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Multiple Mechanosensitive Ion Channels from *Escherichia coli,* Activated at Different Thresholds of Applied Pressure

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Abstract. Mechanosensitive ion channels from *Escherichia coli* were studied in giant proteoliposomes reconstituted from an inner membrane fraction, or in giant round cells in which the outer membrane and the cell wall had been disrupted by a lysozyme-EDTA treatment and a mild osmotic shock. Patch-clamp experiments revealed the presence in these two preparations of an array of different conductances (100 to 2,300 pS in 0.1 M KCl) activated by stretch. The electrical activity induced by stretch in the native membrane was complex, due to the activation of several different conductances. In contrast, patches of proteoliposomes generally contained clusters of identical conductances, which differed from patch to patch. These experiments are consistent with the notion that these different conductances correspond to different proteins in the plasma membrane of *E. coli*, which segregate into clusters of identical channels on dilution involved in reconstitution in proteoliposomes. These conductances could be grouped into three subfamilies of poorly selective channels. In both preparations, the higher the conductance, the higher was the negative pressure needed for activation. We discuss the putative role of these channels as parts of a multicomponent osmoregulatory system.

Key words: Mechanosensitive ion channels — *Escherichia coli* — Bacteria — Osmoregulation — Patch-clamp — Liposomes

Introduction

Mechanosensitive (MS) ion channels, which comprise stretch-activated and stretch-inactivated channels, are gated by mechanical forces exerted on cell membranes. They constitute a class of their own, and can be distinguished from other ion channels which are only gated by voltage or ligand binding. Since their discovery (Guharay & Sachs, 1984), they have been found in a large variety of cells, in animals, plants and bacteria (for reviews, *see* Morris, 1990; Sachs, 1992; Martinac, 1993). Despite the attention devoted to this class of channels, their physiological functions remain in many cases elusive.

The finding that bacteria possess stretch-activated channels is of special interest, given the facilities offered by these organisms for genetic and molecular biology studies. The presence of stretch-activated channels has been documented in Gram-negative (*E. coli*) and Gram-positive (*S. faecalis* and *B. subtilis*) bacteria (reviewed by Martinac, 1993; Zoratti & Ghazi, 1993). Although the groups involved in the patch-clamp study of bacterial cells, agree on the presence in these organisms of MS channels of unusual sizes (*see below*), several discrepancies can be noted between the different reports.

A first problem, of obvious importance for the understanding of the physiological functions of stretch-activated channels, is their localization. *E. coli* is a Gram-negative bacterium and thus possesses an outer and an inner membrane. The outer membrane acts as a molecular sieve, due to the presence of pores, called porins, which have been extensively studied in recent years at the genetic, biochemical and biophysical levels (Nikaido, 1994). The presence of ion channels in the inner (plasma) membrane, was, until recently, not documented. Martinac et al. (1987) attributed to the outer membrane the giant MS channel that they had discovered in giant spheroplasts of *E. coli.* This proposal was substantiated by the same group which observed an identical channel in mutant, giant, round cells in which the outer

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membrane should be intact (Buechner et al., 1990). However, the finding by Zoratti and Petronilli (1988) that Gram-positive bacteria, which lack an outer membrane, possess large MS channels, strongly suggested that such channels could be present in the plasma membrane of Gram-negative bacteria as well. Moreover, when we separately reconstituted in giant liposomes the outer and inner membranes, partially purified from *E. coli*, we observed that the stretch-activated channel activity was preferentially associated with the inner membrane fraction (Berrier et al., 1989). The fact that, in intact cells, the efflux of several metabolites, triggered by hypotonic shock, can be blocked by gadolinium, an inhibitor of these stretch-activated channels, is also in favor of their presence in the plasma membrane. This finding indeed suggests that MS channels might be involved in the control of cell turgor, which is exerted at the level of the plasma membrane (Berrier et al., 1992*b*).

Another question concerns the number of different MS channels in bacteria. In their initial characterization of stretch-activated channels in *E. coli* giant spheroplasts, Martinac et al. (1987) described a single channel of 630 to 950 pS (depending on the polarity of the voltage, in 0.3 M salts). Subsequent studies by Berrier et al. (1989), performed on giant proteoliposomes, revealed the presence in *E. coli* of several different (at least 6) conductances activated by stretch, ranging from 100 to 1,500 pS (in 0.1 M KCl). More recently two MS channels from *E. coli*, a 500 pS channel and a 1,500 pS channel (in 0.1 M KCl), termed MscS and MscL respectively, were solubilized in detergent and functionally reconstituted in giant liposomes by Sukharev et al. (1993). The MscL channel has now been purified and shown to be an oligomer of a small protein, whose gene, *mscL*, has been cloned (Sukharev et al., 1994). *mscL* is thus the first identified gene known to code for an MS channel. In whole cell recordings performed on protoplasts Cui et al. (1995) recently reported the existence of two types of MS channels of 1,100 and 350 pS (in 0.4 M KCl) respectively.

In their investigations with giant protoplasts of *S. faecalis* and *B. subtilis* (Zoratti & Petronilli, 1988; Zoratti & Szabo′, 1991; Szabo′, Petronilli & Zoratti, 1992, 1993), and also of penicillin treated giant spheroplasts of *E. coli* (Szabo′ et al., 1990), Zoratti and coworkers have described how the application of stretch to the membrane patch, resulted in the activation of a whole array of conductances, ranging up to several nanoSiemens in 0.35 M KCl. The abundance of the different conductances led these authors to propose that this multiplicity of stretch-activated conductances might be due to cooperative gating of aggregates of channels with varying stoichiometries.

The number of stretch-activated conductances detected in proteoliposomes reconstituted from *E. coli* membranes, and also the relative fragility of seals made on liposomes upon repeated stretching, had so far prevented us from substantiating our proposal that these conductances correspond to distinct channels (Berrier et al., 1989). We present here an analysis of *E. coli* MS channels reconstituted in liposomes and data obtained directly on the native membrane of *E. coli.* Our results are fully consistent with the notion that the plasma membrane of *E. coli* harbors different MS channels corresponding to different proteins, which can be grouped into three different subfamilies. Each subfamily of channels is activated at a different threshold of applied pressure.

Materials and Methods

Preparation of Giant Proteoliposomes

E. coli strain K12 was grown at 37°C in M9 minimal medium containing 0.2% glucose as the sole carbon source. Cells were harvested at $O.D._{650} = 0.5$ and broken by passages through a French pressure cell. The different membrane fractions (inner membrane, outer membrane, contact zones) were isolated by sedimentation through a sucrose gradient as previously described (Berrier et al., 1989). Inner membrane vesicles were fused with liposomes of azolectin (from soybean, type II-S, Sigma) at a lipid-to-protein ratio of 6, by a cycle of dehydration-rehydration as previously described (Berrier et al., 1989). This procedure yielded proteoliposomes, suitable for patch-clamp recording, with diameters of 5 to 100 μm. For patch-clamp recording, 2 μl of the proteoliposome suspension were deposited on a Nunclon plastic tissue dish and diluted with 1.5–2 ml of 10 mM HEPES-KOH pH 7.4, 100 mM KCl.

