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Fast and slow kinetics of porin channels from *Escherichia coli* reconstituted into giant liposomes and studied by patch-clamp

Catherine Berrier[a], Alain Coulombe[b], Christine Houssin[a] and Alexandre Ghazi[a]

[a]*Laboratoire des Biomembranes, URA CNRS 1116* and [b]*Laboratoire de Physiologie Cellulaire, URA CNRS 1121, Université Paris-Sud, 91405 Orsay Cedex, France*

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E.coli porins (OmpF and OmpC) were purified and reconstituted into liposomes which were enlarged to giant proteoliposomes by dehydration–rehydration and studied by patch-clamp. The porins could be closed by voltage pulses under −100 mV. The kinetics of closure was slow, with closure events of about 200 pS in 0.1 M KCl. Rapid fluctuations (in the millisecond range) of about one third (60–70 pS) of the large closure steps were also observed. The data are interpreted as follows: an increase in membrane potential favours the cooperative transition of multimers towards an inactivated state, while monomers which have not been inactivated can flicker rapidly between an open and a short-lived closed state.

Porin; Ion channel; Liposome; Patch-clamp; *Escherichia coli*

1. INTRODUCTION

The outer membrane of Gram-negative bacteria such as *Escherichia coli* is permeable to low molecular mass compounds (under 600–700 Da). These molecular sieving properties are due to the presence of pores, called porins, which provide specific, as well as non-specific, diffusion pathways for a wide range of solutes (see recent reviews in [1,2]). Porins form trimers which are extremely stable. Structural information about porins has been obtained both from X-ray and electron crystallography studies. Recently the structure of porin from *Rhodobacter capsulatus* at atomic resolution obtained by X-ray cristallography has been reported: each monomer consists of an anti-parallel β-barrel which forms a channel [3]. The structure of phoE, LamB, OmpF, and OmpC from *E. coli*, determined at lower resolutions, are consistent with the basic features of this model, in particular the organisation into trimers of three channels [1,2].

Most of the electrophysiological data on porins have been obtained using purified proteins re-incorporated into planar lipid bilayers. The two major porins of *E.coli*, OmpF and OmpC, have a conductance in the order of 200 pS in 100 mM KCl [4,5]. The voltage dependence of porins is still debated. One group of laboratories found that porins could be closed by increasing the membrane potential [5–10]. The kinetics of closure was slow, with events in the range of seconds. Moreover closures in three steps, each corresponding to about one third of the trimer conductance, have been observed [5–7,9], indicating that each channel may be closed independently. In contrast, another group of laboratories observed that porins are mostly open, and that the rare closure events are independent of the potential applied [4,11].

Recently, patch-clamp, performed either on *E. coli* giant spheroplasts [12,13] or on giant liposomes into which bacterial membranes had been fused [14–16], has started to be used. Delcour et al. described a voltage dependent channel in outer membranes fused into giant liposomes [17]. The activity of this channel was later ascribed to OmpC [18]. Its behaviour is markedly different from that of porins studied in planar bilayers. The channel, which has a conductance of 90 pS in 150 mM KCl, presents a high open probability and a very rapid and frequent gating to closed levels. Depolarization increases the frequency of gating and the cooperativity of closures among ion-conducting units.

In view of these different results, we have studied, by patch-clamp, purified OmpF and OmpC porins reconstituted in giant liposomes. This method allows these pores to be investigated with a high resolution, and reveals that they display a more complex kinetic behaviour than previously reported. Preliminary results have previously been reported in an abstract [19].

2. MATERIAL AND METHODS

E. coli strain K12 was grown at 37°C in M9 minimal medium containing 0.2% glucose as the sole carbon source. The different membrane fractions (inner membrane, outer membrane, contact zones) were isolated by sedimentation through a sucrose gradient as previously described [15]. OmpF and OmpC porins were purified from the outer membrane fraction by salt extraction, using the procedure of

Correspondence address: A. Ghazi, Laboratoire des Biomembranes, Bât 432, Université Paris-Sud, 91405 Orsay Cedex, France. Fax: (33) (1) 69 85 37 15.

