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A patch-clamp study of ion channels of inner and outer membranes and of contact zones of *E. coli*, fused into giant liposomes

Pressure-activated channels are localized in the inner membrane

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Inner and outer membranes of *Escherichia coli* and contact zones were isolated and fused separately with giant liposomes amenable to patch-clamp recording. Different types of large pressure-activated channels were localized in the inner membrane fraction which also contained smaller, pressure-insensitive channels. The outer membrane contained pressure-insensitive channels with large conductances and long opening and closing times which are likely to be porins. Large channels were also observed in contact zones.

Ion channel; Porin; Pressure activation; Patch-clamp; (*Escherichia coli*)

1. INTRODUCTION

Gram-negative bacteria such as *Escherichia coli* are delimited by an outer membrane and an inner membrane. The porins have constituted the first class of prokaryotic channels to be studied. They are located in the outer membrane. Most of them form large, non-specific, water-filled pores, but some porins exhibit a certain solute specificity [1, 2 and 3 for a recent review]. The outer membrane functions as a molecular sieve. The electrophysiological data obtained so far on porins have been mostly obtained on purified proteins incorporated in planar lipid bilayers. In contrast, very little is known concerning the presence of channels in the inner (energy transducing) membrane.

Martinac et al. applied the patch-clamp technique to *E. coli* cells [4]. Since normal bacteria are too small to be studied directly by patch-clamp, they used giant spheroplasts obtained by growth of the cells in the presence of an inhibitor of septation followed by a classical lyzozyme-EDTA treatment. They discovered a pressure-sensitive, voltage-dependent ion channel, which has a large conductance and a poor selectivity. Nevertheless it was not possible to ascertain whether the record was made from the outer or the inner membrane of the bacteria. Although it is not known from planar lipid bilayer experiments whether some porins are activated by pressure, the very large conductance of this channel made the authors suggest that it could be located in the outer membrane rather than in the inner membrane [4,5]. Nevertheless, Zoratti and Petronilli recently observed by the patch-clamp technique, pressure-activated channels in giant spheroplasts of Gram-positive (devoid of outer membrane) bacteria [6].

We present here a new strategy which allows the localization of bacterial channels. Criado and Keller recently described a dehydration-rehydration procedure which allows the fusion of biological membranes and liposomes into giant liposomes of sufficient size to be studied by patch-clamp [7]. We applied this procedure to bacterial membranes. The inner and outer membranes of normally grown *E. coli* K12 were isolated according to the method of Ishidate et al. [8]. This method allows a better separation than previous ones; in particular it yields a third fraction corresponding to the contact zones between the two membranes. The three fractions were separately fused into liposomes. Using patch-clamp, we could localize several types of pressure-sensitive channels in the inner membrane and clearly distinguish them from other channels (points) localized in the outer membrane. We have also observed large conductance channels in the contact zones.

2. EXPERIMENTAL

2.1. *Membrane isolation*

Cells of *E. coli* K12 were grown in minimal medium M9 containing 0.4% glucose as the sole carbon source. Isotopic labelling was performed by adding [^{14}C]leucine (11.9 GBq/mmol, 88 nM final concen-

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tration) to actively growing cultures at an absorbance of 0.3 at 650 nm (1 cm pathlength). Cells were harvested one generation later.

Isolation of the different membrane fractions then strictly followed the procedure of Ishidate et al. [8], except that the last step (density floatation gradient by ultracentrifugation for 72 h) was omitted. After sedimentation through the sucrose gradient, fractions were collected from the top using a Buchler Auto Densi-Flow apparatus. The radioactivity and the refractive index of each fraction were determined on aliquots. From the resulting profile of separation, appropriate fractions were pooled together to yield the three final fractions; inner membrane, outer membrane and contact zone (see fig.1). The different fractions were washed by centrifugation at 90000 rpm in a Tl 100 Beckman centrifuge for 40 min, resuspended in a 10 mM Hepes buffer (pH 7.4) and stored in liquid nitrogen until use.

2.2. *Biochemical characterization of the membrane fractions*

The membrane protein concentrations in the different fractions were calculated from the amount of incorporated [^{14}C]leucine, after prior calibration. Rate of respiration during succinate oxidation was measured polarographically, using a Clark oxygen electrode. 2-Keto-3-deoxyoctonic acid was determined by the thiobarbituric acid method of Weissbach and Hurwitz [9]. 1 mg of lipopolysaccharide was assumed to correspond to 0.45 mmol of 2-keto-3-deoxyoctonic acid [10].