Preparation of Giant Cells and Protoplasts

E. coli strains *lpp ompA*$^-$ (Sonntag et al., 1978) and GC3540 (Vinella, D'Ari & Bouloc, 1992) have a round phenotype. The *lpp ompA*$^-$ and GC3540 strains were kind gifts from Dr. Henning and Dr. Bouloc respectively. They were grown in 50 ml of LB pH 7.2, supplemented, in the case of the *lpp ompA*$^-$ strain, with 30 mM $MgSO_4$. Cultures were grown to an $O.D._{650}$ of 0.12–0.15. Cephalexin, an inhibitor of septation, was then added to a final concentration of 60 μg per ml and the resulting giant round cells (5–7 μm in diameter) were harvested 3–4 hr later. The cells were resuspended in (mM): 250 μl of 10 Tris-HCl buffer pH 7, 100 NaCl, 400 sucrose. For patch-clamp recording of native cells, 2 μl of this suspension were deposited in the patch-clamp chamber and diluted with 2 ml of 10 mM HEPES-KOH pH 7.4, 100 mM KCl, 10 mM $MgCl_2$.

To prepare giant functional ''protoplasts'' we followed the procedure of Birdsell and Cota-Robles (1967), which combines a lysozyme treatment with a mild osmotic shock and EDTA treatment. The concentrated suspension of giant cells in (mM): 10 Tris-HCl buffer pH 7, 100 NaCl, 400 sucrose, was incubated at 37°C for 30 min in the presence of lysozyme (400 μg/ml, final concentration). Two μl of this suspension were deposited in the patch-clamp chamber and covered with 50 μl of (in mM): 10 Tris-HCl pH 7, 1 EDTA, 100 KCl. Two ml of 10 mM HEPES-KOH pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, were then added to the patch-clamp chamber for recording.

Electrical Recordings

Single-channel activity was measured using the methods of Hamill et al. (1981). Patch electrodes were pulled from pyrex capillaries (Corning code 7740) and were not fire polished before use. Whether using proteoliposomes or cells, recordings were performed in the excised mode and the internal face of the membrane patch could be superfused by a flow of solution from one of a series of five piped outlets. The flow rate of the solutions was 50–100 μl/min. Negative pressure (suction) in the pipette was applied by syringe and monitored with piezoelectric pressure transducer (Bioblock Scientific). Unitary currents were recorded using a Dagan 8900 or a Biologic RK-300 patch-clamp amplifier with a 10 GΩ feedback resistance, and stored on digital audiotape (Biologic DTR 1200 DAT recorder). Records were subsequently filtered at 1 kHz (−3 dB point) through a 4-pole Bessel low pass filter, digitized offline at a rate of 2 kHz and analyzed on a Tandon 386 computer, with a program developed by G. Sadoc (Orsay). Data were plotted on a HP Laserjet printer.

Open probability P_o multiplied by the unknown number N of channels in a given patch, *vs.* the applied suction, were fitted with a Boltzmann distribution of the form:

$$N.P_o = N.P_{max}\,(1 + \exp\,\alpha\,(p_{1/2} - p))^{-1} \quad (1)$$

where P_{max} is the maximum probability of channel being open, p is the suction, $p_{1/2}$ is the suction at which the open probability is 0.5 and α is the sensitivity to the applied suction.

In the case of proteoliposomes, for which the orientation of the reconstituted proteins is unknown, the convention for the membrane potential is the same as for the native membrane (assigning zero potential level to the pipette). The contents of all the pipette, bath and perfusion solutions are given in the figure legends.

Results

MS Channels Reconstituted in Giant Liposomes

A fraction highly enriched with inner *E. coli* membranes was fused into giant proteoliposomes by dehydration-rehydration. Several different ion channels were observed by patch-clamping these proteoliposomes. Some of these channels showed no mechanosensitivity. One of these nonmechanosensitive channels, a voltage dependent cationic channel has been characterized in detail (Berrier et al., 1993). This report deals only with MS (stretch-activated) channels, i.e., channels activated by applying negative pressure in the pipette. A general observation concerning MS channels reconstituted in proteoliposomes is that, whatever their conductances, they are closed at all membrane potentials in the absence of applied suction and that, once activated, the channels close upon release of suction. In other words, they display no spontaneous activity (in the absence of applied suction).

Several different conductances activated by stretch, were detected in these proteoliposomes. In assessing whether or not these conductances correspond to different channels, we encountered two difficulties. First, in order to avoid attributing some properties of one channel

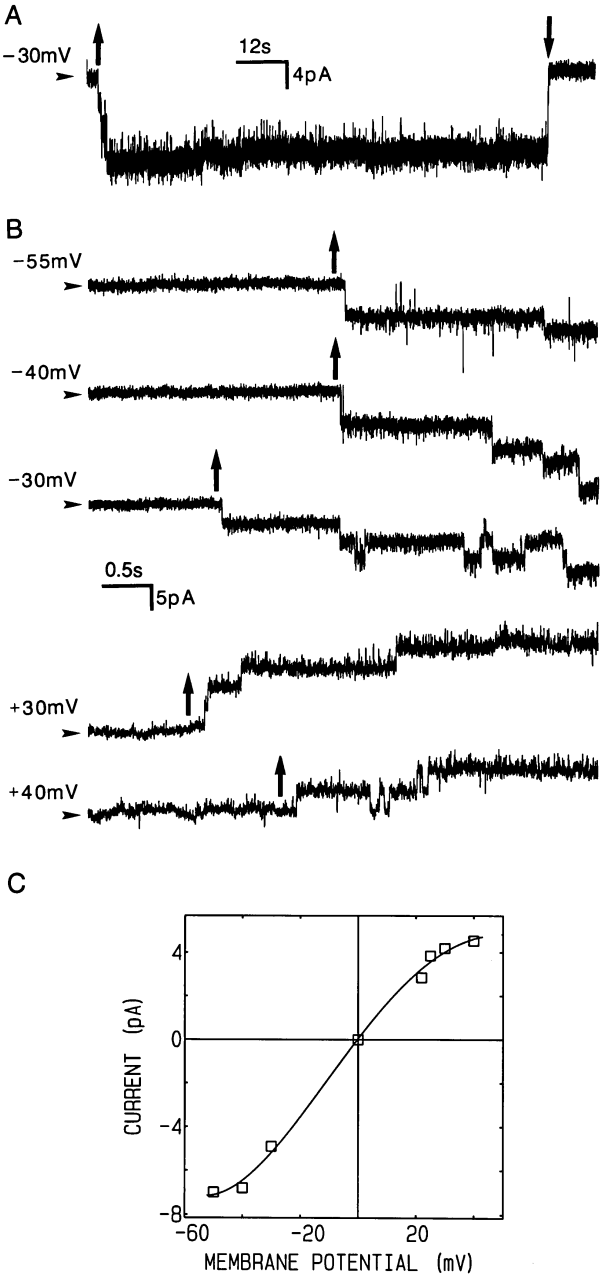

Fig. 1. 100–150 pS MS channels in proteoliposomes. Recordings from an inside-out excised patch of a giant proteoliposome reconstituted with inner membranes from *E. coli* K-12. Bath medium (in mM): 10 HEPES-KOH adjusted to pH 7.4, 100 KCl. Pipette medium: as bath medium with the addition of 2 $CaCl_2$, 5 $MgCl_2$. (*A*) Reversible effect of applied suction. Upward arrow: application of suction (19 mm Hg) in the pipette. Downward arrow: release of suction. Closed level marked by a horizontal arrow. (*B*) Representative traces at various holding potentials as indicated. Upward arrow: application of suction in the pipette (20 mm Hg). (*C*) Corresponding *I-V* curve. Only the most frequent current transition was reported on the curve. In the linear part of the curve the slope is 100 pS. Events of 150 pS were also visible at several potentials.

