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Disruption of polyamine modulation by a single amino acid substitution on the L3 loop of the OmpC porin channel

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Abstract

Structural studies have demonstrated that the extracellular L3 loop of porin constricts the channel and suggest that this loop might be involved in channel selectivity and gating. We previously showed that positively charged polyamines can induce changes in porin gating kinetics by stabilization of closed states. Here we report the effects of the mutation of two different aspartate residues of *Escherichia coli* OmpC porin on the polyamine sensitivity of the channel. Aspartate 105 or aspartate 118 on the L3 loop was replaced by glutamine by site-directed mutagenesis. The gating activity of the wild-type and mutant channels were studied by patch-clamp of liposomes containing reconstituted outer membrane fractions, in the absence or the presence of either polyamine spermine or cadaverine. Porin channels with a D118Q mutation, at the root of L3, still showed some, albeit milder, sensitivity to polyamine modulation. On the other hand, the D105Q mutation, at the tip of L3, abolished the increase in closing frequency which is typically observed in the presence of polyamines. We conclude that aspartate 105 primarily, but not aspartate 118, plays an important role in mediating the polyamine-induced changes in gating kinetics that result in the inhibition of the OmpC channel.

Keywords: Porin; Polyamine; Ion channel; Modulation; Mutant

1. Introduction

Bacterial porins are some of the best characterized ion channels at the structural and biochemical levels [1]. X-ray crystallographic data and secondary structure information have revealed that these outer membrane proteins are trimers of β-barrels, each made of 16 β-strands interspersed by short periplasmic and long extracellular loops [2]. A hydrophilic pore is formed in the center of each monomer, allowing the permeation of solutes of up to 600 Da. A salient feature of all non-selective porins studied so far is the folding of the third extracellular loop, L3, across the channel pore [2–4]. Because of its location within the solute conduction pathway, L3 is postulated to form the constriction zone of the channel where some selectivity is achieved on the basis of size and charge [2]. For example, the anionic preference of the PhoE porin, primarily involved in phosphate transport in vivo, can be attributed to a single lysine residue (#125) of L3 [5], while in the slightly cation-selective OmpF the corresponding amino acid is replaced by glycine.

In recent years, the functional properties of porins have been studied by electrophysiological recordings

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made either on purified porins or on outer membrane fractions reconstituted in planar lipid bilayers or in liposomes [6–12]. Although primarily found in the open state, porin channels can display long-lived closed states that are stabilized by either high transmembrane potentials [6,10,12] or polyamine binding [13,14]. An attractive hypothesis for the voltage- or ligand-driven closures may be that the L3 loop can somehow pivot across the channel to further narrow the ion conduction pathway. Theoretical modeling of electrostatic interactions have suggested that this intramolecular motion might be feasible [15]. Experimentally, it has been shown that the voltage-dependence of this closing activity can be dramatically altered by site-directed mutations of charged residues on L3 or other protein regions [16,17].

In an effort to further our understanding of the relationships between structure and function, we have introduced two mutations on the L3 loop of OmpC, both of which have an aspartate residue replaced by glutamine, and hence remove a negative charge. We have found profound differences in the gating kinetics of the mutants (D105Q and D118Q) which will be evident in this report but will be characterized in details in another publication (Liu et al., manuscript in preparation). In this paper, we have focused our attention on the impact of these mutations on polyamine modulation of porins. The inhibition of the bacterial PhoE porin and the mitochondrial VDAC porin by negatively charged polyanions had previously been described [18–22]. We have recently shown that **polycationic** aliphatic amines (polyamines), such as putrescine, cadaverine, spermidine and spermine, can inhibit porin channels in a voltage-, pH- and concentration-dependent fashion, and that this inhibition can lead to a substantial decrease in outer membrane permeability [13,14,23]. The presence of polyamines in vivo in the vicinity of porins suggests that the modulation by endogenous compounds may be physiologically relevant [24]. A modulation by these compounds has also been demonstrated in a number of eukaryotic channels [25,26]. In the case of porin, the inhibition is achieved by the promotion of short-lived and long-lived (inactivated) closed states [13]. The voltage- and pH-dependence of the effect suggest that ionic interactions play an important role and that the binding of the polyamine might reside somewhere within the pore.

We have made the hypothesis that the L3 loop might be involved in the polyamine-porin interaction, and that the interaction is likely to involve negatively charged residues at the tip of L3 (exposed to the pore interior) rather than at the root of L3 (close to the barrel wall). The resulting inhibition may be due to either a direct binding of the polyamine to negatively charged residues on L3 or through the disruption of the electrostatic field that is created between L3 and the opposite barrel wall [27]. To test this hypothesis we have analyzed the behavior of the D105Q and D118Q mutants with respect to modulation by spermine and cadaverine. The data presented in this paper demonstrate that the replacement of aspartate by glutamine at the root of L3 (D118Q) had little effect on the polyamine inhibition. On the contrary, the D105Q mutant showed relief from the inhibition, suggesting that this residue plays an important role in this type of modulation. Furthermore, the data support the notion that OmpC exists in multiple closed and open states which might be regulated by polyamine binding to distinct sites.

