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Cadaverine induces closing of *E.coli* porins

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We have used the electrophysiological technique of patch-clamp to study the modulation of *Escherichia coli* porins by cadaverine. Porin channels typically have a very high probability to be open, and were not known to be inhibited by specific compounds until the present study. Experiments performed on patches of outer membrane reconstituted in liposomes reveal that cadaverine applied to the periplasmic side increases the frequency of channel closures in a concentration-dependent fashion, and thereby decreases the total amount of ion flux through a porin-containing membrane. The positive charge on cadaverine is important for inhibition, because the effect is relieved at higher pH where fewer polyamine molecules are charged. Modulation is observed only at negative pipet voltages, and therefore confers voltage dependence to porin activity. Cadaverine increases the number and duration of cooperative closures of more than one channel, suggesting that it does not merely block the pore but exerts its kinetic effect allosterically. As a biological assay of porin inhibition, *E.coli* behavior in chemotaxis swarm plates was tested and found to be impaired in the presence of cadaverine. Polyamines are naturally found associated with the outer membrane of *E.coli*, but are lost upon fractionation. We postulate that cadaverine might be a natural regulator of porin activity.

Keywords: *Escherichia coli*/ion channel/polyamine/porin/regulation

Introduction

OmpC and OmpF porins are trimeric channel proteins located in the outer membrane of *Escherichia coli*. Because of their large number and their conduction properties, they represent the main pathway for flux of ions, nutrients and waste through this membrane. Thus, they play an essential role in controlling the overall permeability of the outer membrane. They have been the subject of extensive studies at the biochemical and genetic levels (Mizuno *et al.*, 1983; Mizuno and Mizushima, 1990; Nikaido, 1993, 1994; Ramani *et al.*, 1994). Structurally, they are among the best characterized channels described to date, since their three-dimensional X-ray crystallographic structure has been solved (Cowan *et al.*, 1992). Each monomer contains 16 β-strands that wrap around to form a β-barrel surrounding a central pore. One of the extracellular loops folds back over the barrel and thus defines a constriction zone where size exclusion and selectivity are achieved. Indeed, these general diffusion pores limit passage of solutes to those of molecular weight <600 Da (Nakae, 1976) and are slightly selective for cations (Benz *et al.*, 1985; Delcour *et al.*, 1989b).

Their functional properties have been the subject of more debate. Porins have been described traditionally as permanently open pores, based on the high permeability rates of liposomes containing reconstituted channels (Tokunaga *et al.*, 1979; Nikaido and Rosenberg, 1983) and antibiotic flux assays on live cells (Zimmermann and Rosselet, 1977; Nikaido *et al.*, 1983). Electrophysiological experiments on planar lipid bilayers harboring either purified porins or outer membrane fragments have also confirmed that porins are open most of the time (Benz, 1988). Several studies have documented a voltage sensitivity of porins which tend to close at high voltages (Schindler and Rosenbusch, 1978; Dargent *et al.*, 1986; Xu *et al.*, 1986; Delcour *et al.*, 1989b; Morgan *et al.*, 1990). The physiological relevance of this effect is still unclear since, in some studies, the required voltages seem to be fairly high (Schindler and Rosenbusch, 1978; Dargent *et al.*, 1986; Xu *et al.*, 1986) and permeability rates in intact cells were not sensitive to Donnan potentials (Sen *et al.*, 1988). It seems, however, that the effects are highly sensitive to the reconstitution protocols (Lakey and Pattus, 1989; Saxena *et al.*, 1989) and to the sign of the membrane potential, since the voltage dependence appears to be asymmetric (Delcour *et al.*, 1989b).

The description of the functional state of porins in native and reconstituted systems is complicated further by our observation that porins are mostly closed when giant spheroplasts and giant cells of *E.coli* are studied with the electrophysiological technique of patch-clamp (Buechner *et al.*, 1990). Therefore, its appears that, in electrophysiological experiments, porin open probability is high only when the proteins are removed from their native environment. We have indeed confirmed that when outer membrane fractions are reconstituted in liposomes and subjected to patch-clamp analysis, porins are open 95% of the time (Delcour *et al.*, 1989b). Our working hypothesis is (i) that cell fractionation has removed a modulatory mechanism of porin activity, thus revealing high porin-mediated permeability rates in liposome swelling assays and electrophysiological studies involving reconstituted porins, and (ii) that porins can be opened by as yet undetermined compounds and mechanisms, which would account for the natural fast flow of nutrients inside the cell.

Here, we present evidence that cadaverine, a polyamine normally found associated with the outer membrane of *E.coli* (Koski and Vaara, 1991), promotes closures of porins reconstituted in liposomes, and therefore could be a regulator of porin activity *in vivo*. Modulation of porin

properties has been seen with a variety of factors such as voltage (Schindler and Rosenbusch, 1978, 1981; Dargent *et al.*, 1986; Xu *et al.*, 1986; Delcour *et al.*, 1989b; Morgan *et al.*, 1990), pH (Todt *et al.*, 1992), lipid composition (Lakey and Pattus, 1989; Saxena *et al.*, 1989; Buehler *et al.*, 1991; Ishii and Nakae, 1993) and membrane-derived oligosaccharides (Delcour *et al.*, 1992). However, closing-promoting activity by a specific compound, such as we document here, has not been reported previously. Cadaverine was chosen because it is released during membrane fractionation (Koski and Vaara, 1991), and therefore could be one of the factors responsible for the difference in the probability of finding open porins in spheroplasts and liposomes. It is well known that inhibitors of channel proteins are of great value not only for basic research on channel structure and physiology, but also in the design of therapeutic agents. Our finding of porin inhibition by cadaverine opens the way to investigating a series of compounds with similar inhibitory properties.