Morgan et al. [10]. 1 μg of solubilized porins resuspended in 20 μl of 50 mM Tris-HCl (pH 8) buffer containing the detergent lauryldimethylamine (0.1% w/v) was added to 2.8 ml of an azolectin liposome suspension (1 mg lipids in 10 mM HEPES-KOH, pH 7, 100 mM KCl). The suspension was incubated for 1 h at room temperature, centrifuged at 90 000 rpm for 30 min in a TL 100 Beckman centrifuge, and the pellet was resuspended in 15 μl of 10 mM HEPES-KOH buffer, pH 7.

The liposomes were then fused into giant liposomes using a cycle of dehydration–rehydration, as described [15]. A 2–5 μl drop of the giant proteoliposome suspension was deposited on a nunclon plastic tissue dish and diluted with 1.5 ml of the bath solution (as defined in the figure legends). Single-channel activity was measured using the method of Hamill et al. [20], as previously described [15]. Records were filtered at 1 or 2 kHz (−3 dB point) through an 8-pole Bessel low pass filter and digitized at a rate of 2 or 10 kHz. The membrane potential refers to the potential in the bath minus the potential in the pipette.

3. RESULTS

Porins (OmpF and OmpC), previously purified by salt extraction, were reconstituted into azolectin liposomes obtained by sonication; these proteoliposomes were then enlarged into giant liposomes by a cycle of dehydration–rehydration. Seals were formed on these giant liposomes and the electical activity recorded on excised patches. The electrical activity displayed by the patches between −80 mV and +80 mV is presented in Fig. 1. The channels observed under these conditions were mostly open at all membrane potentials, but showed frequent, very brief closures in the millisecond range. Representation of one of these traces on an expanded time scale shows that most of these closure events have the same conductance, with smaller events appearing as aborted transitions.

Fig. 2 shows the current–voltage relationship obtained by plotting the unitary current corresponding to these resolved events as a function of membrane potential. The unit conductance varied from 50 to 70 pS between −100 mV and +100 mV, in 100 mM KCl symmetrical media. This non-linear, asymmetric relationship was observed in nearly all patches, suggesting that the orientation of porins after reconstitution is not random. In asymmetric KCl media, the reversal potential was −24 mV, for a calculated reversal for K^+ of −28 mV. This corresponds to a ratio of selectivity of potassium to chloride of 20, calculated from the Goldmann–Hodgkin–Katz equation. The channels were also able to discriminate between monovalent cations (Na^+ and K^+), with a preference for K^+.

Brief, resolved transitions of higher conductances than the 50–70 pS unit described above were also observed in these recordings. Their number and sizes increased when the membrane potential was made more negative. A segment of recording at −110 mV shows several of these events (Fig. 3). The closed time of these transitions is similar to that of the smaller transitions, and they appear as discrete events, as exemplified for one of them on an expanded time scale. They are thus likely to correspond to the simultaneous cooperative

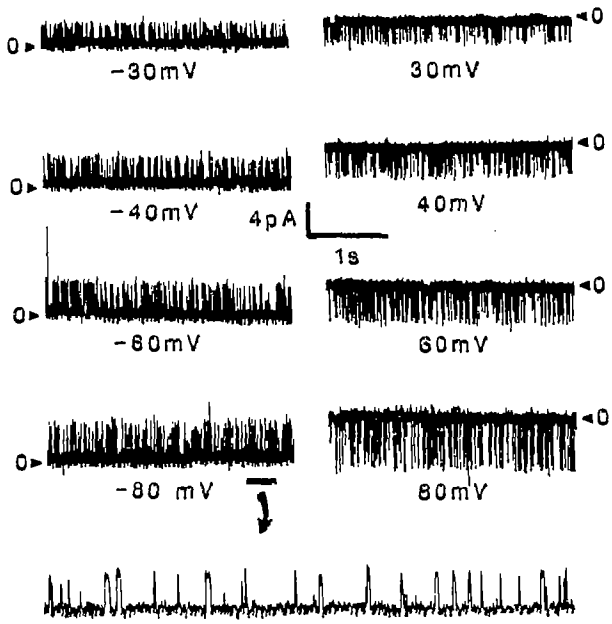

Fig. 1. Fast kinetics of porin channels. Recordings of unitary currents, at various membrane potentials, from an excised patch of giant liposomes reconstituted with purified porins. Bath medium: 10 mM HEPES-KOH ajusted to pH 7.4, 100 mM KCl. Pipette medium: similar to bath medium, with in addition, 2 mM $CaCl_2$, 5 mM $MgCl_2$. O, open level. The lower trace shows a 0.4 s recording of the trace at −80 mV (corresponding to the horizontal bar) on an expanded time scale.