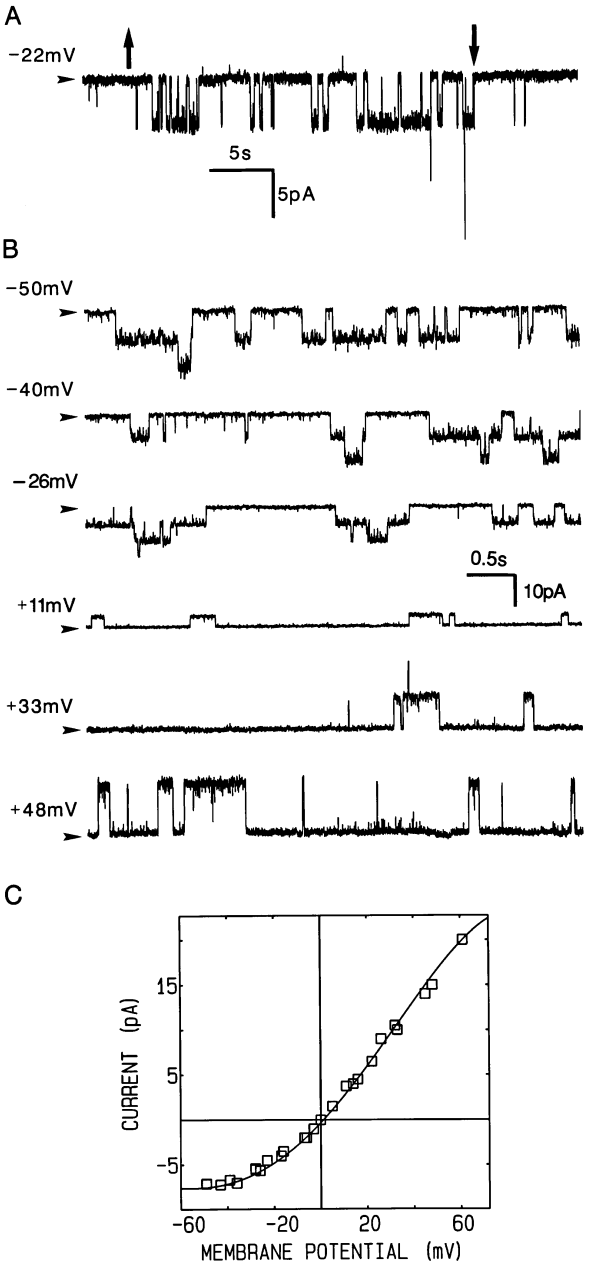

Fig. 2. 300 pS MS channels in proteoliposomes. (*A*) Reversible effect of applied suction. Upward arrow: application of suction (9 mm Hg). Downward arrow: release of suction. (*B*) Representative traces at various holding potentials as indicated. Applied suction (9 mm Hg maintained throughout). (*C*) Corresponding *I-V* curve. In the linear part of the curve the slope is 300 pS. Other conditions as in Fig. 1.

to another, it was desirable to accumulate from the same patch the maximum information concerning the conductance at various potentials and the dependence on applied pressure or on membrane potential of one putative type of channel. However, seals performed on liposomes rarely resist prolonged or repeated suctions. A second difficulty was that the channels observed in liposomes showed unclear dependence on the membrane potential and that the suction needed to activate the channels was highly variable. Thus, for the same channel in the same patch at the same potential, the dependence on negative pressure was observed to vary when suction was repeatedly applied to the patch. In fact, the channels are better distinguished on the basis of their conductance at various membrane potentials.

The following conductances were observed (in 100 mM KCl symmetric media): 100 and 150 pS ($n = 12$), 300 pS ($n = 20$), 500 pS ($n = 16$), 950 pS ($n = 16$), 1,500 pS ($n = 10$), 2,300 pS ($n = 4$). In several cases, the seal broke before a complete *I-V* curve could be obtained and in these cases these values are chord conductances. More or less complete *I-V* curves, recorded from the same patch, could be obtained for the following conductances: 100–150 pS ($n = 2$; Fig. 1), 300 pS ($n = 7$; Fig. 2), 500 pS ($n = 4$; Fig. 3), 1,000 pS ($n = 5$; Fig. 4); 1,500 pS ($n = 6$; Fig. 5). A 2,300 pS conductance was observed in 4 different patches (*not shown*) but no complete *I-V* curve was obtained for this conductance. The current-voltage relationship was practically linear for the other conductances, except for the 300 pS which showed rectification. It is therefore probable that the 2,300 pS conductance corresponded to an independent channel. The 100 and 150 pS conductances were generally observed together, so that it is unclear whether they correspond to different substates of the same channel. The channels were also clearly differentiated on the basis of their kinetics. The 100–150 pS channels showed long opening times, the 1,000, 1,500 and 2,300 pS channels displayed characteristic fast flickerings.

Patches made on proteoliposomes were found either to be devoid of channels (in about 60% of the cases) or to present several MS channels (up to six), which, in most cases (73 out of 78), were identical (Figs. 1–5). Only few patches (5 out of 78) presented two different types of stretch-activated conductances. Three different types of channels were not observed in a same patch. All these observations suggest that the different MS channels segregate into clusters of identical channels. An important point is that, in a given patch, the same types of stretch-activated channels were observed throughout the recording. This was true whether the suction was maintained throughout the recording or whether suction was applied and released several times.

A study of the dependence on suction of the open probability of these channels in liposomes proved to be difficult because of the relative fragility of the patches on liposomes and because of the high sensitivity of the channels to suction. This study was possible for one patch containing several 300 pS channels. The data could be fitted with a Boltzmann distribution of the form given by Eq. 1. Increasing the suction by only 2 mm Hg, increased the open probability by *e*-fold. The steepness