Results

Cadaverine decreases seal currents

By manipulating the protein:lipid ratio during the reconstitution of outer membrane fractions into artificial liposomes, it is possible to obtain patches that contain a large number of porin channels (macropatches). The difference in current in response to a voltage step from 0 to –60 mV, which we call the seal current (I), is a measurement of the flow of ions through the genuine seal (membrane–glass contact), but also through the open channels. In such macropatches, the apparent seal resistance is typically <1 GΩ, due to the large amount of leakage of current through the numerous open porins. For example, a value of 0.48 GΩ is obtained for the experiment shown in Figure 1A in the absence of cadaverine. When increasing concentrations of the polyamine are perfused to the bath-facing side of the excised patch, the seal current is greatly decreased (Figure 1A). Cadaverine does not affect the seal of porin-free patches and, therefore, the observed seal improvement shown in Figure 1A is mostly due to the inhibition of several porin channels. Figure 1B shows that the cadaverine-induced inhibition of porins is concentration dependent, with an IC_{50} of ~18 mM. It appears that the inhibition levels off at 20% of remaining activity, but this is difficult to verify because the perfusion of higher cadaverine concentrations tends to make the patches unstable due to osmolarity effects.

Cadaverine induces porin closures

In order to characterize the inhibitory mechanism, the effect on porin of bath-applied cadaverine was investigated at the single-channel level. Representative traces of porin activity from a patch containing 15–20 porins are shown in Figure 1C. In the absence of cadaverine, porin channels are open most of the time ($P_o > 0.95$), but flicker between ion-conductive and non-conductive states. These closed–open transitions sometimes involve more than one channel, as illustrated on the time-expanded trace. The high degree of cooperativity, a hallmark of porin gating (Schindler and Rosenbusch, 1981), is responsible for the variety of channel amplitudes typically obtained from such traces. This characteristic signature of channel activity is absent in a strain lacking both OmpC and OmpF (A.H.Delcour *et al.*, unpublished results, 1990), but is similar to that of OmpC homotrimers from a strain lacking OmpF (Delcour *et al.*, 1991). It is also observed in patch-clamp experiments on giant cells lacking lipoprotein and OmpA (Buechner *et al.*, 1990). These observations, as well as the high frequency of appearance of this channel activity, strongly suggest that this channel represents one of the major porins, possibly heterotrimers of OmpC and OmpF which would constitute 75% of the porin population in our growth conditions. Because the smallest current amplitude appears to be most frequently observed and corresponds to the smallest difference between higher current amplitudes, we identify it with the current through a single channel. The single channel conductance obtained from current–voltage relationships averages 30 pS ($n = 6$) in control conditions. This value is lower than expected from studies of purified porins reconstituted in planar lipid bilayers (Benz, 1988), a discrepancy that may be ascribed to differing experimental and analysis protocols. Porin properties are known to be highly sensitive to lipid environment (Lakey and Pattus, 1989; Saxena *et al.*, 1989; Buehler *et al.*, 1991), thus reconstitution procedures might affect single channel amplitudes and/or cooperativity, both important in the determination of single channel conductance values. In addition, our algorithm for analysis of single channel current (see Materials and methods) consists of the individual measurement of all events, including those of durations ranging between 0.3 and 1 ms that might have escaped detection in the amplitude histograms typically used in planar lipid bilayer studies.

In the presence of cadaverine, the single channel conductance remains unchanged (data not shown) but, as Figure 1D illustrates, the gating frequency of the channels is dramatically altered. Open–closed transitions occur much more frequently and closed times are prolonged. Cooperative closures are still clearly distinguishable, but tend to involve a larger number of channels. This tendency of each porin channel to spend an increased amount of time in a non-conductive state in the presence of cadaverine will lead to a decrease in the total amount of ions (and thus current) that flows across patches containing many channels. Our observations indicate that the cadaverine inhibition of seal currents is not due to a decreased permeability through individual open porins (which would be reflected by a lower conductance) but to kinetic effects on the channels.

