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Activities of a Mechanosensitive Ion Channel in an *E. coli* Mutant Lacking the Major Lipoprotein

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Summary. The activity of the mechanosensitive (MS) ion channels in membrane patches, excised from *E. coli* spheroplasts, was analyzed using the patch-clamp technique. Outer membranes from a mutant lacking the major lipoprotein (Lpp) and its wild-type parent were examined. The MS-channel activities in the wild-type membrane rarely revealed substates at the time resolution used. These channels showed a stretch sensitivity indicated by the $1/S_p$ (the suction for an *e*-fold increase in channel open probability) of 4.9 mm Hg suction. The MS-channel activities of *lpp* included a prominent substate and showed a weaker mechanosensitivity with an $1/S_p$ of 10.0 mm Hg. Whereas small amphipaths (chlorpromazine, trinitrophenol) or a larger amphipath (lysolecithin) all activated the MS channel in the wild-type membrane under minimal suction, only the larger lysolecithin could activate the MS channel in the *lpp* membranes. After lysolecithin addition, the *lpp* membrane became more effective in transmitting the stretch force to the MS channel, as indicated by a steepening of the Boltzmann curve. We discuss one interpretation of these results, in which the major lipoprotein serves as a natural amphipath inserted in the inner monolayer and the loss of this natural amphipath makes the bilayer less able to transmit the gating force.

Key Words mechanosensitive channel · outer membrane *Escherichia coli* · lipoprotein · membrane protein

Introduction

Since the original discovery of mechanosensitive (MS) channels in muscle membranes (Brehm, Kullberg & Moody-Corbett, 1984; Guharay & Sachs 1984), they have been discovered in some thirty different kinds of cells (Kullberg, 1987; Sachs, 1988; Morris, 1990; Martinac, 1992). Interestingly, MS channels have also been found in microbial organisms including the budding yeast (Gustin et al., 1988), the fission yeast (Zhou & Kung, 1992), the bean-rust fungus (Zhou et al., 1991), the gram-positive bacteria (Zoratti & Petronilli, 1988; Martinac et al., 1992) and the gram-negative bacterium *Escherichia coli* (Martinac et al., 1987).

We have used giant spheroplasts (Long, Slayman & Low, 1978; Ruthe & Adler, 1985) to characterize the MS channel of *E. coli*. In 200-mM KCl solutions, it has a conductance of about 1 nS. It has a slight preference for anions. Its kinetics is generally slow and seems to vary depending on the species of permeant ion. Although this channel is also voltage gated, its most striking characteristic is that its open probability clearly increases with suction or pressure applied through the patch pipette in a manner predicted by the Boltzmann equation, in which the mechanical free energy partitions the channel between open and closed state (Martinac et al., 1987).

It is now generally accepted that MS channels respond to a tension parallel to the plane of the membrane instead of a pressure perpendicular to it. However, how the stretch force is transmitted to the MS channel is still being debated. Some believe that the force is transmitted through the cytoskeleton (Sachs, 1988; Sokabe, Sachs & Jing, 1991). Others believe that it is transmitted through the lipid bilayer (Martinac, Adler & Kung, 1990). The *E. coli* MS channel remains active after bacterial membrane fractions are reconstituted into artificial bilayer in low protein-to-lipid ratios (Delcour et al., 1989). Recent studies showed that such channels even survive solubilization by a detergent followed by dialysis and reconstitution (Sukharev, Martinac & Kung, 1992). In these studies, the chances should be remote in having peptidoglycan, the *E. coli* equivalent of cytoskeleton, being in continuous association with the MS channel throughout these treatments. In a different line of investigation, the MS channel of *E. coli* was found to be activated by a variety of amphipaths, and the time course and effectiveness of channel activation parallel the solubility of these amphipaths into lipids (Martinac, Adler & Kung, 1990). The results were in agreement with the bilayer

couple hypothesis (Sheetz & Singer, 1974). This hypothesis states that the insertion of amphipaths differentially into the two monolayers of biological membranes, which are naturally asymmetric in charge distribution, should cause the bilayer to buckle. The results of Martinac, Adler and Kung (1990) are consistent with the MS channel being a molecular reporter of the mechanical stress in the buckled bilayer. The forces in the bilayer that gate the MS channel resulting from the relative but simultaneous expansion and compression of the two monolayers are treated in a theoretical model by Markin and Martinac (1991).