closure of several 50–70 pS units. This would account for the variability in sizes of these transitions. Putative cooperative closures of 2, 3 and up to 8 or 9 elementary units could thus be observed.

Under these conditions not only did the number of brief cooperative closures increase but also closure events of large conductance and of much higher duration began to appear (Fig. 3). These events correspond to transitions to a closed state different from that of the fast kinetics described above, as evidenced from the wide differences in the closing times. This aspect was more systematically investigated by applying voltage pulses to the patch.

Application of negative membrane potentials (under −100 mV) resulted, in all patches, in a sequence of closure and reopening events of long duration (in the second range) and of large conductance (Figs. 4–6). While a negative membrane potential always promoted closure events, application of positive membrane potential pulses (above 100 mV) had no effect in 75% of the cases. In 25% of the cases, nevertheless, closure events similar to those recorded at negative membrane potentials were observed.

In most cases, as shown in Fig. 4, the fast-kinetic events described above appeared superimposed on the slow kinetics. In other cases (Figs. 5,6), the fast-kinetic events disappeared following the voltage pulses, although this activity had been observed in the same patch at less negative membrane potentials.

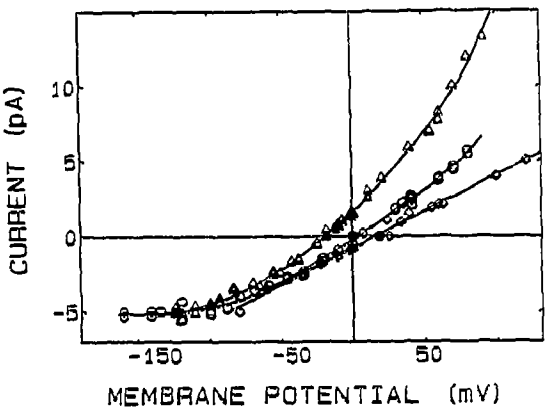

Fig. 2. Current–voltage relationship obtained by plotting the unitary current, corresponding to the smaller resolved events of the fast kinetics, against the membrane potential. (○) Symmetrical KCl media (pipette and bath: 100 mM KCl). (△) asymmetrical KCl media (pipette, 100 mM KCl; bath, 300 mM KCl). (◇) asymmetrical K/Na media (pipette, 100 mM KCl; bath, 100 mM NaCl). pH was adjusted to 7.4 with 10 mM HEPES-KOH, pipette media contained in addition 2 mM $CaCl_2$, 5 mM $MgCl_2$.

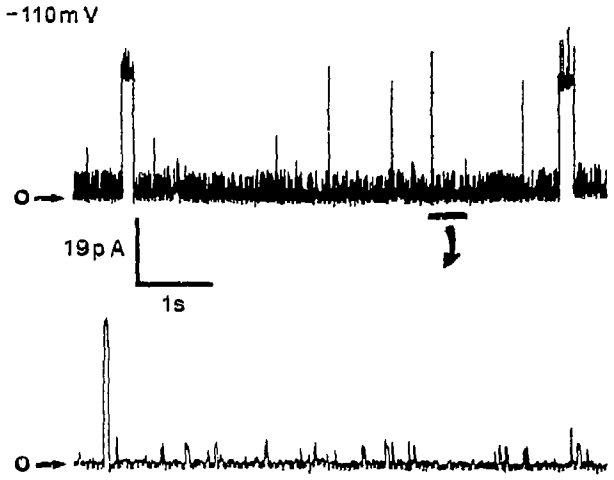

Fig. 3. Cooperative closures with fast kinetics. The lower trace shows a 0.5 s recording of the upper trace (corresponding to the horizontal bar) on an expanded time scale. Same patch and same conditions as in Fig. 1.