In order to document the increased closing activity of porin in the presence of cadaverine, we determined the cumulative closing probability of channels from 40 s records taken from the same patch in four different cadaverine concentrations (Figure 2). Each bar of the histograms represents the cumulative closing probability in 0–30 mM cadaverine, from left to right (see figure legend). Cumulative probability is defined as the probability that at least N channels are closed during the 40 s record, N being given on the x-axis. It is striking that not only does cadaverine greatly increase the probability that one or a few channels will be closed, but it also promotes closure of a large number of channels. Although Figure 2 shows data for nine channels for the sake of clarity, we observed closures of up to 11 channels in control and 18 channels in 10 mM cadaverine. We

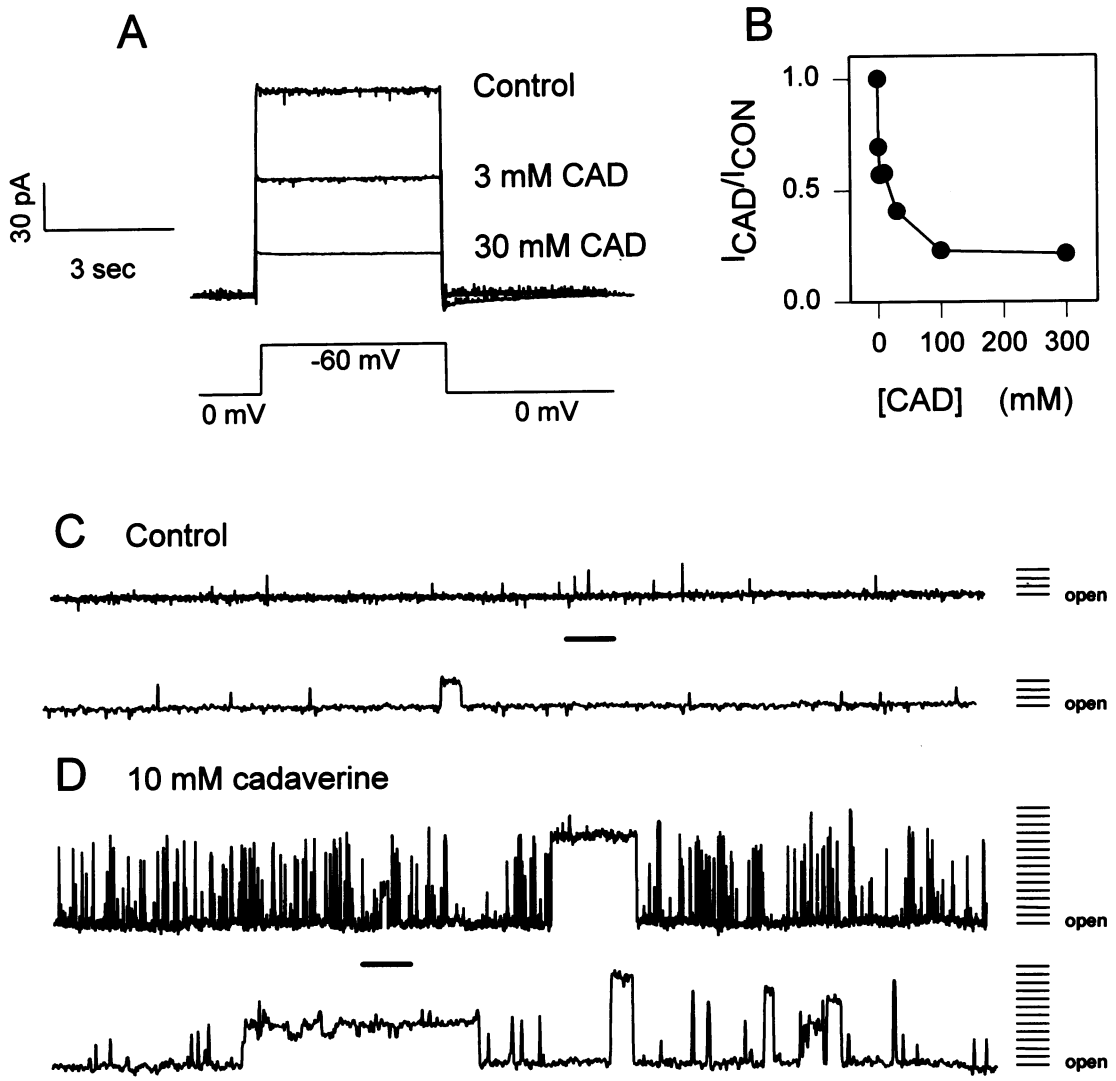

Fig. 1. Cadaverine reduces the current flowing through a porin-containing membrane patch by altering the channel gating kinetics. (**A**) Macroscopic current records were obtained under the indicated conditions when the pipet potential was stepped from 0 to –60 mV as indicated in the voltage protocol (bottom diagram). (**B**) Seal currents were measured as the difference in current levels when the pipet voltage was stepped from 0 to –60 mV in control (I_{CON}) and in the presence of increasing amount of cadaverine in the bath (I_{CAD}). The decrease in the ratio I_{CAD}/I_{CON} with cadaverine concentration suggests that cadaverine binding inhibits porin activity. (**C** and **D**) Single channel current recordings illustrate that cadaverine alters the channel gating kinetics. For each section, a segment of the top trace, underlined by a thick line, is represented in the bottom trace at an expanded time scale. Top trace durations are 12.3 s; bottom trace durations are 410 ms. For all traces, the baseline level corresponding to the current flowing through all open porins of the patch is labeled 'open'. Upward deflections correspond to closures. Closing levels are represented by tick marks and are separated by 1.26 pA, the single channel current amplitude measured for the entire recording. Not all current levels are sampled by the channels in this stretch of data. Pipet voltage was –40 mV.

present the results from a single experiment because of variability in the gating frequency from patch to patch, which is directly correlated with the number of porins in the patches. However, similar observations have been made at various concentrations of this polyamine in >15 patches. This effect is reminiscent of the enhanced closing activity of porins also observed in the presence of membrane-derived oligosaccharides (Delcour *et al.*, 1992).