One way to study the molecular mechanism of mechanosensitivity in the future would be to purify the MS-channel protein and study it in vitro, using bilayers of a single species of phospholipids. This should establish the minimal requirements and the fundamental behavior of this MS-channel protein. Indeed, alamethicin has been shown to function as an MS channel in pure bilayer patches, apparently by forming multi-subunit complexes (Opsahl, Mak & Webb, 1990). However, a bilayer of pure phospholipids is not a natural membrane. From the view point of cell physiology, it is of great interest to examine the natural environment, in which the MS channel functions. An extreme example of environmental effect on MS channel was described by Franco and Lansman (1990) in dystrophic mice. Stretch-activated channels existing in normal myotubes were found to function as stretch-inactivated channels in myotubes of dystrophic mice. Although artificial lipid bilayer can transmit the gating force (Delcour et al., 1989; Sukharev, Martinac & Kung, 1992), the natural membrane-cytoskeletal complex may have adjusted its mechanical properties such that the MS channel can respond to forces that are physiologically relevant. This notion can be tested using the MS channel as a reporter of the membrane's mechanical properties, and, with *E. coli*, using mutations to change or delete components that might be mechanically important (Kubalski et al., 1991).

The MS channel studied here is believed to reside in the *E. coli* outer membrane (*see* Discussion). This membrane is unusual in several respects. It is highly asymmetric, having the highly negatively charged lipopolysaccharides exclusively in the outer monolayer and conventional phospholipids almost exclusively in the inner monolayer. The most abundant protein embedded in this bilayer is the major lipoprotein (Braun, 1975). There are some 7×10^7 copies of this 7,200-MW protein per *E. coli* cell. A third of them are covalently linked to the peptidoglycan layer forming most of the anchors of the outer membrane onto the cell wall. Their N-termini are bound to fatty acids. They are positioned in the inner monolayer and do not protrude into the outer monolayer (Mizushima, 1985; Raetz, 1986). In this study, we monitor MS-channel activity in response to changes in the mechanical properties of the *E. coli* outer membrane caused by a deletion of its major lipoprotein in the *lpp* mutant (Sonntag et al., 1978).

We also present some results from studies of a *lpp ompA* double mutant, which, besides lacking the major lipoprotein, also lacks the OmpA protein (Sonntag et al., 1978). OmpA is a 35,000-MW protein that spans both monolayers, extends into the periplasm and covalently links to the peptidoglycan (Mizushima, 1985; Nakae, 1986).

Materials and Methods

Bacterial Strains

An *E. coli* K12 strain (*aroD*), referred as wild type hereafter, was the parent of strain JE5505, *lpp*, lacking the major lapoprotein (Hirota et al., 1977). The double mutant lacking both the major lipoprotein and the outer-membrane protein A, *lpp ompA*, was derived from JE5505 (Sonntag et al., 1978). All strains were kindly provided by U. Henning.

Preparation of Giant Spheroplasts

E. coli giant spheroplasts were prepared as described previously (Long, Slayman & Low, 1978; Ruthe & Adler, 1985; Martinac et al., 1987). Briefly, cells were grown in modified Luria-Bertani medium (1% Bacto-Tryptone, 0.5% yeast extract and 0.5% NaCl) in the presence of cephalexin, a penicillin analogue, to become filaments 50–150 μm long. These filaments were treated with Tris-EDTA and lysozyme in the presence of 0.8 M sucrose to generate giant spheroplasts (6 μm average diameter). Spheroplasts in aliquots were stored at −20°C.