2. Materials and methods

2.1. Chemicals and strains

E. coli K12 strains AW738 ($OmpF^+$ $OmpC^-$) and AW739 ($OmpF^-$ $OmpC^+$) [28] were used in the mutant construction. Tryptone growth medium (T-broth) contained 1% tryptone (Difco Laboratories) and 0.5% NaCl. Luria-Bertani broth (LB) was from Difco Laboratories. Cadaverine and spermine were obtained from Sigma Chemical Co. as the hydrochloride species, and did not alter the pH of solutions. Azolectin (phosphatidylcholine) was from Sigma, and all other chemicals were from Fisher Scientific. Enzymes used in molecular biology protocols were purchased from either Gibco or Promega. The DNA sequencing and purification kits were from United States Biotechnology and Qiagen, respectively.

2.2. Mutant construction

In order to control the expression of the genes transcribed by the *lacZ* promoter, we cloned an

*Eco*RI fragment bearing *lacI* from pMC9 into the vector pAC9, a low copy number plasmid [29]. The *ompC* gene was amplified by polymerase chain reaction using chromosomal DNA from AW739 and cloned into this modified pAC9 vector at the *Sal*I and *Bam*HI restriction sites. The unique site elimination method (U.S.E. kit, Pharmacia Biotech) was used to generate site-directed mutants. The D105Q and D118Q mutations were confirmed by DNA sequencing.

In order to avoid the use of unstable double mutant strains ($OmpF^-$ $OmpC^-$), we transformed AW739 cells with the plasmid carrying either the wild-type or mutant *ompC* allele. P1 transduction [30] was then used to move an *ompC* deletion from strain AW738 (*ΔompC zei*::Tn*10*) into the chromosome of the plasmid-bearing AW739 strain. Transductants, which are genomically *ompF*$^-$ *ΔompC*, were selected on LB plates containing tetracycline (15 μg/ml) with 0.7 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in the presence of 25 μg/ml kanamycin for plasmid maintenance. *OmpC* expression from the plasmid was confirmed by sensitivity to phage SS4 in the presence of 0.7 mM IPTG and by gel electrophoresis. In all the experiments reported here the expression of either wild-type or mutant *ompC* was from the plasmid-encoded gene.

2.3. Membrane preparation and electrophysiology

The preparation of purified outer membrane fractions was done according to published protocol [31]. Briefly, wild-type or mutant cells were grown to mid-log phase in T-broth at 37°C in the presence of 0.7 mM IPTG, harvested and lysed by two passages through a French press at 16 000 psi. Outer membrane fractions were purified by sucrose gradient centrifugation and stored at −80°C. The determination of protein concentration was done by the bicinchonninic acid method (Pierce).

For electrophysiology, an aliquot of native membrane was mixed with sonicated phosphatidylcholine at a protein-to-lipid ratio of 1:1600 (w:w). The reconstitution proceeded according to a dehydration-rehydration method [31]. The patch-clamp technique was applied to blisters induced from liposomes directly in the recording chamber, as previously described [31]. After patch excision by air exposure, recordings were made in symmetric solutions of the following buffer: 150 mM KCl, 5 mM Hepes, 0.1 mM K-EDTA, 10 μM $CaCl_2$, pH 7.2. Cadaverine or spermine in the same buffer was always applied to the inside-out patch with bath perfusion.

Current measurements were made using standard patch-clamp techniques [32] with an Axopatch 1D amplifier (Axon Instruments). Pipettes had an initial resistance of 10 MΩ. The data was filtered at 2 kHz with a 8-pole Bessel filter (Frequency Devices), and stored on VCR tapes (Instrutech). For data analysis, specific segments of data were refiltered at 1 kHz and digitized at 100 μs sampling interval. Data acquisition and analysis was performed with personally developed software using Axobasic (Axon Instruments).

2.4. Data analysis

A typical record from a porin-containing patch shows a baseline level, which corresponds to the total amount of current flowing through a large number of open porins (see Section 3). It is labeled 'BL' in Fig. 1. For construction of amplitude histograms and kinetic analysis, the 0 pA level has been arbitrarily assigned to this baseline level. Amplitude histograms are obtained by scanning the current record and counting the number of sample points which have amplitudes that fall within ranges of 0.1 pA (0.1 pA bins), spanning from −5 to 10 pA. We have arbitrarily chosen to represent as negative values the amplitudes that correspond to openings, while those of positive values are from closures. Well-defined peaks are obtained in amplitude histograms when the opening or closing transitions are frequent and/or of long duration. In the case of porins, openings, although frequent, are typically unresolved, and show up only as a left shoulder of the 0 pA peak. Many transient closures also have such a short duration that they escape detection in amplitude histograms. For example, in wild-type non-modulated channels, no distinct peaks are observed in the positive range of the amplitude histogram (Fig. 2A) even though closures are visible on the trace (Fig. 1A, 'CON').