A major consequence of the increased gating frequency is an overall decrease in the time that the channels spend in the open state. We have measured the average time that the current trace remains at the baseline level, where all the channels of the patch are open ($<t_o>_{all}$), in the presence and the absence of cadaverine. The results of Figure 3A show the concentration dependence of the decrease in this parameter from a single experiment. The reduction of the average time at the fully open level indicates that cadaverine does not simply permanently inactivate a number of channels. If this were the case, and each remaining channel had conserved its own intrinsic gating frequency, there would be a decrease in the total number of closures per fixed amount of time, simply on the basis of statistical considerations. This would, in turn, result in an increased average time when all the channels are open ($<t_o>_{all}$). Our data clearly indicate that this is not the case. The concentration dependence of $<t_o>_{all}$ is in sharp contrast to the lack of cadaverine effect on the average times that the current spends at various other levels, $<t_{dwell}>$ (Figure 3B). A detailed kinetic analysis is not possible because the patches typically contain

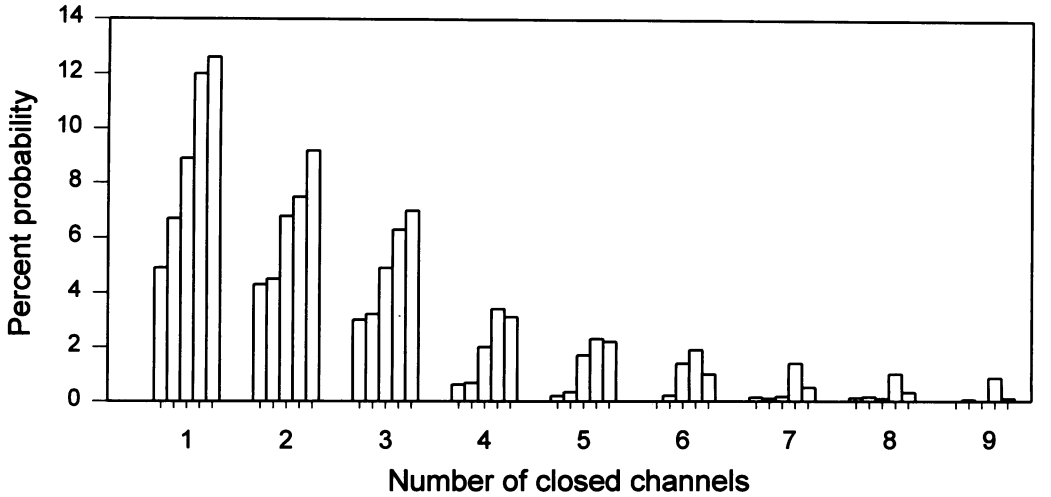

Fig. 2. Concentration dependence of the cumulative closing probability, for up to nine simultaneously closed channels. For the sake of clarity, we have chosen to represent the results in histogram form, rather than multiple scatter plots. For each histogram, the bars represent the cumulative closing probability in the following cadaverine concentrations (indicated by tick marks on the *X*-axis): from left to right, 0, 3, 7, 10, 30 mM. All data originate from the same patch. The pipet potential was –50 mV.

multiple channels due to the trimeric composition of porins and their clustering. Therefore, the dwell times cannot be resolved into openings and closures. Our interpretation of the data is that cadaverine promotes or stabilizes a long-lived non-conductive state, without altering the gating kinetics between the open state and the 'normal' short-lived closed state. The shortening of the average time when all channels are open would be due to higher frequency of transitions to non-conductive states, which include the 'normal' short-lived closed state and the cadaverine-dependent long-lived closed state. This model is supported by the observation of an increased frequency of episodes where one or several channels remain closed for long periods of time, although additional closing events with normal kinetics are seen from this long-lasting closed level (Figure 1D).

Charge effects in the cadaverine inhibition of porin activity

An interesting aspect of the cadaverine inhibition of porins is that it confers voltage dependence to the gating activity. Voltage dependence of porins has been a matter of debate, and certainly appears highly sensitive to the experimental conditions in which the channels are studied (Lakey and Pattus, 1989; Saxena *et al.*, 1989; Buehler *et al.*, 1991). We have ourselves reported some voltage dependence of porins in the voltage range of –60 to –90 mV (negative on the outside) in strains expressing both OmpC and OmpF (Delcour *et al.*, 1989b), and an even milder effect in OmpC homotrimers (Delcour *et al.*, 1991). In the experiments reported here (Figure 4), porin activity in control conditions was not voltage dependent in the pipet voltage range of +60 to –60 mV. However, a well-defined voltage dependence occurs in the presence of cadaverine. The total number of closing events in 40 s recordings is greatly increased in the presence of 3 mM (Figure 4A) or 10 mM (Figure 4B) cadaverine only in the negative pipet voltage range. The asymmetry of the effect suggests that bath-applied cadaverine, which is positively charged at pH 7.2, is more likely to reach its binding site when it is more attracted to the pipet by a larger negative potential. Higher concentrations of cadaverine appear to enhance the effect of voltage on porin activity. These results suggest that charges on the cadaverine molecule may play