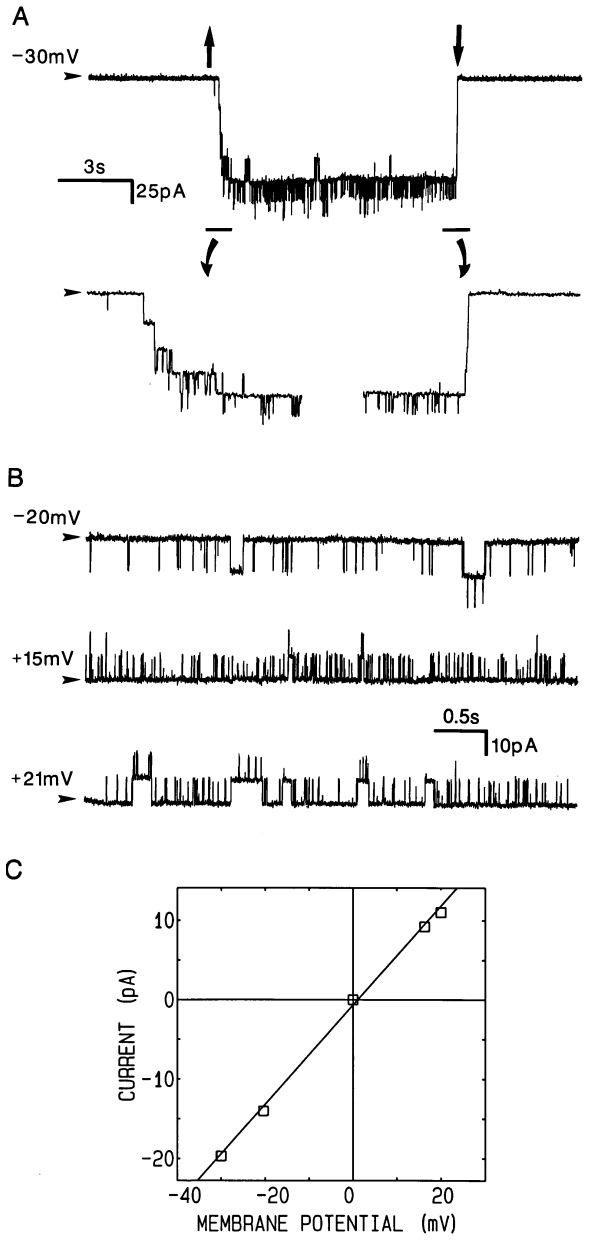

Fig. 3. 500 pS MS channels in proteoliposomes. (*A*) Reversible effect of applied suction. Upward arrow: application of suction (12 mm Hg). Downward arrow: release of suction. (*B*) Representative traces at various holding potentials as indicated. Applied suction (8 mm Hg maintained throughout). (*C*) Corresponding *I-V* curve. The slope is 500 pS. Other conditions as in Fig. 1.

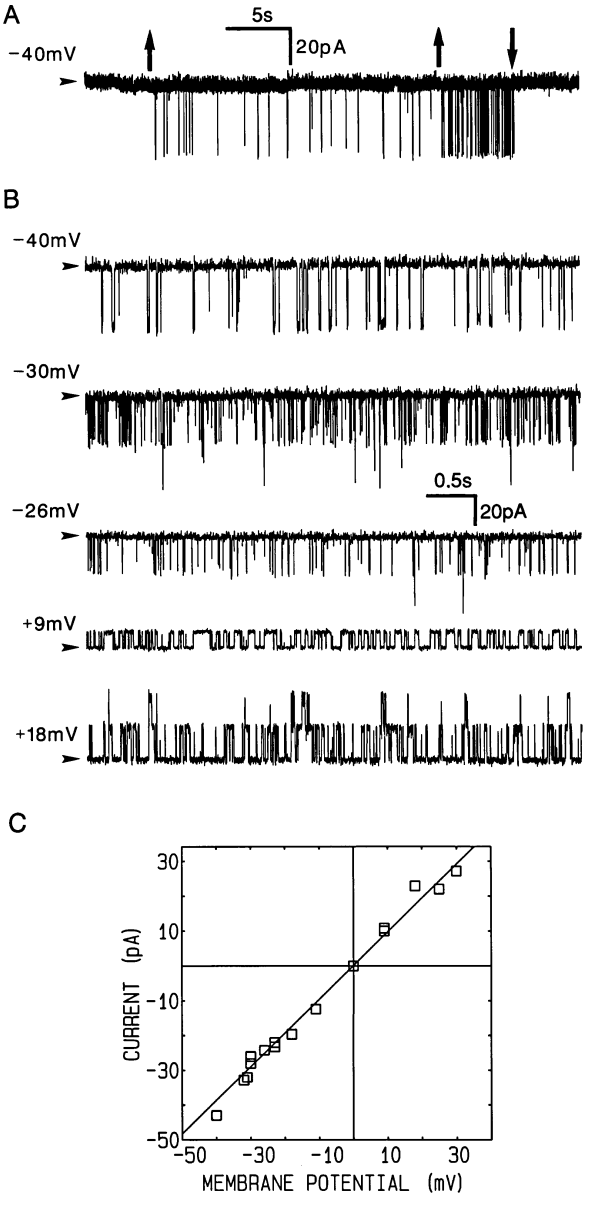

Fig. 4. 1,000 pS MS channels in proteoliposomes. (*A*) Reversible effect of applied suction. First upward arrow: application of suction (39 mm Hg). Second upward arrow: increase in suction (42 mm Hg). Downward arrow: release of suction. (*B*) Representative traces at various holding potentials as indicated. Applied suction (43 mm Hg maintained throughout). (*C*) Corresponding *I-V* curve. The slope is 1,000 pS. Other conditions as in Fig. 1.

of the curve relating probability of opening to applied suction resulted in a threshold effect. We define the threshold of activation as the minimum applied suction needed to activate a channel, when suction is slowly and progressively raised in the pipette. Thresholds of activation were highly variable, even for the same type of channels. However, in two different patches, each containing two different types of channels (a 100–150 pS channel in both cases, associated with a 1,000 or a 1,500 pS channel), we observed that the 150 pS channel was systematically activated for lower values of the applied suction than the 1,000 or 1,500 pS channels (*not shown*).

The voltage dependence of the channels in giant liposomes, unlike in the native membrane (*see below*), was unclear and variable from patch to patch. Slight variations in the membrane tension at constant applied

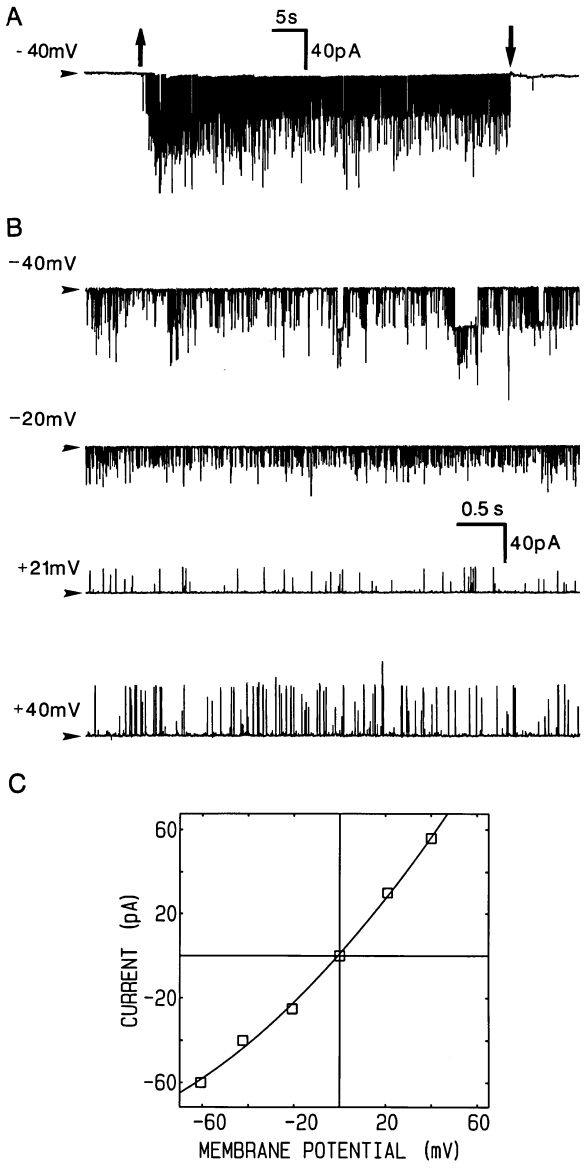

Fig. 5. 1,500 pS MS channels in proteoliposomes. (*A*) Reversible effect of applied suction. Upward arrow: application of suction (30 mm Hg). Downward arrow: release of suction. (*B*) Representative traces at various holding potentials as indicated. Applied suction (30 mm Hg maintained throughout). (*C*) Corresponding *I-V* curve. The slope is 1,500 pS. Other conditions as in Fig. 1.

suction due to changes in the patch geometry, combined with the high sensitivity of these channels in liposomes, could account for this variability.