For these reasons, current amplitudes were typically obtained from inspection of individual closures of a single or multiple channels, and plotted against

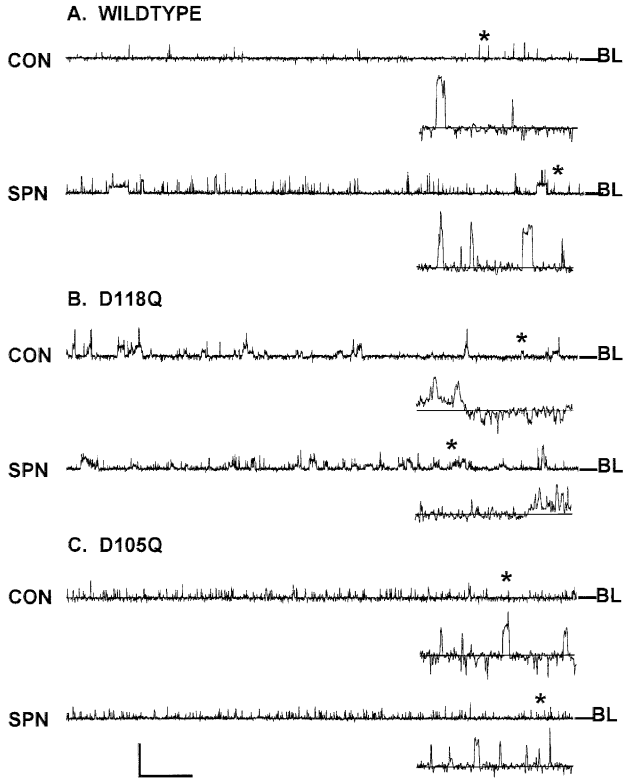


Fig. 1. Patch-clamp recordings of wild-type (A) and mutant (B, D118Q; C, D105Q) OmpC activity in an excised patch sequentially exposed to 0 (CON) or 1 mM spermine (SPN) in the bath. For each 20-s trace, an asterisk marks the location of a 80-ms-segment which is shown below on an expanded scale. 'BL' represents the baseline level on the main traces and is shown as a horizontal line through the expanded traces. Upward deflections from the baseline are closures, and downward deflections are openings of additional channels. The ordinate scale bar is 20 pA for the main traces and 6.7 pA for the expanded traces. The abscissa scale bar is 2 s for the main traces and 27 ms for the expanded traces. The single channel currents were in control conditions: 1.6 pA for wild-type, 1.7 pA for D118Q, 1.2 pA for D105Q. The pipette voltage was −60 mV.

voltage. The single channel conductance was deduced from the best fit through all points, on the assumption that larger current amplitudes are integral multiples of the single channel amplitude. Our working hypothesis is that the smallest unit of conductance represents a porin monomer. It is the most frequent transition that appears as a distinct event and has the size of the smallest common denominator of all current levels. Thus, when we use the term 'channel' in the text, we refer to a single porin monomer. We consistently observe in patch-clamp experiments that the closure of multiple channels extend beyond the

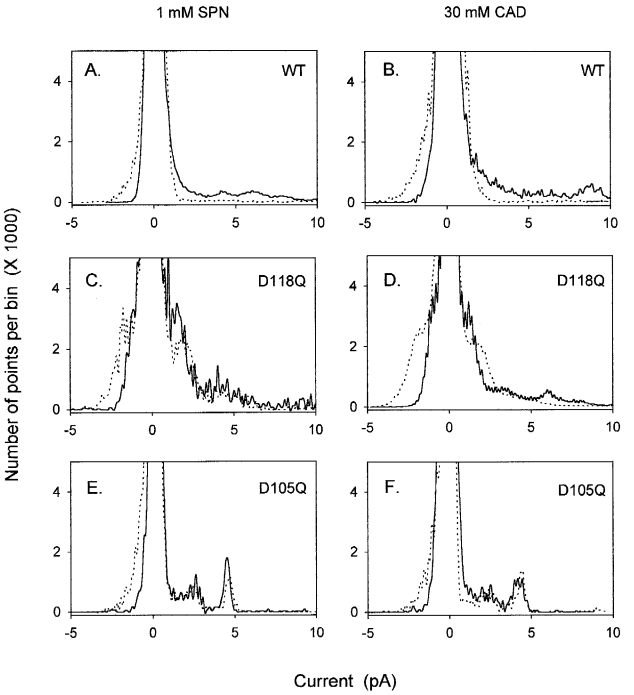


Fig. 2. Amplitude histograms obtained from 25-s recordings of OmpC activity in the absence (dotted line) or the presence (solid line) of polyamine: 1 mM spermine (panels A, C, E) or 30 mM cadaverine (panels B, D, F). The 0 pA level represents the baseline current through many open channels; positive current values represent closures; negative current values represent openings. Except for the D118Q mutant, single channel peaks are not resolved from the baseline peak at 0 pA, but the single channel current can be obtained from measurements of computer-displayed traces. Peaks corresponding to the prolonged closures of multiple channels have the following values (in pA): panel A (solid line): 4.2, 6.0 and 7.8 (2, 3 and 4 channels, respectively); panel B (solid line): 6.0 and 8.8 (3 and 5 channels); panel C (dotted line): 1.7 and 4.0 (1 and 2 channels); panel C (solid line): 1.6 and 4.2 (1 and 2 channels); panel D (dotted line): 1.8 and 3.9 (1 and 2 channels); panel D (solid line): 1.2, 3.3 and 6.1 (1, 3 and 5 channels, respectively); panel E (dotted line): 2.3 and 4.7 (2 and 4 channels); panel E (solid line): 2.3 and 4.3 (2 and 4 channels); panel F (dotted line): 2.4 and 4.5 (2 and 4 channels); panel F (solid line): 2.4 and 4.3 (2 and 4 channels). The I_{500} values are as follows: −1.7 and +1.3 (A, CON); −1.0 and +1.8 (A, SPN); −2.5 and +2.0 (B, CON); −1.4 and +3.5 (B, CAD); −2.7 and +2.5 (C, CON); −1.6 and +2.9 (C, SPN); −3.0 and +3.5 (D, CON); −1.6 and 3.2 (D, CAD); −1.7 and +1.3 (E, CON); −1.3 and +1.0 (E, SPN); −1.8 and +0.8 (F, CON); −1.1 and +1.5 (F, CAD). The pipette voltage was −60 mV. Bins are 0.1 pA.