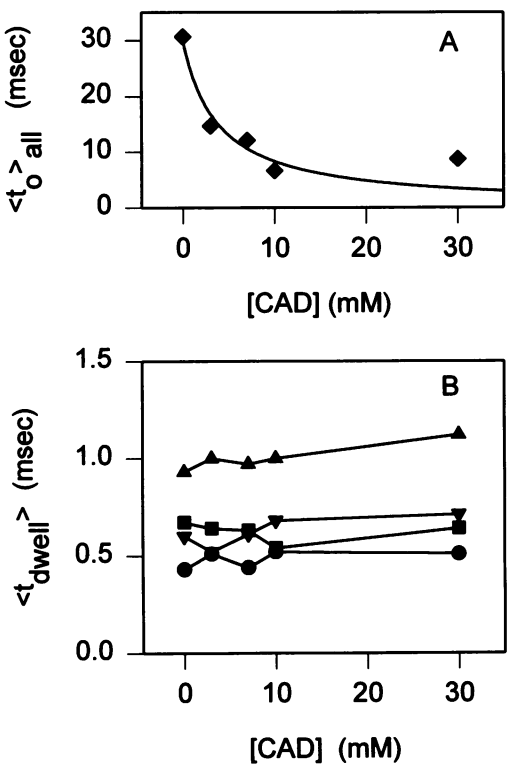

Fig. 3. Concentration dependence of average dwell times. (**A**) Average dwell time at the baseline level where all channels are open ($<t_o>_{all}$). Although the data points were fitted to a binding isotherm for the purpose of illustrating the inhibitory trend, the apparent binding constant is not accurate since the average open time corresponds to that of many open porins. (**B**) Average dwell times ($<t_{dwell}>$) at current levels corresponding to the closure of one channel (●), two channels (■), three channels (▲) and four channels (▼). The pipet voltage was –50 mV.

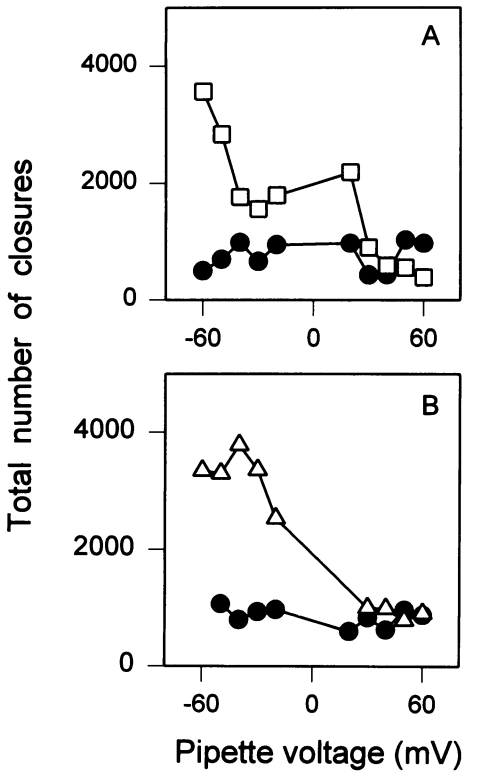

Fig. 4. Cadaverine promotes voltage-dependent gating. The total number of closures in 40 s recordings is calculated in two separate experiments comparing gating kinetics in control (●) with that in either (**A**) 3 mM (□) or (**B**) 10 mM (△) cadaverine.

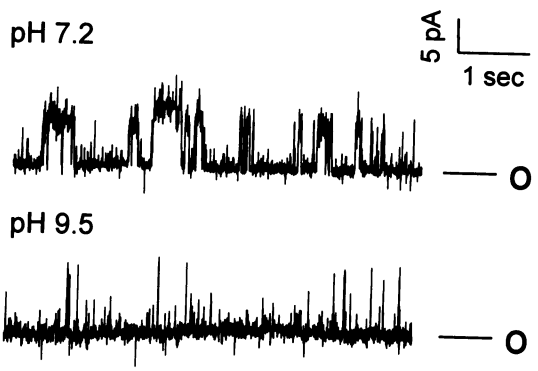

Fig. 5. Cadaverine inhibition of porin is decreased at higher pH. Current traces were obtained from the same patch at the pHs indicated, in the presence of 10 mM cadaverine. Pipet voltage was −70 mV. The baseline level corresponding to the current flowing through all open porins of the patch is labeled 'O'. Upward deflections correspond to closures.

an important role in conferring voltage dependence to porin gating.