Patch-Clamp Experimental Protocols, Recordings and Data Analysis

The methods of single channel recordings were essentially as published (Martinac et al., 1987). Briefly, aliquots of frozen spheroplasts were thawed at room temperature before use. All experiments were done at room temperature using a List Medical EPC7 patch-clamp amplifier. Single channel recordings were obtained from inside-out excised patches which were formed by brief air exposure of the tip of the pipette. Bath solution was, in mM, 250 KCl, 90 $MgCl_2$, 10 $CaCl_2$, 5 HEPES, pH 7.2. Pipette solution was 200 KCl, 40 $MgCl_2$, 10 $CaCl_2$, 5 HEPES, pH 7.2. Unitary conductances were calculated for symmetric K^+ conditions. Mouth suction applied to patch pipettes was monitored by a pressure transducer (differential type, ±5 psi, Omega Engineering, Stamford, CT) calibrated with a mercury manometer. Single channel data were stored on tape (PCM-1-video tape system, Medical System, Greenvale, NY), digitized and analyzed off-line on a PDP-11 computer (Indec System, Sunnyvale, CA)

with a program developed by Dr. Y. Saimi. The open probability, P_O was calculated by integrating the total current passing through all active channels during the recording time and dividing this integral by the number of active channels and the single channel current. Since subconductances occurred in mutants, the open probability was also calculated using amplitude histogram according to the formula:

$$NP_O = (n_o^1 + 2n_o^2 + \cdots Nn_o^N)/(n_c + n_o^1 + n_o^2 + \cdots n_o^N)$$

where $n_o^1 \cdots n_o^N$ are numbers of open events at each conductance level; n_c is the number of events at the closed level, and N is the number of conductances. Data points were plotted and fitted to single exponential functions using Lotus 1-2-3 program (Lotus, Cambridge, MA).

In the experiments reported here, each patch was first verified to contain at least one active MS channel by applied suction. We then established the minimal suction at which we could reliably observe channel activity. This suction varied from patch to patch, presumably reflecting the differences in patch geometry and residual biological material attached to the patch. Since it varied from 0 to 60 mm Hg, it is not meaningful to compare this suction between wild type and mutants.

After the minimal suction was first established, suction was then increased and channel activities recorded for 2 min. Afterward, the suction was released for about 2 min before the next test suction was applied. At the end of the experiment, activities at the minimal suction and at least one other suction were again recorded for comparison. Should the activities at the final check differ more than 10% from the activities in the body of the experiment, the complete data set would be discarded, since changes in patch geometry were indicated. Suction was not sustained over the 10-min intervals. Because of the slow rate of amphipath insertion into the membrane, these experiments require observation over 1 hr. Only about 30% of the patches remained intact and stable throughout the various manipulations over this long duration. Only the results of these experiments are shown here. Results from fragmentary experiments are nonetheless entirely consistent with the experiments shown.

Significance of differences in the results was determined with t-test at $P < 0.05$.

Outer-Membrane Preparation and Gel Electrophoresis

E. coli membranes were separated essentially as described (Delcour et al., 1989). Briefly, cells of each strain were grown at 35°C in tryptone broth (0.5% NaCl and 1% tryptone, Difco, St. Louis, MO) to an OD_{590} of 0.5–0.6. After centrifugation, cells were resuspended in 50 mM potassium phosphate buffer (pH 6.6) containing 5 mM $MgSO_4$ and 1 mM dithiothreitol. Membrane vesicles were obtained by passing the cells through a French press (American Instrument, Silver Spring, MD) twice at 16,000 lb/in^2 (Smit, Kamio & Nikaido, 1975). Inner- and outer-membrane fractions were separated through sucrose gradient centrifugation (Osborn et al., 1972). All membrane fractions were stored at −80°C.

Outer membrane proteins of different strains from thawed samples were separated by SDS-polyacrylamide electrophoresis (Laemmli, 1970) except that the 9% separation gel contained 8 M urea. Urea was removed from the gel before staining with Coomassie brilliant blue by incubating overnight at room temperature in 5% methanol and 7.5% acetic acid. Each lane was loaded with 30 μg of proteins. Protein concentration of the samples was determined by the method of Smith et al. (1985) using the Pierce micro-protein BSA assay reagent (Pierce Chemical, Rockford, IL).

All chemicals were of reagent grades. Amphipaths were purchased from Sigma (St. Louis, MO).