trimeric unit and that the closures of trimers are not the favored events. This interesting phenomenon is likely due to clustering of channels and extensive contact between trimers.

Because of the unresolved nature of the opening transitions, kinetic analysis was done only for the closures with an algorithm that uses the half-amplitude criterion to classify events as closures of 1, 2, . . . , N channels, and includes as genuine transitions events lasting for more than 300 μs. This means that every time the current amplitude remains larger than half of the single channel current for at least three consecutive sample points (taken every 100 μs), the program counts this deviation as a closing event of 1 channel. For multiple channels, multiple of half the single channel amplitudes are used as criteria. The events involving 1, 2, . . . , N simultaneously closed channels are counted and represented in a histogram form ('closure histograms' of Fig. 3). This analysis is more sensitive to event detection than amplitude histograms since it tallies events even if they are of very short durations. This gives an explanation for the apparent discrepancy between amplitude histograms and closure histograms. Closures of 1 channel are very short and frequent, and thus they do not appear as a distinct peak in amplitude histograms, but are the dominant bar in closure histograms. On the other hand, closures of multiple events are less frequent than single channel closures but more prolonged (especially in the case of modulated channels), and thus appear as a peak in amplitude histograms, but have a smaller size bar in closure histograms. As a corollary, some rare events corresponding to the closures of large numbers of channels are detected in the closure histograms, but do not appear in the amplitude histograms. For example, the largest peak of the histogram obtained in the presence of spermine in Fig. 2A (solid line) is due to the closing of 4 channels, but the closures of up to 9 channels could be detected in the closure histogram (gray bars in Fig. 3, WT). Depending on the interplay between frequency and duration, some events may appear dominant in one histogram type but not the other. For this reason, our conclusions on the modulation of closing kinetics is based only on the closure histograms.

For the calculation of the overall open probability, NP_o, we assumed that in all conditions the seal

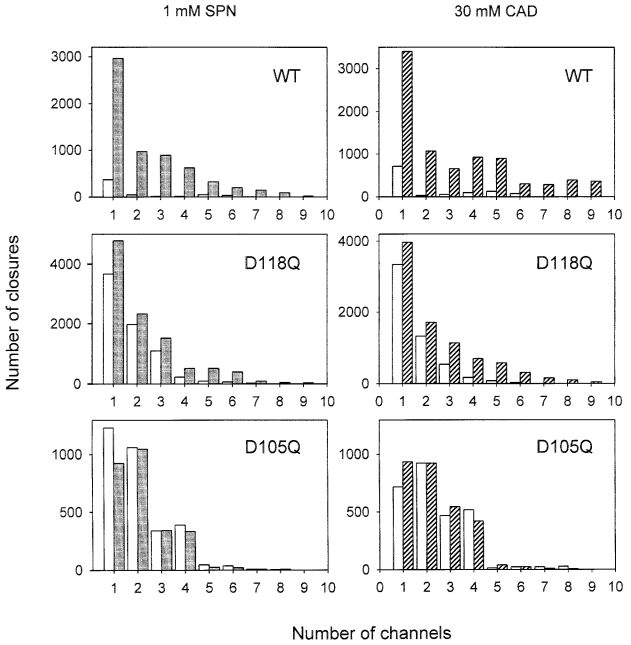


Fig. 3. Number of closures computed from 40-s recordings of wild-type and mutant OmpC activity in the absence of polyamine (open bars) or the presence of 1 mM spermine (gray bars) or 30 mM cadaverine (hatched bars). The *X*-axis represents the number of channels closed at the same time during individual events. For sake of clarity, we are representing only the values for up to 9 channels, although closures of more channels were typically observed for WT and D118Q channels in the presence of polyamines. The difference in the number of closures observed in control conditions for the two set of experiments is due to the variability in the number of open channels in the patch at the onset of the experiment. The pipette voltage was -60 mV.

current between the bilayer and the glass is negligible (seals on blisters made form porin-free bilayers are reproducibly greater than 50 GΩ) and that all observed current is due to open porins. NP_o was calculated as the ratio I(cond)/I(max). We used the baseline level as the maximum current flowing through the patch and integrated this value over a constant recording time to obtain I(max). I(cond) was calculated as the integrated current flowing through the patch during the same amount of time in a specific condition of voltage and polyamine concentration. This calculation takes into account the fact that high polyamine concentrations often lead to substantial decreases in the baseline current level due to the inactivation of a large number of channels.