MS Channels Recorded in the Native Membrane

A lysozyme-EDTA treatment of *E. coli* cells combined with a mild osmotic shock gives structures in which the outer membrane is ruptured, exposing large areas of the cytoplasmic membrane to the environment. Electron microscopic study of these structures by Birdsell and Cota-Robles (1967) showed that the outer membrane forms tight coils which remain attached to the cell, albeit to a small area of the surface. These authors have called these EDTA-lysozyme spheroplasts ''functional'' protoplasts to emphasize the absence of a closed outer membrane.

To record from the native membrane, we prepared giant protoplasts from giant round cells. Giant round cells can be obtained by growing cells which have a round phenotype, in the presence of cephalexin, an inhibitor of septation (Buechner et al., 1990). The advantage of using giant round cells is that their geometry makes them in principle amenable to patch-clamp recording in their native state, so that intact cells and treated cells can be compared. Several genotypes lead to a round phenotype for *E. coli* cells. We have used with equal success the *lpp ompA* double mutant (Sonntag et al., 1978) and strain GC3540 (Vinella, D'Ari & Bouloc, 1992). The lysozyme-EDTA treatment of giant round cells of both strains, following the procedure described in methods, yielded structures which could be clearly distinguished in phase contrast microscopy (i.e., under conditions of patch-clamp recording) from the untreated cells. The untreated cells appeared as dark, refractive structures of somewhat irregular form. The lysozyme-EDTA treatment gave larger, nonrefractive, grey spheres (Fig. 6). The treated cell suspension also contained structures which more closely resembled untreated cells and which were probably cells which had not fully undergone the lysozyme-EDTA treatment. Increasing the incubation time of the cells with lysozyme markedly increased the proportion of grey spheres.

We compared the rate of seal formation on untreated cells of the *lpp ompA* double mutant and on the two populations of cells obtained after lysozyme-EDTA treatment (those appearing as grey spheres and those resembling the untreated cells). Seals were difficult to form with untreated cells: only 10% of the attempts were

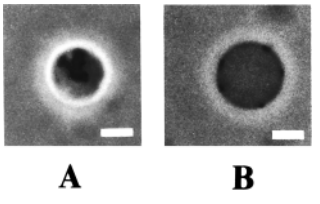

Fig. 6. Phase-contrast micrographs of giant round *E. coli* cells in the native state (*A*) and after a lysozyme-EDTA treatment followed by a mild osmotic shock (*B*). The giant round cells were obtained by growing the *lpp ompA*$^-$ strain in the presence of cephalexin. Note the difference in shape and refractivity between the two cells. The black dots observed at the periphery of the treated cells might correspond to coils of outer membranes as described by Birdsell and Cota-Robles (1967). Bar: 3 μm.

successful. The lysozyme-EDTA treatment dramatically improved the success of seal formation: with the two populations of treated cells the rate of seal formation was identical, in the order of 50–80%. Interestingly in nearly all cases, including untreated cells, when a tight seal could be obtained, MS channels were present in the patch. All the findings described below are from treated cells appearing as grey spheres since there is little ambiguity as to the nature of the patched membrane in these structures.

When seals were obtained with these structures, application of suction in the pipette resulted in the activation of stretch-activated ion channels of different conductances ranging from 50 to 1,500 pS in 0.1 M KCl. The electrical activity was in general more complex than in proteoliposomes, due to the presence in the same patch of several different conductances (Fig. 7).

However, different subgroups of conductances were identified and corresponded with those observed in proteoliposomes. A 100–150 pS conductances ($n = 22$) with slow kinetics were clearly distinguished from all other channels. Again these two conductances were generally observed together. 50 pS ($n = 4$) and 200 pS ($n = 6$) conductances, with the same type of kinetics were also occasionally observed. A second subgroup comprised conductances of 300 ($n = 43$) and 500 pS ($n = 6$). A third subgroup comprised channels of 1,000 ($n = 5$) and 1,500 pS ($n = 17$) with fast kinetics.

The thresholds of suction (as defined above) needed to activate these channels were extremely variable between patches. Even in the same patch the threshold could vary (increase or decrease) for the same channel, when pressure was applied and released several times. However and most importantly, when different conductances were present in one patch, we systematically observed that the higher the conductance, the higher was the negative pressure needed for its activation. In other words, when the applied suction was progressively raised, the different channels appeared by increasing order of conductances: first the 100–150 pS conductances, then the 300 or 500 pS, and the 1,000 or 1,500 pS conductances for still higher values of the applied suction (Fig. 7).

In contrast to what was observed with proteoliposomes, the channels of 100 to 500 pS could be spontaneously active (in the absence of suction in the pipette) in some patches, especially at positive voltage. In these cases, an increase in suction invariably resulted in an increase in the probability of opening of these channels, indicating that they were mechanosensitive. The threshold pressure needed for activation of these channels ranged from 0 to 200 mm Hg. For channels whose conductance was 1,000 pS or more, the threshold of activation was between 60 and 220 mm Hg. Also in contrast with the situation in proteoliposomes, some channels which had been activated by suction remained active after the release of suction. This hysteresis phenomenon might explain the existence of spontaneously active channels. They may be channels activated during seal formation which, with protoplasts, often required the application of negative pressure as high as 100 mm Hg, for several minutes.

The relative frequencies of observation of the different channels are likely to reflect their relative proportions in the native membrane, except for the 1,000 and 1,500 pS channels: for these channels the suction needed for their activation probably often exceeded the mechanical capacity of the patch. For the same reasons, still higher conductances, such as the 2,300 pS conductance observed in proteoliposomes, may have remained undetectable. Most of the patches which could sustain high applied suction were observed to contain the three most frequent types of conductances (100–150, 300 and 1,500 pS) but some patches contained clusters of only one type of channel (Figs. 8 and 9). The 100–150 pS channels were generally only easily detectable at low applied suction. At higher suction they remained open most of the time and their activity was obscured by that of the 300 pS channels.

The pressure dependence of the 300 pS and 1,500 pS channels was analyzed in patches, containing only these channels. These channels were less sensitive to pressure in the native membrane than in proteoliposomes. The open probability distributions of the two channels could be fitted with a Boltzmann distribution of the type given in Eq. 1 (Figs. 8 and 9). No clear differences could be observed between the sensitivity α of the two types of channels to applied suction. In 6 independent experiments performed with the two types of channels, the amount of pressure needed to increase the open probability by *e*-fold, varied from 8 to 23 mm Hg. The probability distributions of the two types of channels differed only markedly in the $p_{1/2}$ parameter, reflecting the fact that the 1,500 pS is activated at higher values of the applied suction.

The MS channels in *E. coli, B. subtilis,* and *S. faecalis* have been reported to be voltage dependent (Martinac et al., 1987; Szabo′ et al., 1992, 1993). Both the 300 and 1,500 pS were indeed observed to be more frequently open at low positive values of the membrane potential than at negative potential. However, in contrast to these previous reports, in the case of the 300 pS channel, an analysis of its voltage dependence showed that it does not follow a Boltzmann distribution. As evidenced in Fig. 8, the distribution was more complex, going systematically through a maximum around 30 mV. Also, above 40 mV, the membrane potential very often altered the kinetics of the 300 pS channel, by inducing a characteristic 150 pS substate (*see* the trace at 50 mV on Fig. 8).