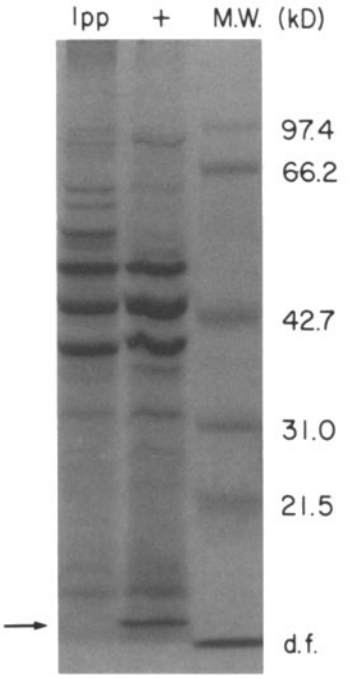

Fig. 1. Electrophoretic pattern of outer-membrane proteins of the *lpp* mutant (left lane), and that of its wild-type parent (center lane). Right lane shows standards with molecular weights marked. *d.f.* is the dye front. The molecular weights of various bands of *E. coli* proteins can only be estimated because of the presence of 8 M urea in the samples. Arrow points to the major lipoprotein present in the wild type but absent in the *lpp* mutant.

Results

Outer-Membrane Proteins of the *lpp* Mutant

Since the major lipoprotein is inserted into the inner monolayer of the outer membrane and forms an important link to the peptidoglycan, we wished to examine its contribution to the mechanical properties of the outer membrane. This was achieved by comparing the activities of the mechanosensitive (MS) channel in the outer membrane of the *lpp* mutant *vs.* those of its wild-type parent. To confirm that this mutant lacked lipoprotein, fractions highly enriched with outer membranes from each strain were obtained and examined by SDS polyacrylamide gel electrophoresis. Figure 1 shows that a band at ap-