The impact of charges in the inhibitory mechanism is substantiated by the data presented in Figure 5. The influence of cadaverine on porin gating kinetics is compared between solutions of cadaverine at pH 7.2 and 9.5, applied to the same patch. From the pK_a value of each amine group, one can calculate that the ratio of concentration of divalent to monovalent cadaverine molecules is decreased by a factor of ~200 when cadaverine is titrated from pH 7.2 to 9.5. The decrease in the concentration of the most highly charged species has the direct effect of relieving the inhibition of porin activity, as shown by the traces of Figure 5. A quantitative analysis reveals that increased pH by itself did not affect porin gating, and that the total number of closed events in 20 s in the presence of cadaverine at pH 9.5 decreased by a factor of 2.6 from its value at pH 7.2, to reach a number similar to that of control conditions.

Cadaverine inhibition of swarming ability

It is expected that the regulation of porin closing activity would have a direct impact on the overall permeability of the outer membrane, and thus influence metabolic, growth and chemotactic properties of the bacteria. Ingham *et al.* (1990) have shown that a porin double mutant (*ompF*$^-$ *ompC*$^-$) is unable to perform chemotaxis, because the lower permeation rate of the chemoattractants through the outer membrane decreases the number of molecules bound to the chemoreceptors on the cytoplasmic membrane. Thus, we would expect that the lower number of open porins in the presence of cadaverine would reduce the chemotactic ability of *E.coli*. In order to investigate the physiological relevance of the cadaverine inhibition of porins, we have performed chemotaxis swarm plate assays in the presence of various concentrations of cadaverine. Figure 6 shows photographs of swarm plates, where motile bacteria were inoculated in soft agar containing 0, 30 or 100 mM cadaverine. The rings observed in the control plates (Figure 6A) are formed by bacteria which, after having consumed all the serine and aspartate around the inoculum and thereby created a gradient of these chemicals, have migrated through the plate in response to these powerful chemoattractants. The size of the rings is correlated both with growth and chemotaxis rates and, thus, the ability of nutrients and attractants to permeate through the outer membrane. The diameter of the rings is greatly reduced in the presence of cadaverine (Figure 6B and C). This result is not due to a possible repellent effect of cadaverine, because the polyamine does not produce clear zones in negative chemotaxis plug assays.

Discussion

When *E.coli* porins are reconstituted in artificial bilayers, they display a high probability of being in an open, ion-conducting state. Our results indicate that cadaverine, a polyamine naturally found associated with the bacterial outer membrane, is able to modulate open porins by promoting occupancy of a closed, non-ion conducting state. Although charged and of relatively low molecular weight, cadaverine does not appear to permeate the channels because we do not see any shifts in reversal potential in its presence. We cannot exclude, however, the possibility that the rate of permeation of cadaverine through porin would be so slow that the cadaverine flux contributes to the recorded current a minute amount below detection sensitivity. It seems, however, that the main effect of cadaverine is to alter the gating kinetics of porin and thereby decrease the flow of ions through a porin-containing membrane.

The characteristic signature of channel activity which we find here modulated by cadaverine is absent in a strain lacking both OmpC and OmpF (A.H.Delcour *et al.*, unpublished, 1990), and altered in a OmpC mutant carrying

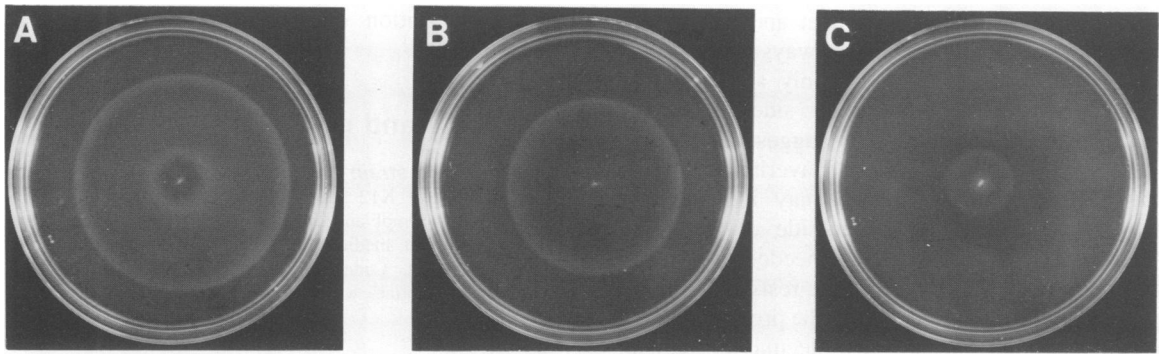

Fig. 6. Cadaverine inhibits *E.coli* swarming ability. Swarm plates were made in soft agar in the absence (**A**) or the presence of 30 mM (**B**) or 100 mM (**C**) cadaverine. The diameter of the outer ring in (A) was 5.7 cm; the diameter of the rings in (B) and (C) was 4.5 and 1.6 cm, respectively.

a single mutation in the pore region (Delcour *et al.*, 1991). These results strongly suggest that the observed channels are from these major porins. Even though several types of OmpC and OmpF homo- and heterotrimers might be represented, the observed channel activity is always uniform in any given patch (although some kinetic variability does exist from patch to patch). Both the noise level associated with many open channels and the low ionic strength of the buffers make it difficult to assign individual transitions to a porin monomer of a specific type with only an expected 10% difference in channel amplitude between OmpC and OmpF (Benz *et al.*, 1985). We therefore assume that the observed current traces represent the activity of a mixture of indistinguishable OmpC and OmpF channels. This interesting question of how a mixture of homo- and heterotrimers gives rise to such a homogenous channel behavior, in terms of gating and current step size, deserves further attention. However, it does not mitigate the significance of the results presented here, that cadaverine inhibits porin under the general conditions where both OmpC and OmpF are expressed in similar amounts. The wild-type strain was chosen for the reported studies because it has been the object of most of our previous work and its porin channels are well characterized in patch-clamp experiments. The determination of the specific sensitivities of the individual porin types will, however, be necessary for a more complete understanding of porin modulation in various environmental conditions which influence the ratio of OmpC and OmpF. So far, experiments in progress on strains expressing either only OmpC or OmpF suggest that both channel types are sensitive to polyamines.