3. Results

3.1. Kinetic signatures

Patches made on blisters containing reconstituted outer membrane fractions usually contain a large number of porins. Because of the high opening probability of these reconstituted channels in the absence of polyamines, a typical trace recorded from such a patch represents the total amount of current flowing through 15–30 open porins (Fig. 1A, CON). Since the record never dwells at the zero current level, all of our analysis is based on the deflections that originate from the observed current level. Thus, we have chosen to call this observed level a baseline (labeled 'BL' in Fig. 1). Two types of deflections arise from this baseline and are best seen in the expanded traces of Fig. 1: those that correspond to a reduction of total current and thus represent transient channel closures (upward spikes), and those that correspond to an increased total current and thus represent openings (downward spikes). Most closing transitions correspond to the total, but transient, closure of one or several cooperative channels. For example, 3 channels are involved in the large closure seen on the expanded trace of Fig. 1A (CON). The baseline level is also interrupted by frequent, extremely fast, transient openings of additional channels, that, even on an expanded time scale, still appear as unresolved spikes (downward deflections on the expanded control trace in Fig. 1A). This unconventional type of behavior of porin channels has been described in previous publications [10,12,13].

In the presence of 1 mM spermine in the bath solution, a dramatic change in the gating kinetics of wild-type channels is readily observed (20-s traces of Fig. 1A): the frequency of closures is increased, the number of channels cooperatively involved in such transitions is larger, and the average dwell time in closed states is lengthened. All of these effects are similar to those reported for the inhibition of porins by cadaverine [13]. In addition, the baseline becomes more quiet because of the decrease in the frequency of unresolved openings, as seen on the expanded trace in Fig. 1A (SPN) and further documented below. Thus, the overall effect of the polyamine is to inhibit porin activity by enhancing the closing probability and by stabilizing closed states. This type of modulation has been observed with both OmpC and OmpF and the four polyamines putrescine, cadaverine, spermidine and spermine ([13,14], and Iyer and Delcour, manuscript in preparation).

The behavior of the two porin mutants, D118Q and D105Q, in the absence and the presence of 1 mM spermine in the bath is shown in Fig. 1B and Fig. 1C, respectively. The kinetic signature of the mutants in control conditions is clearly different from that of wild-type. A detailed analysis of the implications of this change in gating pattern based on a number of mutations will be presented elsewhere (Liu et al., manuscript in preparation). In this report, we wish to focus solely on the effects of mutations with respect to polyamine modulation. However, it is important to point out that the two mutations at different aspartate residues of the L3 loop have introduced profound modifications in the gating kinetics. Both of them have enhanced the intrinsic gating activity, seen as a larger frequency of closings and openings. However, each mutation has modified this activity in a distinct way. The D118Q mutant shows a mixture of transient and prolonged closures, the latter being increased in number as compared to wild-type; the average dwell time of the additional openings from the baseline is also lengthened (see expanded trace in Fig. 1B, CON). The D105Q mutant displays a greatly increased probability of short-lived cooperative closures of multiple channels (seen as upward spikes in Fig. 1C 'CON'), but no prolonged closures; the additional openings from the baseline are more frequent than in wild-type but still extremely short (see expanded trace in Fig. 1C, CON).

The two mutants are further distinct by their sensitivity to spermine. The D118Q mutant increases its closing activity in the presence of the polyamine (Fig. 1B). The D105Q mutant in the presence of spermine displays a gating pattern similar to that in control conditions, and appears resistant to polyamine modulation at this concentration (Fig. 1C): there is no apparent increase in closing frequency, and no occurrence of prolonged closures involving a large number of channels. This difference in sensitivity has been observed reproducibly in more than 10 patches for each strain in two separate membrane preparations. It is also observed when the channels are studied in the presence of cadaverine, as documented below.

3.2. Modulation of opening kinetics

It is noteworthy that the diminished number of openings from the baseline in the presence of either polyamine is still observed in both mutants, at all concentrations of polyamines and negative voltages tested. This effect is apparent on the expanded traces of Fig. 1, but more clearly documented in Fig. 2. Because these opening transitions are so fast, they cannot reliably be analyzed as individual events on the basis of the half amplitude criterion (see Section 2). However, their existence is demonstrated when amplitude histograms are constructed from current traces. Thus, we chose to use amplitude histograms to illustrate effects that pertain to modulation of the opening kinetics **only**. Effects of the closing kinetics are hinted but not adequately represented in amplitude histograms (see Section 2). Conclusions on the modulation of the closing kinetics will be drawn mostly from the closure histograms described in the next section.

Fig. 2 shows the amplitude histograms obtained for the wild-type and the two mutants in the presence or the absence of 1 mM spermine (panels A, C and E) or 30 mM cadaverine (panels B, D and F) at a pipette potential of −60 mV. For sake of clarity, only the bottom parts of the histograms are shown, and hence only the foot of the largest peak is seen. This peak is made up of the points of the baseline level, and has been arbitrarily assigned the value of 0 pA. Note, however, that it really represents the total current flowing through porin channels that are predominantly in the open state. For each strain, the amplitude histogram in the presence of the polyamine (solid line) is overlaid on top of the control histogram (dotted line). Points of negative current values correspond to opening events, while those of positive values are obtained from closures.