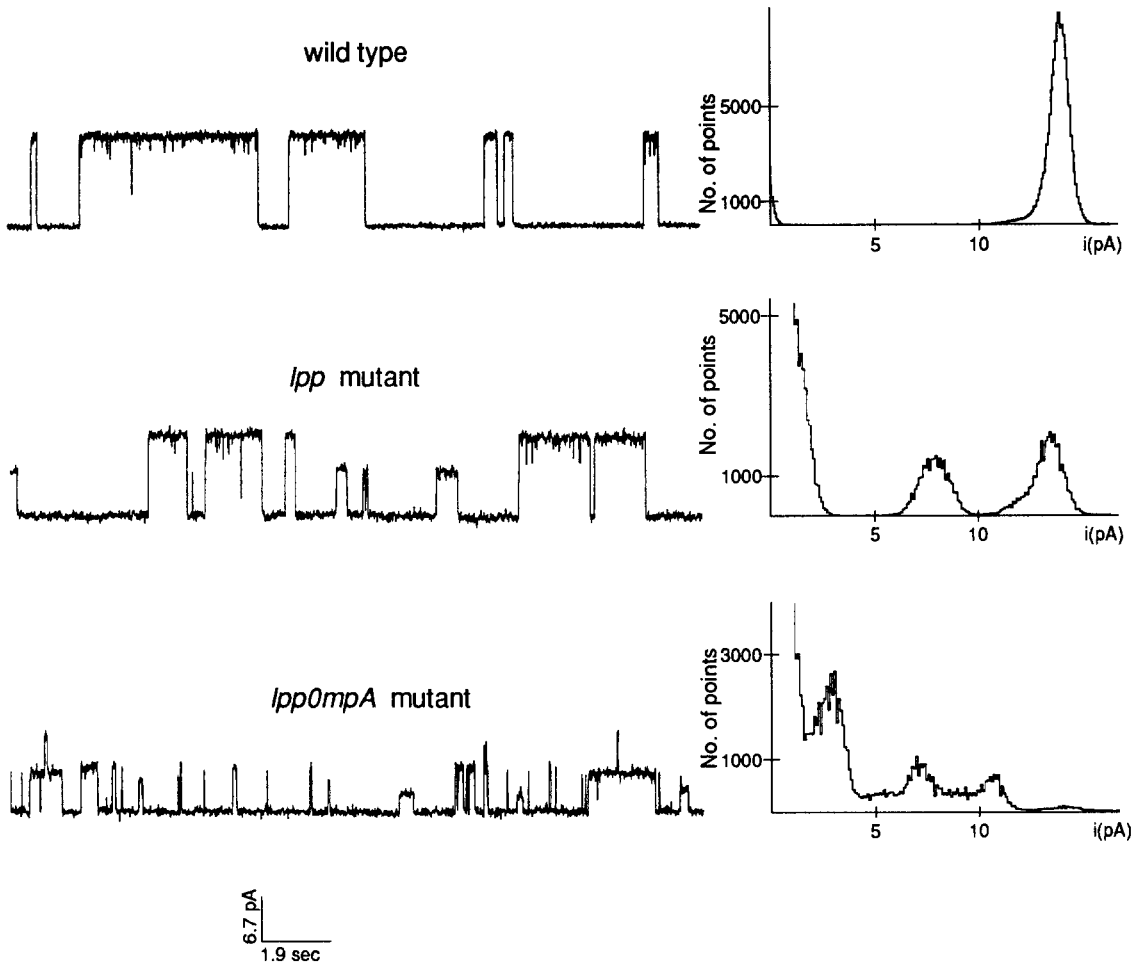

Fig. 2. Activities of the mechanosensitive channels in the wild-type parent (top), *lpp* mutant (middle), and *lpp ompA* double mutant (bottom). All recordings were at pipette voltage of +10 mV and applied suction of 40 mm Hg. Left half of the figure shows single MS-channel activities. Right half of the figure shows amplitude histograms. It is apparent that the wild-type MS channel shows one predominant conductance; the *lpp* mutant shows two conductance states; the *lpp ompA* double mutant shows four such states.

proximately 7,000 MW, prominent in the wild-type parent was missing in the mutant (arrow). The differences in other bands in the low molecular-weight region (below 24.5 kD), where the bands of other lipoproteins are expected, were minor and were within the variation one encounters from different extracts of the same strains. The major membrane proteins migrated between 30 and 50 kD. These included the various porins and OmpA. There was no difference in banding positions or densities between *lpp* and its parent, with respect to these proteins, which constitute the bulk of the outer membrane. In the region above 50 kD, however, several bands appear darker in *lpp* than in its wild-type parent, reflecting their overexpression (Sonntag et al., 1978). These appear to be high molecular-weight transport proteins that are expected to span both monolayers of the outer membrane (*see* Discussion).

Activities of Single MS Channels in the *lpp* Membrane *vs.* Those in the Wild-Type Membrane

As reported previously (Martinac et al., 1987), the activities of MS channels were readily encountered in patches of spheroplast membranes. The present study includes observations on activities of these channels in 15 patches of wild-type membranes and 13 patches of *lpp* membranes.

The wild-type MS channel has a unit conductance of approximately 1 nS (Fig. 2, top, left) and is occasionally opened at substates. However, as shown by the amplitude histogram, the occurrence of open substates was so rare that it escaped detection in this histogram (Fig. 2, top, right). The activities of the MS channels in the *lpp* membrane were quite different. In addition to unit open state, the