This reduction of current through an ion channel by an amine compound has been observed elsewhere. There are numerous examples of amine-induced blocks of different types of ion channels in a variety of eukaryotic cells (Hille, 1992). Polyamines as well have been reported recently to modulate ion channels of heart (Ficker *et al.*, 1994; Lopatin *et al.*, 1994), pituitary tumor cell lines (Weiger and Hermann, 1994), neurons (Williams *et al.*, 1991; Scott *et al.*, 1993), and to exert effects on other ionic membrane pathways (Votyakova *et al.*, 1993; Hughes *et al.*, 1994; Lapidus and Sokolove, 1994). In many cases, the inhibitory action produced by the amine is due to blocking an open channel pore, resulting in decreased overall permeability. At the single channel level, this phenomenon is characterized, depending on the drug binding rate, either by a decrease in the single channel conductance and/or by a rapid flicker of the current trace as the drug binds and unbinds from the channel. Voltage dependence is often a hallmark of open-channel block, since the charged blockers typically bind to sites that are buried deep within the membrane. Although the cadaverine effect is sensitive to the transmembrane potential, we do not propose that cadaverine is *solely* an open-channel blocker, in which case we would expect either a decrease in single channel conductance or a large amount of flickering at all current levels and between all current levels. Neither of these are observed in our experiments. In fact, we see an increased frequency in *cooperative* closures, during which it is unlikely that many channels are plugged by blocker molecules all at exactly the same time. Although we cannot rule out that some block might be occurring, we favor a model whereby the binding of the polyamine to a site within the transmembrane field has an effect on porin conformation, thus promoting occupancy of a long-lived closed state. This model is in agreement with the results of Kobayashi and Nakae (1985), who reported that spermidine can bind to OmpF trimers at a few carboxyl groups in the pore interior and cause a conformational change in the protein. Hancock and coworkers (1991) also confirmed the existence of a divalent cation binding site on OmpF, although they believe it to be closer to the surface of the protein.

An interesting aspect of the modulatory action of cadaverine on porin is that it confers voltage dependence to the gating kinetics. The presence of a positive charge is important for the inhibitory mechanism, as suggested by the experiments carried out at different pHs. The greater negative potential bias at the pipet side of the patch will attract cadaverine to its binding site and enhance the inhibitory effect. This type of modulation is similar to the voltage dependence and rectification conferred onto heart inward rectifier K^+ channels by polyamines (Ficker *et al.*, 1994; Lopatin *et al.*, 1994). It is also reminiscent of that exerted by polyanions on mitochondrial voltage-dependent anion channels (VDAC) (Mangan and Colombini, 1987). In this case, the authors proposed that the intrinsic voltage dependence of VDAC was amplified by accumulation of polyvalent anions presented on either side of the membrane and by their electrostatic interactions with the channel gating charges. We have not been able to test this possibility by applying cadaverine on either side of the porin channel in the same patch, because it is

technically difficult to perfuse the pipet and retain high quality recordings. In addition, porin always reconstitutes in the same orientation and we are only able to study excised patches with the periplasmic side facing the bath. Some preliminary experiments suggest that higher resistance seals are obtained when cadaverine is present in the pipet from the start, which may indicate that modulation is achieved from either side of the porin channels. Additional experiments are needed to confirm this finding. The isolation of cadaverine-resistant mutants in porin genes will also help to identify the precise location of the polyamine binding site and its molecular mechanism of action.

It is important to point out that modulation is observed at relatively low membrane potentials in absolute value. At this time, it is not known whether outer membrane potentials do exist and fluctuate. It is, however, possible to imagine scenarios where, depending on the composition of the external medium and the adapted state of the bacterium, either Donnan potentials (negative periplasm) or transient depolarizations (positive periplasm) might take values of <100 mV. Polyamines have been shown to be components of the outer membrane (Koski and Vaara, 1991), and their local concentrations might be fairly high because of their probable association with negatively charged lipopolysaccharides. It is not known whether their concentrations on either side of the outer membrane change in different environments. Cytosolic concentrations of putrescine have been reported to decrease when cells are grown in high osmolarity media (Munro *et al.*, 1972). This efflux, as well as the periplasmic location of arginine decarboxylase responsible for putrescine synthesis (Buch and Boyle, 1985), might lead to high levels of putrescine in the periplasm. The presence of the polyamines in the vicinity of porin channels suggests that they may have an important role *in vivo*.