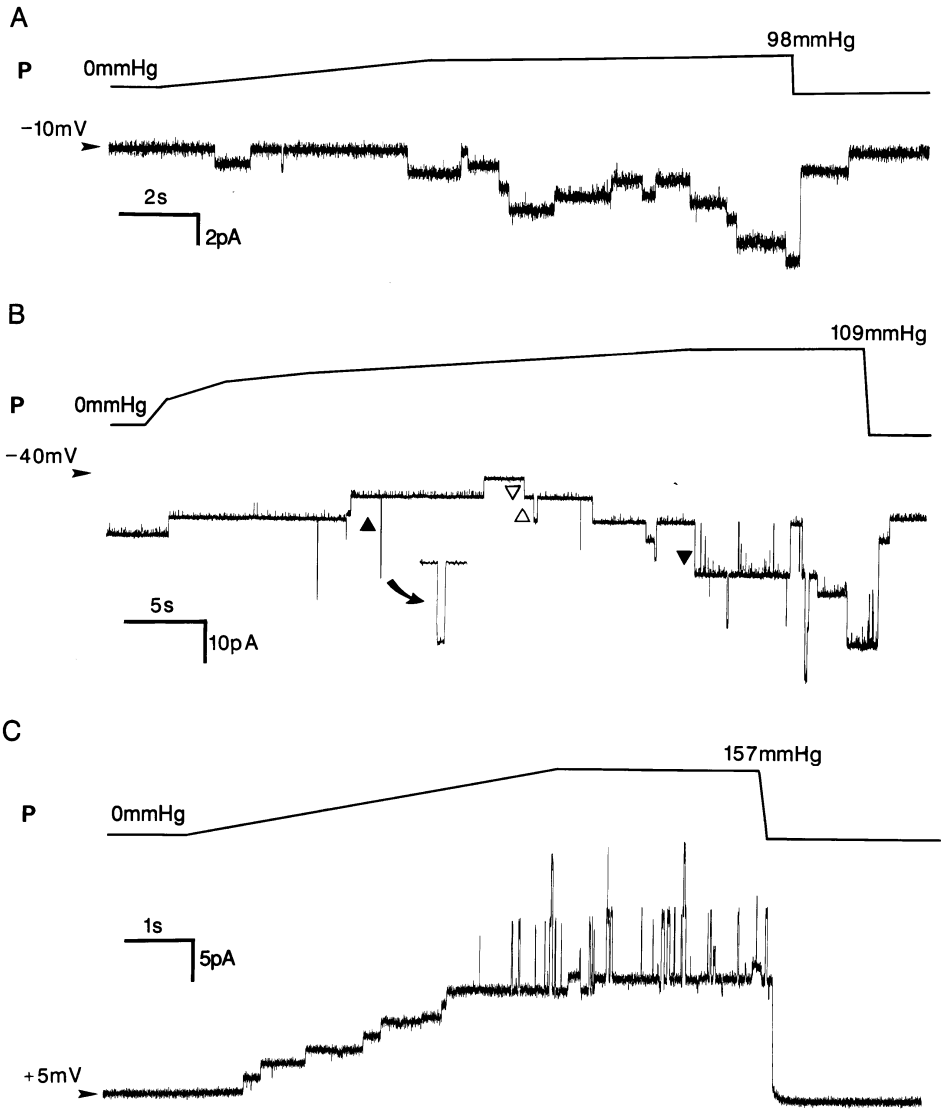

Fig. 7. Multiplicity of MS channels recorded in the native membrane, and activated by order of conductances, as the applied suction is progressively raised. Recordings from inside-out excised patches on giant *lpp ompA*$^-$ cells after lysozyme-EDTA treatment. Bath medium (in mM): 10 HEPES-KOH adjusted to pH 7.4, 100 KCl, 10 $MgCl_2$. Pipette medium: as bath medium with the addition of 20 $CaCl_2$. For each trace, the upper curve indicates the profile of the applied suction. (*A*) Activation of 100 and 150 pS conductances. (*B*) Same patch as in (*A*). 100–150 pS channels were spontaneously active. Increasing the suction in the pipette triggered the opening of 300 pS channels. (▽): 100 pS transition, (△): 150 pS transition, (▼): 300 pS transition. (▲): a 500 pS channel is also present in the patch, as shown on an expanded time scale. Also in the same patch, 1,500 pS channels were activated at still higher values of the applied suction. (*C*) Different patch, containing 300 pS channels, and 1,500 pS channels activated at a higher applied suction.

The MS channels of *E. coli* show little ion selectivity. The ion selectivity of the most frequent channels was analyzed in asymmetric media (100 mM KCl in the pipette and 300 mM KCl in the bath, and 100 mM KCl in the pipette and 20 mM KCl in the bath). In agreement with the report of Sukharev et al. (1993), the 1,500 pS channel was unselective ($n = 6$) and the 300 pS was found to be weakly anion selective, both in proteoliposomes and in the native membrane ($P_{Cl}/P_K = 1.7 \pm 0.3$, as calculated from the GHK equation, $n = 7$). The selectivity of 100 pS conductance was determined in the native membrane and found to be slightly cation-selective ($P_K/P_{Cl} = 2.4 \pm 0.7$, $n = 3$).

Discussion

The localization of stretch-activated channels in Gram-negative bacteria has been debated. Direct recording of