2.3. *Formation of giant proteoliposomes*

The dehydration-rehydration method described by Criado and Keller [7] was used with modifications. Asolectin (from soybean, type II-S, Sigma), further purified according to Kagawa and Racker [11], was sonicated for 5 min at 10 mg/ml in 10 mM Hepes (pH 7), 100 mM KCl, using a bath sonicator to yield small multilamellar liposomes. 100 μl of this liposome suspension was mixed with biological membranes from one of the three previously described fractions. In a standard experiment, 200 μg of membrane proteins were used. This corresponds to a theoretical lipid/protein ratio of 6, taking into account endogenous lipids. The suspension of liposomes and biomembranes was centrifuged at 90000 rpm for 30 min in a Tl 100 Beckman centrifuge and the pellet was resuspended in 20 μl of 10 mM Hepes (pH 7.4). 6–7 μl of this suspension were deposited in the well of a 96-well culture dish (Nunclon) and dehydrated for 30 min at room temperature in a desiccator, using a vacuum pump. The dehydrated film was covered with 10 μl of 10 mM Hepes (pH 7), 100 mM KCl. The culture dish was covered with a lid and kept overnight at 4°C. Giant proteoliposomes (5–100 μm) were usually formed within 2–3 h.

Attempts to obtain giant proteoliposomes directly by dehydration-rehydration of bacterial membranes in the absence of added exogenous lipids using the method described in [12] were not successful. Giant proteoliposomes could also be obtained by freeze-thaw of a mixture of liposomes and bacterial membranes, as already described for other types of membranes [13]. This method was not further used, since we found that giga-seals were more readily formed using proteoliposomes obtained by dehydration-rehydration.

2.4. *Single-channel recording*

A 2–5 μl drop of the giant proteoliposome suspension was deposited on a Nunclon plastic tissue dish (35 mm in diameter) and diluted with 1.5 ml of the bath solution (as defined in the figure legends). All experiments were conducted at room temperature. Single-channel activity was measured using the methods of Hamill et al. [14]. Patch electrodes were pulled from Pyrex capillaries (Corning 7740) and were not fire-polished before use. The resistance of the pipette was 10 MΩ. A Dagan 8900 patch-clamp amplifier with a 10 GΩ feedback resistor was used. Application of the pipette to the surface of a liposome and gentle suction generally resulted in the formation of a GΩ seal. The patch was then excised and unitary currents were recorded and stored with a VCR device (Sony). Records were subsequently filtered through an 8-pole bessel low pass filter 920 LPF (Frequency Devices Inc.) at 1 KHz (–3 dB point) for data representation and analysis. Membrane potentials are given in terms of potential of bath solution, assigning zero potential level to the pipette. When control patch-clamp experiments were conducted on protein-free asolectin liposomes, most of the patches were electrically silent, but in some instances, rare square channel-like fluctuations of small conductances (10–20 pS) were observed. In any case, given the size of the channels reported in this paper, this artifactual electrical activity is of no consequence.

3. RESULTS

3.1. *Fractionation of* E. coli *K-12 cell envelopes*

E. coli K-12 cells, previously labelled with [^{14}C]leucine, were subjected to the procedure described by Ishidate et al. [8]. Fig.1 displays the radioactivity profile of the different fractions collected after the sedimentation gradient. Three major peaks are observed. Peaks I and III (of apparent buoyant densities of 1.12 and 1.24 g/ml) correspond to the inner and outer membrane fractions, respectively, as previously described [8].

The extensive analysis of peak II by Ishidate et al. [8] led them to identify this fraction with the contact zones (or attachment sites) that link the inner membrane to the murein outer membrane layer and which were originally described by Bayer [15]. This fraction contains closely apposed membranes of the two types. The lipopolysaccharide contents and the respiratory activities of the three fractions are given in table 1. These results show that in our preparations the contamination of outer membrane in the inner membrane fraction (as deduced from the 'spill-over' of lipopolysaccharide in this fraction) was about 8%. No respiratory activity was observed in the outer membrane fraction, so that contamination by inner membrane of this fraction was considered to be less than 2%. Characteristically, the attachment site fraction showed both a high lipopolysaccharide content and a sizeable respiratory activity.

3.2. *Patch-clamp recording of the inner membrane*

Inner membranes and asolectin liposomes were fused to give giant liposomes with a lipid/protein ratio of 6.

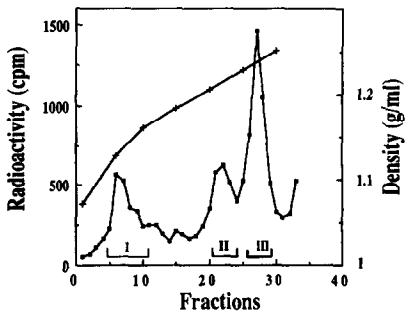

Fig.1. Membrane isolation. Radioactivity profile (■) and density profile (+) of the fractions collected after the sedimentation gradient. Final fractions I, II, III, were obtained by pooling the fractions as indicated in the figure.