The kinetic effects which we have observed with electrophysiology appear to be significant enough to influence the growth and chemotaxis of bacteria in soft agar plates. Ingham *et al.* (1990) have reported that, in the absence of OmpC and/or OmpF, chemotaxis is impaired due to decreased influx of chemoattractants to the periplasmic space. We interpret the results of our swarm plate assays as an indication of a substantial decrease in the overall outer membrane permeability due to the presence of cadaverine. At concentrations up to 100 mM, cadaverine affects viability only minimally, but retards growth. Although cadaverine is a polycation, its effect on outer membrane physiology is clearly distinct from that of polycationic antibiotics studied to date. In order to exert their bactericidal actions, polycationic antibiotics need to cross the outer membrane, apparently not through the porins but by disruption of the outer membrane integrity leading to an increased permeability (Hancock *et al.*, 1991). Although our experiments do not establish whether cadaverine affects porin gating by altering the lipid environment, the observed decreased outer membrane permeability is in sharp contrast to polycationic antibiotic effects. Because of their unique structure, porins would be ideal targets for new types of antibiotics. Further studies with different natural and synthetic polyamines will provide clues on the structural determinants involved in the inhibition of porins and their biological consequences.

Materials and methods

Materials and strain

Escherichia coli K12 strain AW737 (Ingham *et al.*, 1990), which expresses both OmpF and OmpC porins, was used throughout this study. Tryptone growth medium (T-broth) contained 1% tryptone (Difco) and 0.5% NaCl. Cadaverine dihydrochloride (CAD) and azolectin (phosphatidylcholine) were from Sigma, and all other chemicals were from Fisher.

Chemotaxis swarm plate assay

Single colonies of AW737 cells were inoculated in the center of a tryptone soft agar plate (0.3% agar in T-broth) containing either no cadaverine, or increasing concentrations of the polyamine. Plates were incubated at 35°C and chemotactic rings were visualized after 5–8 h.

Membranes and liposome preparation

The procedure for isolation of outer membrane fractions and reconstitution into liposomes is essentially as described (Delcour *et al.*, 1989a), and summarized as follows. Bacteria were grown in T-broth to mid-log phase, harvested and passed twice through a French press at 16 000 p.s.i. Outer membrane fractions were purified by sucrose gradient centrifugation, and stored at −80°C. Protein concentrations of these fractions were determined by the bicinchoninic acid assay (Pierce). Reconstitution into artificial liposomes was done by mixing outer membrane fractions with azolectin at protein:lipid ratios of 1400–1600, followed by a dehydration–rehydration procedure.

Electrical recording

Patch-clamp experiments were performed according to standard protocols (Hamill *et al.*, 1981) on blisters induced from giant liposomes containing the reconstituted outer membrane fractions (Delcour *et al.*, 1989a). Patch pipets (Boralex, Drummond) had a resistance of 10 MΩ, were coated with nail enamel, and filled with a solution of 150 mM KCl, 5 mM HEPES, 0.1 mM K-EDTA, 0.01 mM $CaCl_2$, pH 7.2. We routinely formed seals of ~0.6–1.2 GΩ, due to the presence of multiple open porin channels in the patch. After patch excision by air exposure, control experiments were done in symmetric solutions. Cadaverine in the same buffer at pH 7.2 or 9.5 was then applied to the inside-out patch with bath perfusion. Currents were filtered at 2 kHz (Frequency Devices) and recorded with an Axopatch-1D amplifier (Axon Instruments). Continuous recordings were made on VCR tapes (Instrutech). For analysis, the data were re-filtered at 1 kHz and digitized at 100 μs sampling intervals. Data acquisition and analysis were done with personally developed programs using Axobasic (Axon Instruments).

Data analysis of single channel records

Because the patches contain multiple, mostly open, porins, we used as a baseline the current flowing through all the open channels (labeled 'open' in Figure 1C and D). Deflections from the baseline represent transient closures, and we assigned as level 1, level 2, ..., level N, current levels corresponding to the closures of 1, 2, ..., N channels. Amplitude histograms typically show only a small number of well-defined peaks, and misrepresent the population of current amplitudes that are evident by visual inspection of current traces. This is because many events are of such short duration (<1 ms) that they escape detection in amplitude histograms. Therefore, channel amplitudes were typically obtained from inspection of individual transient closures, rather than from all-point histograms. Indeed, there are enough individual events with well-defined square-top shapes and duration in excess of ~1 ms that can be used for size measurement and averaging. Current–voltage relationships were plotted for all levels (corresponding to single and multiple closures). A 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. The peaks observed in amplitude histograms typically represent the amplitude of several channels that either remained closed for long periods or were visited frequently. Although the closure of three channels or their multiples is sometimes favored, this pattern is not observed consistently over many patches.

Kinetic analysis was performed 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 >300 μs. The number and average durations are computed individually for opening events involving all the channels (all open level = level 0), and closing events corresponding to 1, 2, ..., N channels (levels 1, 2, ..., N). The closing probability of N channels is calculated as the ratio of total time that the current dwells at level N to the total recorded time. Cumulative closing probability is computed as the ratio of total time that at least N channels are closed to the total recorded time.

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