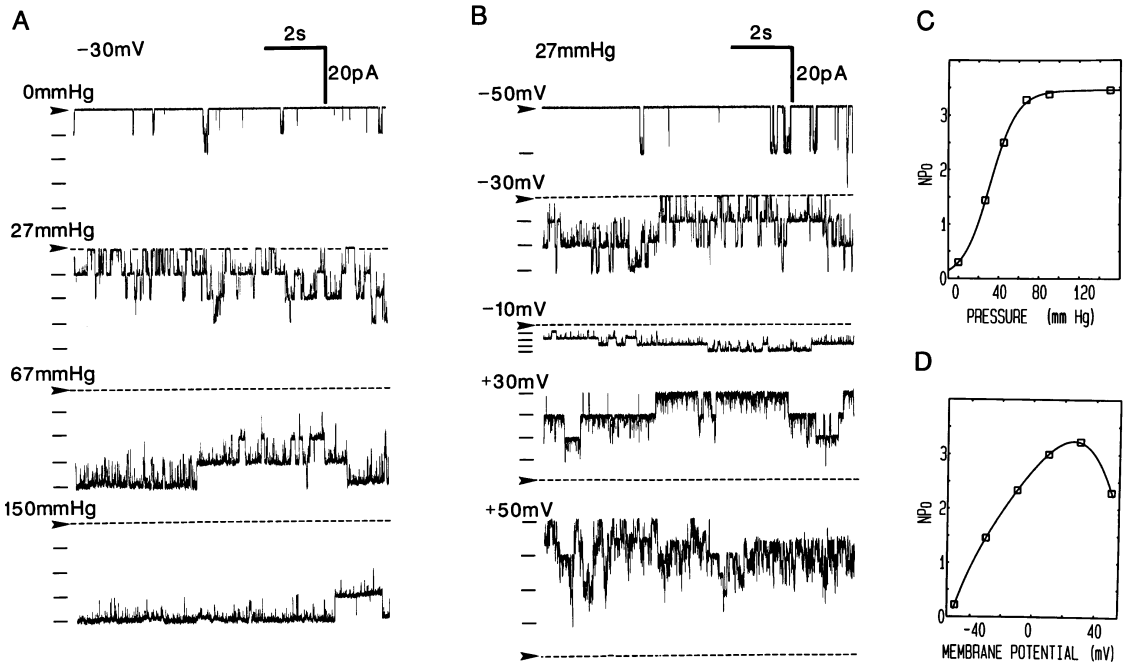

Fig. 8. Pressure dependence and voltage dependence of the 300 pS channel in the native membrane. Recordings from an inside-out excised patch made on a giant *lpp ompA*$^-$ cell after lysozyme-EDTA treatment. Conditions as in Fig. 7. (*A*) Representative traces at various values of applied suction, as indicated. The membrane potential was −30 mV. (*B*) Representative traces at various membrane potentials, as indicated. The applied suction was 27 mm Hg. (*C*) Open probability P_o multiplied by the number *N* of the channels in the patch *vs.* the applied suction, at fixed membrane potential (−30 mV). *N* P_o was calculated by integrating the current through open channels in a given segment of recording (at least 30 sec) and dividing the integral by the time of recording multiplied by the current through a single channel. The data were fitted to Eq. 1. 1/α was 14.5 mm Hg, $P_{1/2}$ was 32 mm Hg and *N*. P_{max} was 3.45. (*D*) Open probability P_o multiplied by the number *N* of the channels in the patch *vs.* the applied membrane potential, at fixed applied suction (27 mm Hg). The data were fitted to a polynomial.

electrical activity in normal *E. coli* cells is precluded by the size of this bacterium. Martinac et al. (1987) circumvented this difficulty by growing cells in the presence of an inhibitor of septation. Under these conditions, one gets very thin and long filamentous cells. A lysozyme-EDTA treatment, which partially destroys the cell wall, yields ''giant spheroplasts'' amenable to patch-clamp recording. This procedure led to the detection of a high conductance MS channel which was tentatively ascribed to the outer membrane. In these structures it was unclear whether the patch pipette was sealed onto the outer or the inner membranes. The main argument in favor of this proposal came from experiments in which a similar ion channel was detected in patch-clamp experiments performed on mutant giant round cells, in the absence of treatment (Buechner et al., 1990). It is however open to doubt whether a gigaohm seal can be formed on the outer membrane. The outer membrane is a molecular sieve due to the presence in this membrane of some 10^5 porins per cell (Nikaido & Vaara, 1987). Given the conductance of porin channels, the formation of a gigaohm seal on this membrane would require that practically all the porins of the membrane be closed, a proposal difficult to reconcile with the function of these pores.

In an attempt to clarify this issue, we previously compared the electrical activity of giant proteoliposomes reconstituted with either outer or inner membrane fractions isolated from *E. coli*. The MS channel activity was mainly present in the inner membrane fraction (Berrier et al., 1989). However, although these data argue in favor of these channels being present in the plasma membrane, they do not constitute definitive evidence because unavoidable cross contaminations exist between the different fractions and because the efficiency of reconstitution of these fractions is not known. In this paper, we report on experiments performed with structures obtained by subjecting giant cells to the procedure described by Birdsell and Cota-Robles (1967) which have been shown by electron microscopy to yield cells largely stripped of outer membrane. This protocol yields large grey spheres which can be clearly identified under the light microscope, and which are probably the same as the structures described by Kubalski et al. (1992) as true protoplasts in electrophysiology experiments. In patch-clamp experiments performed on these structures, we detected MS channels in nearly all patches. This indicates that MS channels are present in the plasma membrane of *E. coli.* While this paper was submitted, Cui, Smith and Adler

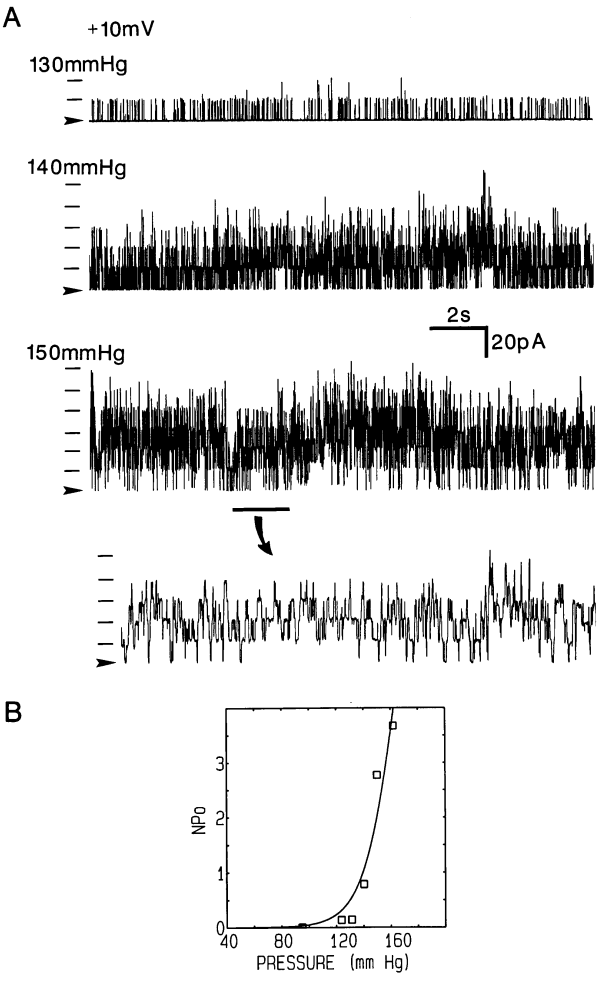

Fig. 9. Pressure dependence of the 1,500 pS channel in the native membrane. Recordings from an inside-out excised patch on a giant *lpp ompA*$^-$ cell after lysozyme-EDTA treatment. Conditions as in Fig. 7. (*A*) Representative traces at various values of the applied suction, as indicated. The membrane potential was +10 mV. The inset shows a 2 sec recording on an expanded time scale. (*B*) Open probability P_o multiplied by the number *N* of the channels in the patch *vs.* the applied suction, at fixed membrane potential (10 mV). The data were fitted to Eq. 1. 1/α was 12.7 mm Hg, $P_{1/2}$ was 176 mm Hg and *N*. P_{max} was 16.

(1995) reported experiments consistent with this notion: they performed whole cell recordings on protoplasts similar to the ones described here and detected two types of MS channels.

The problem of the localization of MS channels in bacteria is evidently linked to their function. Zoratti and coworkers have documented high stretch-activated conductances, very similar to those described here, in the plasma membrane of *B. subtilis* and *S. faecalis* (Zoratti & Petronilli, 1988; Zoratti & Szabo′, 1991; Szabo′ et al., 1992, 1993). Since these Gram-positive bacteria do not have an outer membrane, there is no ambiguity in these cells as to the patched membrane. The plasma membrane of Gram-negative and Gram-positive bacteria perform similar physiological functions and share many similarities in their transport systems and bioenergetics apparatus. On this basis, it would be expected that any membrane component present in both types of bacteria would be located in the plasma membrane. The results reported here are fully consistent with this notion.