In wild-type (Fig. 2A and B), the presence of the polyamine has led to changes in the appearance of the **foot** of the baseline peak: there is a narrowing on the negative side, and a widening on the positive side. Quantitatively, this effect can be documented by comparing the abscissa of points that have the same ordinate value and are taken from the two curves. For example in Fig. 2A, an ordinate of 500 points per bin is shared by points on the control curve (dotted line) that have an abscissa of −1.7 and +1.3 pA, and by points of the spermine curve (solid line) that have an abscissa of −1.0 and +1.8 pA. Values of this parameter, which we call I_{500} (for current I at the 500-points mark) are given for all amplitude histograms in the legend of Fig. 2. The shift of this parameter is an indication of changes in the frequency of short-lasting transitions. In the example of Fig. 2A mentioned above, the shift of I_{500} from −1.7 pA (dotted line) to −1.0 pA (solid line) is due to the disappearance of unresolved openings in spermine conditions. The shift of I_{500} from +1.3 pA (dotted line) to +1.8 pA (solid line) is due to the introduction of fast gating to closed levels of low amplitudes (closures of one or two channels) in the presence of spermine. In addition, the appearance of frequent and prolonged closures involving a large number of channels can lead to the rise of some additional well-defined peaks of positive value. For example, the peak at +6 pA in Fig. 2A represents the current level attained during the simultaneous closure of 3 channels. Current values for such peaks are given in the legend of Fig. 2.

Four main observations can be made from the amplitude histograms obtained with the D118Q mutant (Fig. 2C and D): (1) the control histogram shows a broader baseline peak than in wild-type channels (the width at the 500-points mark is 3 pA (panel A) and 4.5 pA (panel B) for wild-type, and 5.2 pA (panel C) and 6.5 pA (panel D) for the D118Q mutant); (2) several well-defined peaks of larger amplitudes are evident even in the control histograms; (3) the left shoulder on the baseline peak disappears in the presence of polyamine due to the inhibition of channel opening; (4) the peaks corresponding to closing events are increased in magnitude in the presence of the polyamine, due to the stabilization of closed states. The first two observations are due to the increased opening and closing activities in this mutant with respect to wild-type, but despite these changes in gating kinetics, the D118Q mutant shows a polyamine sensitivity that is qualitatively similar to that exerted on wild-type channels.

In all experiments done with the D118Q mutant, we have observed a slight decrease in the single channel conductance in the presence of the polyamine. This is documented by a rightward shift in the position of the first peak of positive value in the amplitude histograms (see current values of peaks in the

legend of Fig. 2). This type of effect is typically not seen in wild-type channels and may indicate a change in the residency time of the polyamine in the mutant channel as compared to wild-type. Indeed, a decrease in the single channel conductance is a hallmark of 'fast blockers' that block and unblock the channel on a time scale that is much faster than that of the channel's intrinsic gating kinetics. The flickering of the conductance between the normal and the blocked value is so rapid that the bandwidth of the amplifier limits the full-scale deflection that represents the current through the open unblocked channel [33].

In the D105Q mutant, the decreased number of additional openings from the baseline level is still evident in the presence of the polyamines, as can be seen by the narrowing of the baseline peak in the negative value range (Fig. 2E,F; see legend for values of I_{500}). However, there is no substantial difference in the height of the peaks representing channel closures, testifying that the closing activity from the predominantly open state is not altered in the presence of the polyamine. This is in sharp contrast with the effects observed in wild-type and D118Q mutant channels. Note, in addition, that the number of peaks of positive current values is greater that in wild-type channels, due to the kinetic changes introduced by this mutation, even in the absence of modulation.

3.3. Modulation of closing kinetics

Fig. 3 documents that polyamine modulation is readily observed as an increase in the frequency of closures in wild-type and the D118Q mutant, but not in the D105Q mutant. The number of closing events of one or multiple channels was computed for a 40-s record at a pipette voltage of -60 mV in the absence (open bars) or the presence of 1 mM spermine (gray bars) or 30 mM cadaverine (hatched bars). This number is plotted on the *Y*-axis against the number of channels which are simultaneously closed. This type of plot highlights two effects of the polyamines on wild-type or D118Q channels: (1) single channels close more frequently, and (2) the unlikely simultaneous closures of a large number of channels in the control has become greatly enhanced in the presence of either polyamine. The D105Q mutant does not display such behavior. For all levels considered, the number of closures remains essentially unchanged in the presence of the polyamine.

This lack of effect on the D105Q mutant has been observed consistently in several independent patches tested with either spermine ($n = 10$) or cadaverine ($n = 7$) at four voltages. A hallmark of polyamine inhibition is its voltage-dependence [13,14]. In wild-type channels, a greater modulation is observed when the polyamine is applied to the bath and the pipette potential is made increasingly more negative. This asymmetric voltage-dependence indicates that the channels are all oriented in the same way in the patch, and there is evidence that the channels have conserved the native configuration, i.e., with the extracellular side facing the inside of the pipette [10,34]. This voltage-dependent effect is clearly seen in Fig. 4 which shows the fold increase in total number of closures at four pipette potentials. We chose to represent the relative increase rather the absolute number of closures to facilitate the comparison between the wild-type and mutant channels, since the three channel types display drastically different gating kinetics even in the absence of modulation. The D118Q mutant displays a mild voltage-dependence, with a greater modulatory effect at more negative pipette potentials, although the magnitude of the enhance-