Fig. 5 also illustrates the effect of voltage pulses of different magnitude on the kinetics of closure. Many reopening events of the same amplitude as the closure events were observed, but eventually the channels appeared to get locked in a long-lived, inactivated state which is thus clearly different from the reversible closed state. The more negative the membrane potential, the faster this inactivated state was reached.

Nevertheless the channels could be reopened, by imposing a less negative membrane potential to the patch. A voltage pulse from −20 mV to −160 mV was applied four times to the same patch (Fig. 6A). The closure of three levels of conductance at −160 mV, followed by their reopening at −20 mV were systematically observed. The representation of the second pulse, on an expanded time scale, shows that reopenings were much faster than closures (Fig. 6B).

The sizes of the current transitions of the slow kinetics were variable. Most of the patches showed transitions of 190–230 pS (Figs. 4–6). Some patches exhibited transitions of 150–170 pS (Fig. 7), however, intermediate values between 150 and 250 pS were sometimes observed in the same patch. Larger transitions were also recorded, which in some cases could be ascribed to the simultaneous closure of smaller levels. An example can be seen in Fig. 6B, where the last closure, which appears as a discrete event of 460 pS, clearly results from the simultaneous cooperative closure of the last two 230 pS levels.

In several studies of porins reconstituted in planar lipid bilayers the application of large voltage pulses resulted in slow closure in three steps, each corresponding to about one third of the trimer conductance [5–7,9]. The conductance of a trimer is about 200 pS, under the ionic conditions used here (0.1 M KCl), as infered from the stepwise increases of the conductance of planar lipid

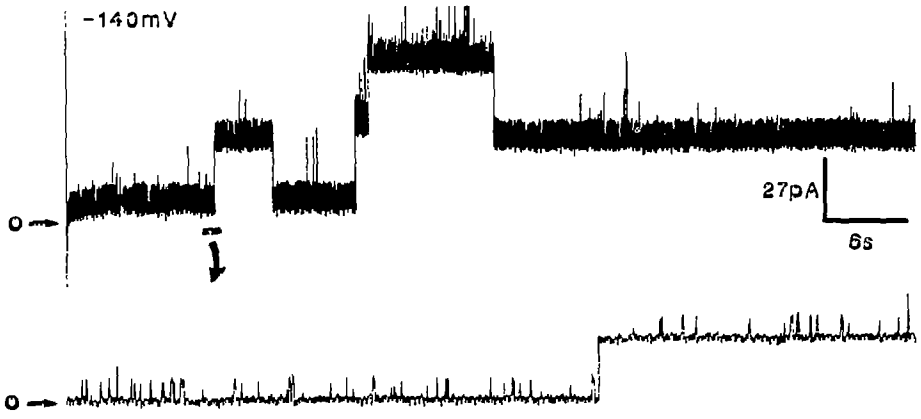

Fig. 4. Slow and fast kinetics of porin channels. Closures were elicited by a 60 s test pulse from 0 mV to −140 mV. The lower trace shows a 1 s recording of the upper trace (corresponding to the horizontal bar) on an expanded time scale. Other conditions are as in Fig. 1.

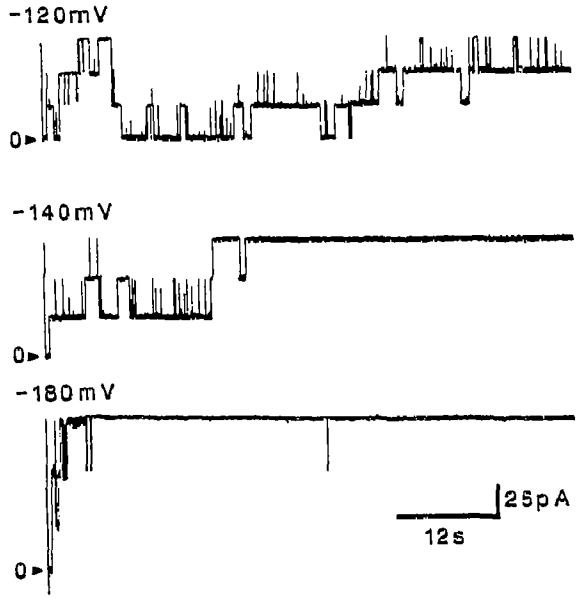

Fig. 5. Effect of the membrane potential on the slow kinetic closures. Closures were elicited by 60 s test pulses from −20 mV to the various membrane potentials indicated on the figure. Other conditions are as in Fig. 1.