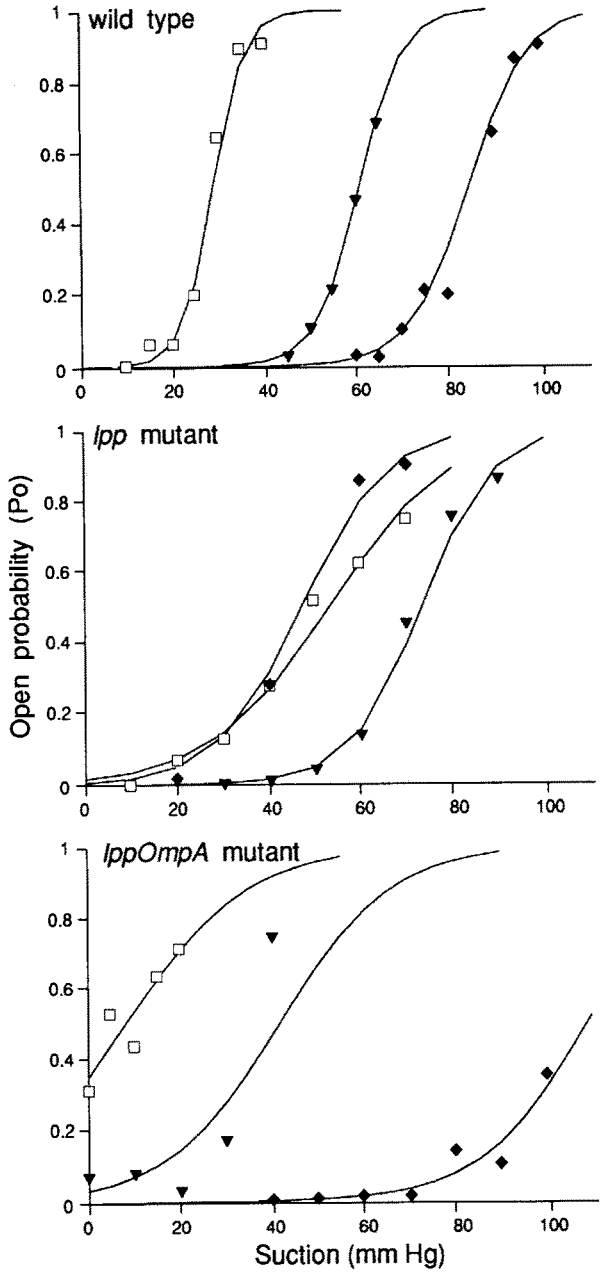

Fig. 3. The mechanosensitivity of the MS channels in different strains. Open probability of the MS channels is plotted against applied suction and the data are fitted with Boltzmann curves (*see text*). Each Boltzmann curve represents a single experiment from an individual patch. The curves for the wild type (squares) are much steeper than those of the *lpp* (middle) or the *lpp ompA* mutant (bottom), indicating the ineffectiveness of the mutant membranes in transmitting the stretch force. $1/S_p$ values are as follows. Top, wild-type curves, 3.4 mm Hg (squares), 4.8 mm Hg (triangles), and 6.4 mm Hg (diamonds); middle, *lpp* mutant curves, 13.1 mm Hg (squares), 9.3 mm Hg (diamonds) 7.9 mm Hg (triangles); bottom, *lpp ompA* mutant curves, 13.3 mm Hg (squares), 9.1 mm Hg (triangles) and 11.6 mm Hg (diamonds). The origins of these curves are the minimal suctions to obtain channel activity. The differences in the minimal suctions in different patches most likely reflect the geometry of the patches and not the property of the channels themselves.

