really distinct. It is possible that the macroscopic current reduction involves a different mechanism than that inducing kinetic changes. For example, in the case of polyamines and cationic porins, macroscopic current reduction still occurs in mutants that have lost the kinetic response to polyamines [13]. It is possible that this aspect of modulation involves a different set of molecular determinants, that may or may not overlap with those that dictate modulation of kinetics.

The patch-clamp technique allows the detection of fast events of small amplitude and is well suited for the measurement of rapid current fluctuations resulting from interactions of ion channels with blockers and modulators [23]. The difference in kinetic signatures of OmpF modulated by spermine and PhoE modulated by ATP suggests that the molecular basis for the interaction between the porins and the modulators might be different. Higher concentrations of ATP enhance the flickering activity of PhoE, rather than prolong non-conducting states. This concentration-independent behavior of the flicker dwell times is consistent with a blocking mechanism, where the off-rate, directly related to the average dwell time of the blocked state (i.e. the flickers), is concentration-independent. Although large, ATP has a molecular weight that is slightly below the 600 Da cut-off for porins and might still permeate through the PhoE pore, where it would act as a blocker of ionic current. In some respect, this behavior is similar to that of ATP during its transport through VDAC [26], although a direct comparison is not feasible because our experiments were not done on single PhoE pores.

The comparison between the kinetic signatures of PhoE and OmpF in presence of their respective modulators is informative because it emphasizes that polyamines do more than simply blocking the OmpF pore as they permeate through it. The OmpF kinetic pattern in the presence of spermine is more complex, with a mixture of high frequency flickers and prolonged closures involving many unit pores. The high level of cooperativity that is associated with the closing and reopening transitions of the prolonged closures is difficult to reconcile with a rapid and simultaneous binding and unbinding of blocker molecules to a large number of pores. We and others have documented a cooperative behavior that extends beyond trimers, even in the absence of polyamines [20,27–29]. Given the natural large density of porins and the fact that we use outer membrane fractions rather than purified porins, it is not surprising to find a great extent of clustering in these macropatches, and protein-protein interactions need not be restricted to monomers within individual trimers. Such interactions between adjacent trimers has been proposed in studies of two-dimensional OmpF/phospholipid crystals by atomic force microscopy [30]. Thus, we propose that, in addition to producing some amount of block (hence the flickers), polyamine molecules interfere with the intrinsic gating machinery of OmpF and OmpC. This interference then results in modulation of gating kinetics seen as a stabilization of closed states [10,11].

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