bilayers upon insertion of porin trimers [4]. We thus would have expected to observe closures (and reopening events) of some 60–70 pS (instead of ca. 200 pS) for the slow kinetics. Transitions of this size were observed, but more rarely and only as isolated events. Fig. 7 shows the consecutive closures of three different levels of conductance, which may correspond to different multiples of a 60 pS conductance.

4. DISCUSSION

Reconstitution of porins into giant liposomes obtained by dehydration–rehydration, following the method originally devised by Criado and Keller [21], provides a simple and easy approach to the study of these pores by patch-clamp. We confirm, by this technique, the voltage-dependence of porins [5–10]. A notable difference between reconstitution into planar lipid bilayers and into giant liposomes amenable to patch-clamp recording is that, with the latter technique, the proteins are incorporated in the membrane before any electrophysiology experiment. The bath and the pipette media are devoid of porins, so that no incorporation of proteins can occur during the experiment. An important

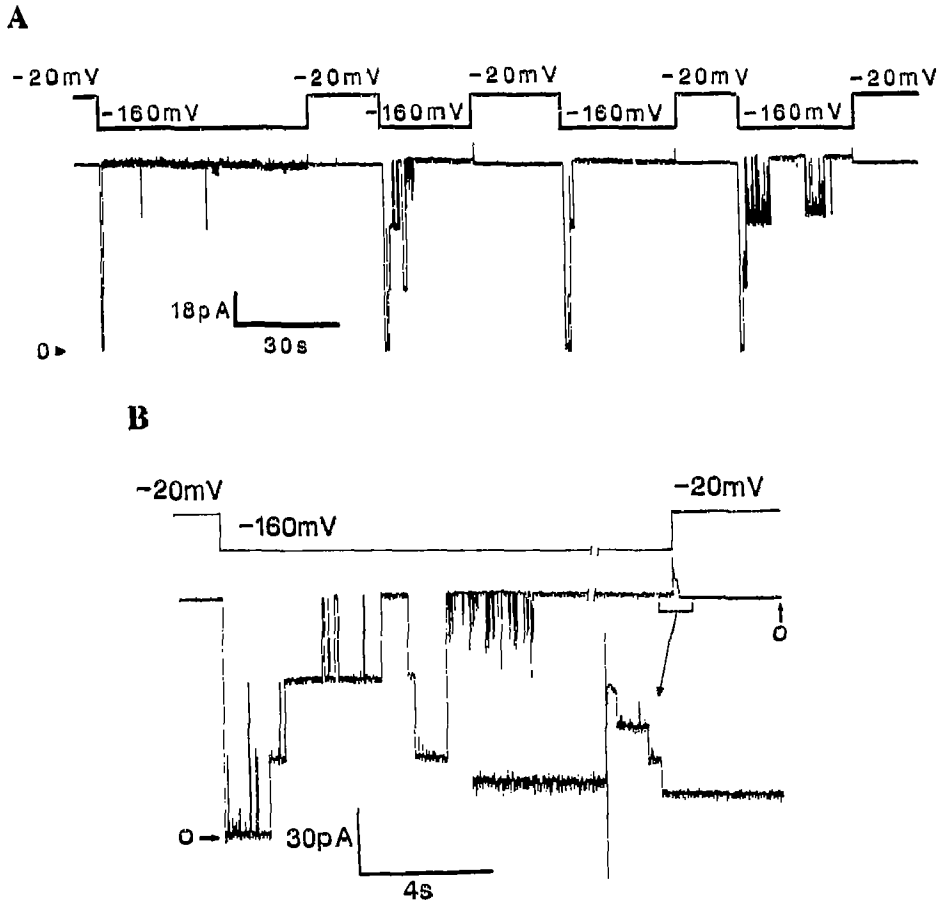

Fig. 6. Reversibility of porin closure. (A) A voltage pulse from −20 mV to −160 mV, was applied four times to the same patch, as indicated. (B) The second cycle is represented on an expanded time scale. Other conditions are as in Fig. 1.