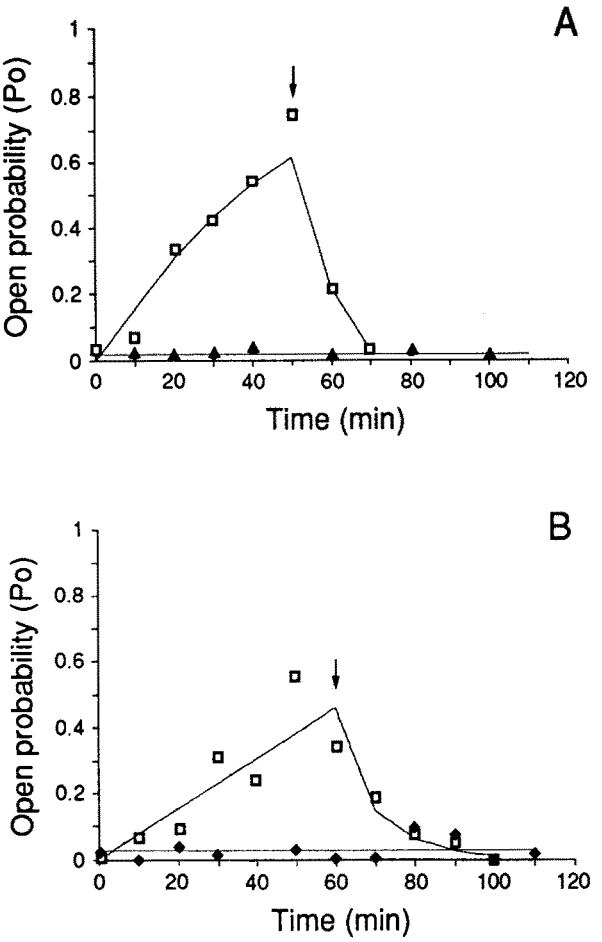

Fig. 4. The abilities of two smaller exogenous amphipaths, chlorpromazine, and trinitrophenol, in the activation of the MS channels in different strains. Pipette voltage was +10 mV. (*A*) 20 μM chlorpromazine (CPZ) activated the MS channel from the wild type (squares), but was ineffective in activating the channels in the *lpp* mutant (filled triangles). Arrow indicates the start of CPZ washout. The minimal suctions were 40 mm Hg for both the wild-type and the *lpp* patch. (*B*) 0.5 mM trinitrophenol (TNP) activated the wild-type MS channel but did not activate the *lpp* mutant MS channel. Arrow indicates the start of TNP washout. The minimal suctions were 25 mm Hg and 20 mm Hg for the wild-type and the *lpp* patch, respectively.

channel dwelled in a substate of about 500 pS for about half the total open time (Fig. 2, middle).

The Force-Transmitting Effectiveness of *lpp* Membrane

Although the dwell times at different open states are different in the two strains, we believe that the same MS-channel proteins underlie the activities described here (*see* Discussion). Thus, we were able to use these MS channels as sensors to report on the stretch forces applied to the membrane. MS-channel

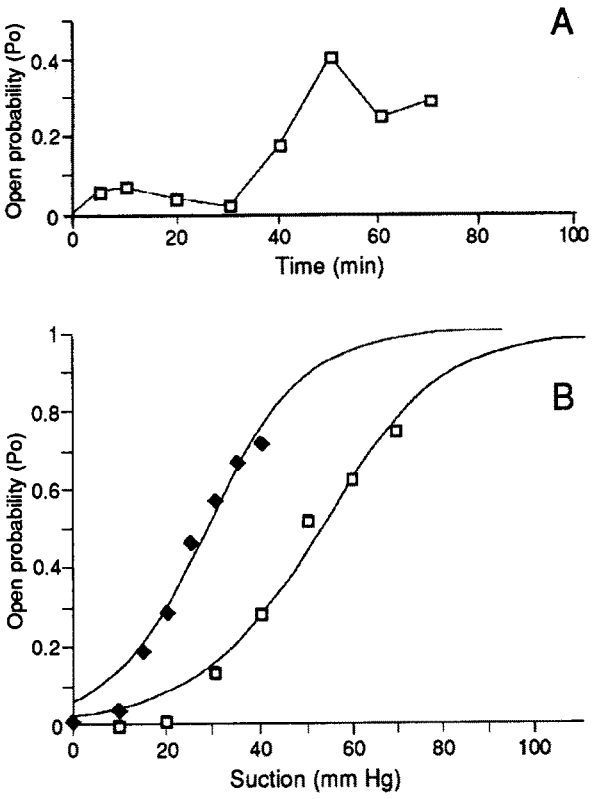

Fig. 5. The ability of a larger exogenous amphipath, lysolecithin, in the activation of the MS channels in different strains. (*A*) Activation of the *lpp* mutant MS channel by 1 mM lysolecithin within one hour of recording. Pipette voltage was +10 mV. The minimal suction was 20 mm Hg. (*B*) Boltzmann curves of the *lpp* mutant from the experiment shown in *A*, before (squares) and 60 min after the application of 1 mM of lysolecithin (filled diamonds).

open probability, P_o, increased with the suction applied through the patch-clamp pipette. As described in Martinac et al. (1987), the results can be fitted with a Boltzmann distribution

$$P_o = \{\exp[(p - p_{1/2})/S_p]\}/\{1 + \exp[(p - p_{1/2})/S_p]\}$$

in which p is the suction, $p_{1/2}$ is the suction at which the channel is open half of the time, $1/S_p$ is the slope of the plot of $\ln[P_o/(1 - P_o)]$ *vs.* suction. For the wild-type membrane, increasing the suction by 4.9 ± 1.1 mm Hg ($n = 7$) increases the open probability by e-fold. These results are almost identical to those reported previously on a different wild-type strain (Martinac, Adler & Kung, 1990). The *lpp* membrane was clearly much less effective in transmitting the stretch force to the MS channel. The average $1/S_p$ of the channel in this membrane was 10.0 ± 2.7 mm Hg ($n = 6$), and it was significantly larger than the $1/S_p$ for the wild type ($P < 0.05$). Results from three different patches of wild-type membrane and three different patches of *lpp*-mutant membrane are shown in Fig. 3, top and middle, respectively.