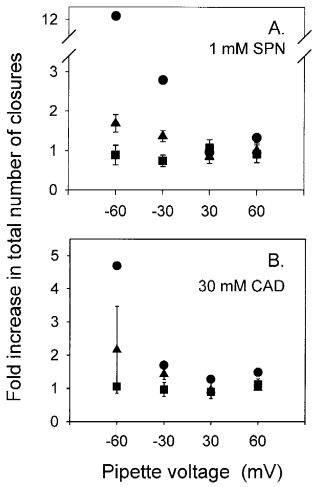


Fig. 4. Increase in total number of closures induced by 1 mM spermine (A) or 30 mM cadaverine (B) in 40-s recordings at the voltages indicated. Symbols are: circles for wild-type channels, triangles for D118Q channels and squares for D105Q channels. Error bars are SD ($n = 3$); only 1 experiment was performed with wild-type channels.

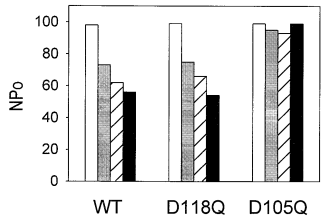


Fig. 5. Relative NP_o values obtained for a single experiment on OmpC channels from each strain, where the following concentrations of cadaverine were tested: 0 mM (open bar), 10 mM (gray bar), 30 mM (hatched bar) and 300 mM (black bar). The pipette voltage was −60 mV, and the recording time was 40 s.

ment is not as large as in the wild-type channels. In contrast, the closing kinetics of the D105Q mutant remains insensitive to polyamine even at a pipette potential of −60 mV (Fig. 4) or −100 mV (data not shown).

Modulation of the D105Q mutant channel cannot even be rescued by very high concentrations of polyamines. Fig. 5 represents the relative open probability calculated for a 40-s recording at a pipette potential of −60 mV in 0, 10, 30 or 300 mM cadaverine. The wild-type and D118Q mutant show a clear concentration-dependent modulatory effect. However, even in the presence of as high a concentration of cadaverine as 300 mM, the D105Q mutant remains insensitive. The lack of modulation remained even when the pipette potential was stepped to −100 mV. For this experiment, the closing activity was also not increased by the presence of 100 mM cadaverine (data not shown). Although a single experiment is represented because of variability from patch to patch, the lack of concentration dependence in the D105Q mutant was also observed in two other experiments.

In high concentration of cadaverine, we have observed shifts in reversal potentials for the D105Q mutant only. Preliminary work has indicated that this mutant is much more permeable to chloride than the wild-type [17]. The reversal potential shift observed in 300 mM cadaverine, for example, can be readily ascribed to the large chloride current that develops due to the presence of an asymmetric chloride distribution (the cadaverine applied to the bath is of the cadaverine-2HCl form). These shifts lead to larger current amplitudes at positive pipette potentials, but the channel kinetics remain unaffected.

4. Discussion

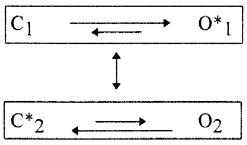
Polyamines have recently appeared as a new type of modulator of channel activities in eukaryotes and prokaryotes [13,14,25,26]. We have shown that cadaverine and other polyamines are negative regulators of bacterial porins [13,14]. Although not precisely understood at this point, a physiological role is suspected for this modulation, since polyamines are associated with the outer membrane [24] and their concentration can be modulated by environmental factors [35,36]. The modulation of porins by these compounds, originally discovered by electrophysiology, is significant enough to alter the permeability properties of the outer membrane [23].

A combination of electrophysiology and site-directed mutagenesis can be used to achieve a complete understanding of the molecular mechanisms underlying this type of modulation. For example, initial mutant studies on heart inward rectifier potassium channels have implicated specific aspartate and glutamate residues [37,38]. The involvement of negatively charged amino acids in polyamine inhibition is not surprising, considering the polycationic nature of these compounds at neutral pH. Our previous studies demonstrated that the inhibition by cadaverine is voltage-dependent and hinted that the binding site is within the pore [13]. Examination of the constriction zone of porin reveals the presence of titrable groups on the L3 loop and on the opposite side of the barrel. Modeling of the electrostatic potential has, however, suggested that not all titrable charges are ionized [27]. The two residues that we have mutated in OmpC (asp105 and asp118) correspond to OmpF aspartate residues that do not experience substantial shifts in their pK_a and thus are presumed to carry a full negative charge (asp113 and asp126).

The kinetics of porin gating and its modulation by polyamines are complex. The difficulty in analysis is compounded by the fact that it is hard to obtain single channel patches, even in reconstituted systems. The trimers are known to cluster [39] and are probably held together by strong protein-protein, protein-lipid and possibly protein-cell wall interactions. Further addition of lipids leads to a decreased probability of obtaining porin-containing patches rather than a ‘dilution’ of porins. Our patches typically contain at least 15 channels at a protein-to-lipid ratio of 1:1600

(w:w). These 15 channels are mostly open and carry a large current that corresponds to the observed baseline level. In addition, the patches contain an unknown number of channels which are mostly closed and whose very rapid transitions to open states lead to unresolved current spikes from the baseline level. The opening and closing transitions appear to represent the activity of the same channel type, namely OmpC porin, for the following reasons: (1) the kinetics of closures and openings are *both* changed by single amino acid substitution in OmpC only; (2) although an accurate conductance measurement is hard to obtain from the opening transitions, the opening and closing events appear to have the same reversal potentials in asymmetric conditions; (3) in the D118Q mutant which displays longer and better resolved openings, the same single channel conductance can be obtained from analysis of opening and closing transitions; (4) both openings and closures are sensitive to polyamine modulation in a complementary way.