Fig. 7. Variable sizes of the transitions of the slow kinetics. A voltage pulse from +20 mV to −180 mV induced the consecutive closures of three different levels of conductance: 170, 110, 60 pS. Other conditions are as in Fig. 1.

consequence is that the large transitions with slow kinetics are true opening and closure events and cannot be ascribed to insertion into, or de-insertion from the bilayer, of porin proteins.

Most of the transitions of the slow kinetics had a conductance of about 200 pS. Events expected to reflect closure of monomer channels were more rarely observed, in contrast with previous experiments using planar lipid bilayers [5–7,9]. An explanation for this might be that we used ionic conditions (0.1 M KCl) different from those used in these experiments (0.5 or 1 M salt). Buehler et al. have recently reported that, in planar bilayer experiments, decreasing the ionic strength from 1 to 0.1 M KCl led to the appearance of additional subpopulations of larger channels [22]. This was ascribed to a plasticity of porin channels. The possibility that the different transitions observed here reflect different states of a monomer channel cannot be ruled out, but implies considerable variations in the size of the channel. An alternative, but not necessarily exclusive, possibility is that some of these transitions correspond to the concerted closure and reopening of multimers. It is conceivable that decreasing ionic strengths increase the cooperativity between channels. The 200 pS transitions are likely to represent closures of trimers, but cooperative closure of a dimer, or of a trimer and of a monomer of a second trimer cannot be excluded. Concerted closures of two levels of large conductance, i.e. possibly of two trimers, were observed, indicating that this type of cooperative behaviour could extend beyond the three channels of a trimer. Similar conclusions as to the cooperative behaviour of porin channels have been reached by Xu et al. [8] from their studies of ompF reconstituted into planar bilayer under conditions of moderate ionic strength (0.1 M KCl or NaCl).

We observed the manifestation of fast-kinetic events with a unit conductance of about one third of that of a trimer. It is unclear why this fast kinetics has not been documented in previous studies performed with planar bilayers. One should note, however, that Morgan et al. in a recent study mentioned the existence of rapid (less than 1 ms) opening and closing events ([10], see also [23]). In our experiments, fast-kinetic events frequently appeared superimposed on the slow kinetics. At low absolute values of the membrane potential these events were the only manifestation of the presence of the channels. In view of its conductance we tentatively interpret this substate as the flickering of monomer channels between open and closed states. Fast-kinetic transitions of higher conductances were also observed. If our interpretation of the fast kinetics is correct, they correspond to the cooperative closure of several monomers, favoured by high negative membrane potentials. The maximum size of these transitions (up to 8 or 9 times the conductance of the most frequent transition) is consistent with cooperative interactions between monomer channels of different trimers.

Delcour et al., using whole outer membranes of *E. coli* fused into giant liposomes, studied by patch-clamp, have described a cationic channel with a conductance of 90 pS (in 150 mM KCl), which is characterized by a high open probablility and frequent and rapid gating to closed levels [17]. Increasing positive potentials favoured the cooperative closure of several 90 pS units, with the same fast kinetics. This electrical activity was later ascribed to OmpC on the basis of modifications of its conductance and gating in a strain carrying a single mutation in the *ompC* gene [18]. It probably corresponds to the fast-kinetic events described here, with an inverse polarity of reconstitution for the channels. However, the slow kinetics observed here was not described by these authors: this may be due to the fact that they did not apply high (above 100 mV) potentials to the patches.

In conclusion, the results presented in this paper indicate that porins have a less static behaviour than origi-

nally thought and emphasize the cooperative behaviour of these pores. Porins have at least three different closed states, and a simple interpretation of our data is that an increase in membrane potential favours the cooperative transitions of multimers towards closed and inactivated states; monomers or multimers can also gate rapidly to a different, very short-lived closed state. Future studies should aim at relating these properties to the structure of porins.

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