Table 1

Biochemical characterization of the membrane fractions

	Inner membrane	Contact zones	Outer membrane
Lipopolysaccharide (mg/mg protein)	0.15	1.5	2.1
Respiratory activity with 20 mM succinate (nmol O_2/mg protein per min)	80	10.5	<1.5

After formation of the seal and excision of the patch, application of suction through the pipette resulted, in 50% of the cases (36 out of 74 patches), in the opening of channels which closed abruptly upon release of suction (fig.2). Different elementary conductances (in 0.1 M KCl), 140 (fig.2A), 330 (fig.2B), 490 (not shown), 950 pS (fig.2C), corresponding to different types of channels could be commonly observed. Higher conductances (1500 and 2000 pS) were more rarely obtained. After repeated suctions, seals have a tendancy to break down. In consequence, complete current-voltage curves from a single patch could only be obtained for the 490 and 950 pS channels. Fig.3 displays current-voltage curves obtained by pooling all the data obtained from different patches (excepting the 1500 and 2000 pS conductances). These pressure-dependent channels could also be differentiated on the basis of their kinetics: slow kinetics were associated with the 140, 330, 490 pS channels (fig.2A,B) and rapid kinetics with channels of higher conductances (fig.2C). In several instances, substates could be clearly identified (fig.2B). Despite the dilution brought up by the reconstitution (as compared to native membranes), these channels were often associated in clusters of identical conductances (fig.2B,C), but association of different types of pressure-dependent channels were also observed (fig.2D). Reconstitution at a higher lipid/protein ratio dimished the frequency of apparition of the pressure-dependent channels in proportion, but the functioning in clusters was still observed.

The inner membrane fraction also contained several different channels which were not pressure-dependent. The most frequently observed conductances were in the range 50–150 pS. Recordings of one of these channels and the corresponding current-voltage curve are shown in fig.4.

3.3. *Patch-clamp recording of the outer membrane*

In most of the patches obtained from outer membranes fused into liposomes, single-channel currents, characterized by long opening and closing times, were observed (fig.5). Most of the conductances were in the

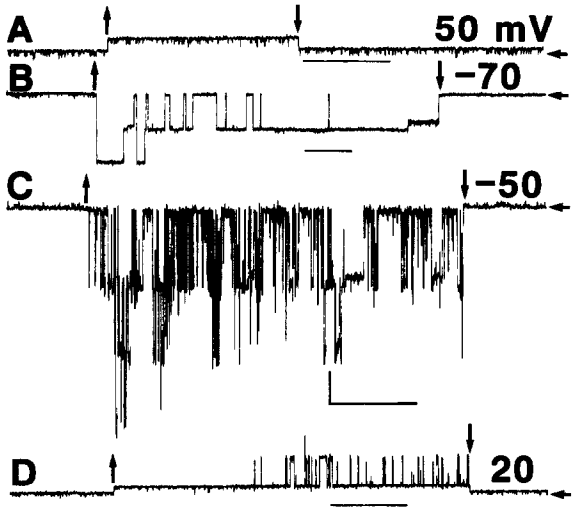

Fig.2. Pressure-dependent channels in the inner membrane. Recordings of unitary currents from inner membrane proteoliposomes. Lipid/protein ratio: 6. Bath medium and pipette medium (in mM): Hepes/KOH 10 (pH 7.4), KCl 100, $CaCl_2$ 2, $MgCl_2$ 5. The patch holding potential is indicated on the right of each recording. The closed levels are indicated by horizontal arrows. Upward arrow: suction (10 mm Hg) on. Downward arrow: suction off. Vertical bar, identical for all traces: 40 pA. Horizontal bar: 0.5 s.

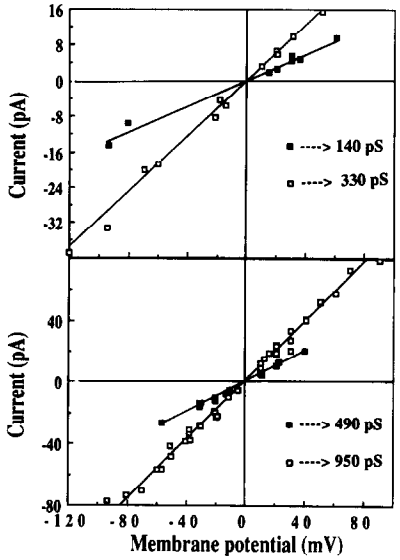

Fig.3. Current-voltage relationships of pressure-dependent channels. Data from unitary currents obtained in different patches, under conditions described in fig.2, were pooled together.