Bacterial MS channels have been implicated in osmoregulation (Berrier et al., 1992*b*). Bacteria have developed complex mechanisms to cope with variations in the osmolarity of their environment. When the osmolarity of the medium rises, bacteria respond by accumulating potassium and osmoprotectants synthesized in the cytoplasm or pumped from the external medium. Transport of potassium and osmoprotectants involves well-studied active transport systems of the plasma membrane whose activity is regulated by the membrane turgor (for reviews, *see* Csonka and Hanson, 1991; Booth, 1993; Bakker, 1993). When the bacteria are shifted to a medium of lower osmolarity, they release ions (principally potassium) and small molecules (metabolites and/or osmoprotectants) so as to reduce the membrane turgor (Epstein & Schultz, 1965; Tsapis & Kepes, 1977; Berrier et al., 1992*b*; Schleyer, Schmid & Bakker, 1993). Bacterial stretch-activated channels, activated by an increase in membrane turgor, may provide the exit pathway for these effluxes. This proposal is based on the observation that gadolinium, an inhibitor of bacterial and other MS channels, is able to block the release of metabolites and slow the exit of potassium, triggered by an osmotic down-shock (Berrier et al., 1992*b*). If such is the function, or one of the functions of these channels, it implies that they be located in the plasma membrane since the membrane turgor is felt at the level of the plasma membrane and is dictated by the cellular content, in particular by potassium and/or osmoprotectants.

Both the data obtained with a reconstituted inner membrane fraction and with the native membrane agree with the existence in *E. coli* of a whole array of stretch-activated, weakly selective, conductances. In protoplasts the channels are sometimes spontaneously active, and the effect of pressure is not always reversible, two phenomena which are not observed in proteoliposomes. This may be explained both by the existence of an offset tension and by a lack of elasticity of the membrane patch of protoplasts as compared to liposomes, possibly due to remains of the cell wall. There is little ambiguity as to the identity of the conductances observed in the two preparations. Three groups of conductances could be somewhat arbitrarily distinguished: relatively low conductances (100–150 pS) with slow kinetics, 300 and 500 pS conductances with intermediate kinetics, and higher

conductances (from 1,000 pS to 2,300 pS) with fast kinetics. Sukharev et al. (1993) recently reported two different channels, a 500 pS and a 1,500 pS channel (in 0.1 M KCl), recorded both on the native membranes and after solubilization and reconstitution in giant liposomes. The 500 pS channel, named MscS (S for small) is weakly anionic. The 1,500 pS channel named MscL (L for large), is a nonselective channel with fast kinetics, activated at higher applied suction than the smaller one. These channels clearly belong to two of the three groups identified here. However, the situation is still more complex. In what may be called the MscS and MscL subfamilies, different conductances are detected and the lower conductance channels (100–150 pS) with slow kinetics constitute a third subfamily which we shall refer to as MscM (M for mini) to be consistent with the above terminology. In whole cell recordings performed on protoplasts Cui et al. (1995) recently reported the existence of two types of channels corresponding to MscM and MscS, but MscL were not observed; this may be due to the fact that in this recording configuration, high pressure could not be applied.

In their study of MS channels in Gram-positive bacteria (*B. subtilis* and *S. faecalis*), Zoratti and coworkers have documented the existence in these organisms of a complex electrical activity, with a multiplicity of stretch-activated conductances ranging from 100 pS to 3 nS (in 0.35 M KCl) (Zoratti & Petronilli, 1988; Zoratti & Szabo′, 1991; Szabo′ et al., 1992, 1993). These authors favor an interpretation in which the larger stretch-activated conductances arise from the cooperative gating of a small number of lower conductance channels. This interpretation is in part based on the observation that upon repeated suction the larger conductances often run down into lower conductances with different kinetics (Szabo′ et al., 1992, 1993). Our results with *E. coli* proteoliposomes do not favor this hypothesis. While the electrical activity observed in protoplasts is sometimes complex, due to the simultaneous activity of different conductances, we generally observe only one type of conductance in a given patch of proteoliposomes. It thus appears that the dilution inherent in the reconstitution procedure separates the different channels, which segregate into clusters of identical conductances. In these patches, no change in the conductance was observed during recordings. Thus each of these conductances presumably corresponds to a stable ion channel protein. This proposal is reinforced by the finding that the 500 pS and 1,500 pS channels from *E. coli*, solubilized by Sukharev et al. (1993) correspond to separate molecular species since they can be separated by gel filtration. The MscL-1,500 pS has been purified and its corresponding gene has been cloned (Sukharev et al., 1994). In a strain in which this gene has been deleted, the 1,500 pS channel is no longer detected but the 500 pS channel is still present (Sukharev et al., 1994).

If each of the stretch-activated conductances that we observe corresponds to a different protein, it is however possible that the number of involved genes is much more reduced: the combination of a limited number of different subunits into oligomers might result in a large number of different MS channels, as documented for other ion channels. The *mscL* gene corresponds to a protein of 136 amino acid residues with a predicted relative molecular mass of 15,000, and has been shown to code alone for the 1,500 pS channel whose relative molecular mass was estimated to be 70,000 by gel filtration experiments (Sukharev et al., 1994). The 1,500 pS channel is thus probably a homomultimer. Different degree of multimerization and/or heteromultimerization might account for the variety of MS channels reported here.

The threshold of activation for each channel was variable between patches in proteoliposomes and spheroplasts but none of these systems truly represent the native structure in which the exoskeleton (i.e., the cell wall) controls and limits the membrane tension. Moreover, the relevant parameter is not the measured applied pressure but the membrane tension, related, by Laplace's law, to the pressure via the radius of curvature of the patch. The radius of curvature may vary from patch to patch and with the applied pressure (Sokabe & Sachs, 1990). For all these reasons, the absolute values of the thresholds of activation obtained in patch-clamp experiments probably have little significance. Nevertheless, the existence of these thresholds and the fact that, in a given patch, they increased with the conductance of the channels, might be significant, physiologically. A multiplicity of MS channels with different sizes and different thresholds of activation may be advantageous for a species confronted with a variable environment. It could correspond to the possibility of a graded response of the cells to the osmotic stress. However, preliminary experiments from our laboratory indicate that efflux of several osmoprotectants (glutamate, trehalose, glycine betaine) triggered by osmotic shock, are very similar in wild-type and *mscL*$^-$ strains. It is possible that the multiplicity of MS channels reflects a redundancy corresponding to the importance of their function.

Given their conductances and relative lack of ion selectivity, the opening of these channels certainly results in the collapse of the proton-motive force. However, under physiological circumstances, this opening may be very brief: less than two seconds is necessary to empty a cell of its potassium content (Berrier et al., 1992*b*). Upon restoration of a normal turgor and closing of the channels, repolarization of the membrane by high-capacity proton pumps can be very fast. Thus, the presence of these high conductance channels in the plasma

membrane is not incompatible with the maintenance of a proton-motive force, because these channels are tightly gated, and presumably open under relatively rare circumstances corresponding to osmotic stress.

In conclusion, we have described a set of poorly selective MS channels from the plasma membrane of *E. coli*, which may be part of a multicomponent osmoregulatory system. Although there are reports of other cells possessing more than one type of stretch-activated channels (Davidson, Tatakis & Auerbach, 1990; Ruknudin, Sachs & Bustamante, 1993), it appears that the high multiplicity of these channels together with their large conductances are characteristics of bacterial mechanosensitive channels.

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