Because the kinetics that govern the opening and closing transitions from the baseline level are very different, we propose that, even in control conditions, the patches contain a mixture of porin channels in two favored states: a stable open state (O_1) and a stable closed state (C_2). The flow of ions through channels in the stable open state O_1 yields a total current that correspond to the observed trace. Frequently, channels depart from the O_1 state into closures of an average duration of 0.7–1 ms, which represent the C_1 state. These O_1-C_1 transitions are seen in Fig. 1 as upward deflections from the trace. Silent channels in the stable closed state C_2 are revealed when they depart into a very short-lived open state O_2. In wild-type, these openings are too fast to be analyzed with our current algorithms, but show up as downward spikes, as best seen on the expanded traces of Fig. 1A. Because we do not see favored transitions that correspond to the openings and the closings of three or multiples of three channels, we cannot propose that these states represent only the trimeric forms. At this point, we also cannot ascertain whether these two states correspond to two populations of channels that are readily interconvertible, or whether these two populations represent different molecular entities, such as might arise from covalent modification or post-translational processing. If we assume that these two populations represent conformational states that can be visited by all channel monomers, we can propose the following minimum scheme, where the asterisks denote the two favorite states:



We have shown that, in wild-type, cadaverine does not act as a mere blocker of the open pore because no changes in single channel conductance are observed and because the channels continue to behave as cooperative units [13,14]. Thus, we propose that the presence of polyamines shifts the equilibria in favor of the closed states, and possibly introduces additional ones. The hypothesis that multiple closed and open states might exist in porin is substantiated by the behavior of the D105Q mutant in the presence of the polyamine. It appears that the mutation has not completely abolished polyamine sensitivity, since the opening kinetics, governed by transitions between the C_2 and O_2 states, is still modulated. However, the mutation has altered the sensitivity of the O_1 to C_1 transitions, since the frequency of closures from the stable O_1 state is not increased by the presence of the polyamine. We propose that modulation of the O_1 to C_1 and O_2 to C_2 transitions involves binding of polyamines to two separate sites, and that only the site affecting the O_1 to C_1 transition has been modified by the mutation. It is easily conceivable that the D105 at the tip of L3 is readily exposed to the binding of polyamine when the channels are in the favored open state O_1. On the other hand, this residue would be less, or not at all, accessible for binding when the channels populate C_2, the other favored state.

The mutation at D118 did not abolish polyamine modulation. This was expected since this residue is located at the foot of L3, and does not appear to be exposed to the channel interior where the binding of polyamines was anticipated. The homologous residue in OmpF (D126) is also involved in two salt bridges

to arginines 100 and 168 [27], which would make the residue less available for ionic interactions with cations penetrating inside the pore. The mutant, however, displays less of an increase in closing frequency in the presence of polyamines than wild-type and appears to have a milder sensitivity to these drugs. It is possible that the fact that the mutated channel spends a reduced amount of time in the open state makes the normal polyamine binding site inside the pore less available, and that this accounts for the following two observations that we have made: (1) a reduced sensitivity of the D118Q mutant to polyamine; (2) an apparent decrease in the dwell time of the drug inside the channel which leads to a decreased single channel conductance [33].

In the case of the D105Q mutant, the effects of the mutation on the polyamine-induced inhibition are more severe, even though the closing probability of the two mutants are comparable in the absence of modulation. Because of the disappearance of modulation and because of the location of the D105 residue in the channel interior, we favor an interpretation where the lack of sensitivity of the D105Q mutant is primarily due to a disruption of the binding site, possibly in combination with a decreased availability of the site due to kinetics changes. The closing probability of the D105Q mutant in the absence of polyamine is still much smaller than that of modulated wild-type channels, and hence it is unlikely that the lack of modulation of this mutant by polyamines is due to the closed state already maximally stabilized in the absence of drugs.

Thus, to summarize, our model proposes that a binding site for polyamine is revealed in the O_1 state and involves primarily D105, and that another binding site for these compounds is responsible for the modulation of the C_2-O_2 transitions and does not involve D105 nor D118. Confirmation of this hypothesis awaits the measurement of binding constants for various polyamines on wild-type and mutated porins.

It is not surprising that multiple molecular determinants take part in the binding of polyamines and their subsequent modulation. Complex effects have been demonstrated on neuronal glutamate receptors, where four sites of action of spermine have been postulated on the NMDA receptor on the basis of pharmacological and electrophysiological experiments [25]. In the inward rectifier potassium channel IRK1, mutations at the two critical amino acids still leave the channels with some rectification properties and polyamine sensitivity [38]. The results described here on porins represent our initial effort at deciphering the relationships between channel structure and polyamine sensitivity. It is clear that a more accurate picture will require the analysis of a larger number of mutants, and the isolation of polyamine-resistant mutants in porins is presently underway.

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