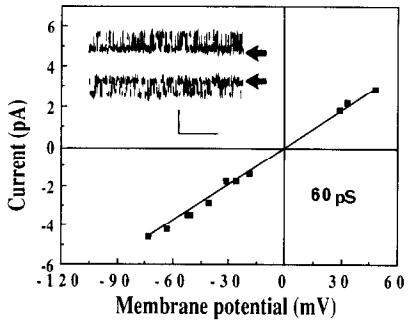

Fig.4. Current-voltage relationship of a pressure independent channel of the inner membrane. Inset: corresponding segment recordings, pipette and bath medium as in fig.2. Holding potential: 48 mV (upper trace), -50 mV (lower trace). Vertical bar: 5 pA. Horizontal bar: 250 ms.

range 150–300 pS (fig.5A,B) but much higher conductances (700–1100 pS) were also observed (fig.5C).

From their kinetics and conductances, it seems reasonable to identify these channels with the porins of the outer membranes, which have been studied extensively in planar lipid bilayers [3]. Patches which contained only one channel were only obtained when fusion was achieved with a high (20) lipid/protein ratio (fig.5A). Channels with smaller conductances and fast kinetics were also observed in this fraction (fig.5D). Importantly, all of these channels were insensitive to application of suction to the pipette.

Pressure-sensitive channels were only observed in two patches from a series of 30 patches, obtained from proteoliposomes reconstituted at a lipid/protein ratio of 6, for which suction was systematically applied at different membrane potentials. The conductances of these channels were 500 and 1500 pS, respectively. In other experiments for which the lipid/protein ratio was lower, this type of channel was never observed. It is noteworthy that no porin-like channel was observed in the two patches containing a pressure-dependent channel. It is also noteworthy that porin-like channels were not observed in patches obtained from liposomes reconstituted with the inner membrane fraction.

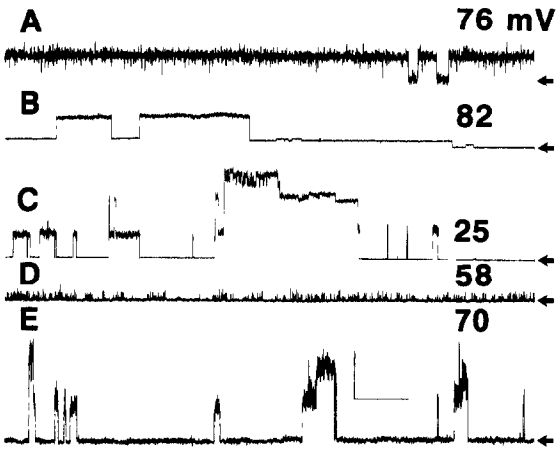

Fig.5. Recordings of unitary currents from outer membrane proteoliposomes (A–D) and from contact zone proteoliposomes (E). Pipette and bath medium as in fig.2. The lipid/protein ratio was 6, except in A (20). Vertical bar: 40 pA (A–C,E), 20 pA (D). Horizontal bar, identical for all traces: 1 s.

3.4. *Patch-clamp recording of the contact zones*

Upon a cycle of dehydration/rehydration, membranes from the contact zone fraction mixed with asolectin liposomes yielded giant liposomes very similar in size and shape to that obtained with the two other fractions. Some patches exhibited porin-like activities which were not sensitive to suction. Two patches (out of thirteen) contained pressure-dependent channels with 300, 500 and 1500 pS conductances. Characteristically, the two types of activities were not observed in the same patch, suggesting that the record was made from either one or the other membrane fused with the liposomes. Fig.5E displays a recording of a very large pressure-independent channel (700 pS) observed in this fraction.

4. DISCUSSION

This paper demonstrates that an efficient separation of the membranes [8], combined with the formation of giant proteoliposomes by dehydration-rehydration [7], allows the study of separation of the different fractions of the bacterial cell envelopes by patch-clamp. A wide range of channel activities were observed in the three fractions under study. At this stage no complete description could be attempted and further studies will be needed to fully characterize the different channels. This method, which allows the variation of the lipid/protein ratio in the reconstitution process thus enabling one to obtain patches containing only one channel, should prove very helpful in this respect.

Pressure-activated channels can be easily reconstituted in liposomes. We show here that *E. coli* strain K12 possesses several different pressure-activated channels. The pressure-activated channel described by Martinac et al. [4] in giant spheroplasts is similar (in conductances and kinetics) to the 330 pS or the 490 pS (in 0.1 M KCl) channel observed here. Multiple conductances of pressure-sensitive channels have also been recently observed in *E. coli* giant spheroplasts (Zoratti, M., personal communication).

In order to localize the various channels, the purity of the different fractions was assessed and reconstitution was achieved at a similar lipid/protein ratio for each fraction, to ensure meaningful statistics. This study demonstrates without ambiguity that the pressure-sensitive channels are present in the inner membrane: on the one hand, pressure-activated channels were observed in half of the recordings from the inner membrane fraction (to be compared with one out of fifteen for the outer membrane fraction); on the

other hand the electrical activity which we found characteristic of the outer membrane and which we identified with porin activity, was insensible to suction and was not observed in patches from the inner membrane. Interestingly, the contact zone fraction did not seem to be significantly enriched in pressure-activated channels. The two patches obtained from the outer membrane fraction and which displayed a pressure-sensitive channel activity could, in principle, be ascribed to a contamination of the outer membrane fraction by inner membranes. However, this contamination was determined to be below 2%. Thus it is not excluded that the outer membrane also harbours pressure-sensitive channels. The density of such channels in the outer membrane would be in any case much lower than in the inner membrane.

The localization of the pressure-sensitive channels in the inner membrane of *E. coli* is fully consistent with the recent findings of Zoratti and Petronilli. These authors, applying the patch-clamp technique to giant spheroplasts obtained from *Streptococcus faecalis*, reported the existence of pressure-dependent channels of high conductances in this bacteria [6]. Thus the cytoplasmic membranes of both Gram-negative and Gram-positive bacteria contain such channels. Our results also rule out the possibility that pressure-activated channels could have been induced by the different growth conditions necessary to obtain giant spheroplasts [4,6] since, here, membranes were isolated from normally grown bacteria. It is also clear from this study, that when giant spheroplasts of *E. coli* are patched, the recording is made from the inner membrane. Indeed, electrical recordings from the inner membrane fraction reconstituted in liposomes and from giant spheroplasts are very similar and can be clearly distinguished from those obtained from the outer membrane fraction. This is of importance, since in future studies both the spheroplast procedure and the liposome procedure will probably be used.

Finally, the localization of the pressure-sensitive channels in the cytoplasmic membrane raises several questions concerning their physiological function. Obviously such a channel could help to control turgescence, especially during osmotic downshock. Given the high conductance of these channels, their apparent high density in the membrane and the small size of the bacterial cell, the concerted opening of these channels should result in a complete collapse of the protonmotive force.

A transient collapse of the protonmotive force is of no consequence for the cells, but an excessive sensibility of these channels resulting in a permanent abolition of the protonmotive force should prove harmful to the bacteria, so that it cannot be ruled out that opening of these channels is under the control of other elements, possibly lost during spheroplast formation or isolation of the membranes.

Some nine different porins of the outer membrane of *E. coli* have been characterized up to now [3]. Of these, only the two larger ones, the general diffusion porins OmpC and OmpF, should be present in significant amount in *E. coli* K12, under our conditions of culture. These porins, when reconstituted in lipid bilayers, have a conductance of some 200 pS in 0.1 M KCl. We report here the existence in the outer membrane of channels displaying conductances up to 1100 pS (in 0.1 M KCl). This could imply that the outer membrane contains other porins than those reported so far. It has to be mentioned that the very large porins are expected to be rare and/or to have a low probability of opening. Otherwise this would contradict the fact that the outer membrane of *E. coli* has a low permeability to macromolecules with molecular masses higher than 600–800 Da (which corresponds to the exclusion limit of the 200 pS general diffusion porins) [3]. Aggregation of porins of lower conductances is also a possibility.

Reconstitution of the contact zones was also attempted. This fraction is of special interest since it has been suggested that contact sites between the two membranes could be implicated in the transfer of macromolecules across the two membranes [16]. Very large channels were observed in this fraction. While it is evident that channels belonging to the two membranes can be found in this fraction, it is not yet possible to decide whether it contains specific channels.

While this manuscript was in preparation, Blöbel and coworkers reported the existence of a 115 pS channel in inverted vesicles of *E. coli* fused with planar lipid bilayers [17]. This channel was claimed to be located in the inner membrane, although no criteria for the purity of the inner membrane preparation was given. Given the size of this channel, the authors hypothesized that it could be implicated in protein translocation. It is clear from this report, that *E. coli* membranes contain a wide variety of channels, some of wich are of a larger size than the one reported in [16]. To determine which of these are protein-conducting channels should prove to be a challenging prospect in the study of bacterial channels.

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