The Effects of Small Amphipaths on the MS-Channel Activities in Different Membranes

Addition of amphipathic molecules in the bath gradually increased the open probability of the MS channels in *E. coli* membrane patch at a minimal test suction (Martinac, Adler & Kung, 1990). This increase, which was reversible, occurred over tens of minutes, consistent with their slow partitioning into the lipid bilayer. In six different patches, 20 μM chlorpromazine activated the MS channels in the wild-type membrane. Figure 4*A* (squares) shows one such experiment, in which 20 μM chlorpromazine activated the MS channel over 50 min. Similarly, in four patches, 500 μM trinitrophenol also slowly activated them (Fig. 4*B*, squares). In contrast, these amphipaths at these concentrations were ineffective in activating the MS channel in the *lpp* membrane (Fig. 4*A* filled triangles (*B*) filled diamonds). For each amphipath, four *lpp* patches were tested, each was verified to contain MS channels by application of suction. Even a higher concentration of chlorpromazine (50 μM) did not activate the MS channel in the mutant (*not shown*).

The Effect of a Large Amphipath, Lysolecithin, on the MS-Channel Activities in *lpp* Membrane

Lysolecithin (1 mM; molecular weight approx. 780), like chlorpromazine (MW 355) and trinitrophenol (MW 229), activated the MS channel in the wild type without affecting the $1/S_p$ significantly (*data not shown*, Martinac, Adler & Kung, 1990). The same concentration of lysolecithin also activated the MS channels in the *lpp* mutant (Fig. 5*A*). Furthermore, after lysolecithin was added, *lpp* membrane apparently transmitted stretch force more effectively, since the $1/S_p$ decreased. In the case shown in Fig. 5*B*, for example, the $1/S_p$ decreased from 13.5 mm Hg to 10.1 mm Hg per e-fold change in P_o. This effect was reversible; after lysolecithin was washed out, $1/S_p$ was 12.6 mm Hg. Similar results were obtained from three other *lpp* patches—$1/S_p$ changed from 9.1, 7.1, and 7.9 mm Hg in the control to 7.7, 6.3, and 3.2 mm Hg, respectively, in the presence of lysolecithin.

The MS Channel in a *lpp ompA* Double Mutant

The MS channel in the outer membrane lacking both the lipoprotein and the outer-membrane protein A

was also investigated. Six patches of *lpp ompA* membranes showed that, although the channel still visited the unit open state of about 1 nS, it did so only rarely. (Note a small but significant peak in the amplitude histogram at the far right of Fig. 2, bottom.) Besides a substate of approx. 500 pS that was evident in the *lpp* single mutant, there were at least two other substates at 750-pS and 200-pS conductance (Fig. 2, bottom). The channel dwelled mostly in 200-pS substate (right). The effectiveness of the *lpp ompA* membrane in transmitting the stretch force to the MS channel, however, did not differ significantly from that of the *lpp* membrane (Fig. 3, bottom) and had a $1/S_p$ value of 11.5 ± 1.8 (n = 4).

Discussion

The MS-Channel of *E. coli*

The location of the MS channel studied here is still being debated. Fractions enriched in outer or inner membrane were reconstituted into liposomes. Patch-clamp examination revealed MS-channel activities in both types of liposomes. In one study, far more activities were encountered in liposomes with fractions enriched with inner membrane than ones with the outer membrane (Berrier et al., 1989). These results were taken to mean that this MS channel is located in both membranes of *E. coli*, but mostly the inner one (Berrier et al., 1989). This interpretation relies on a judgment on the percentages of MS channel containing patches from each type of reconstituted liposomes *vs.* the known percentages of membrane cross contamination in the enriched fractions. A similar study by different investigators showed the MS-channel activity to be mostly in the outer-membrane fraction and not the inner-membrane fraction (Martinac et al., *unpublished result*).

Several lines of evidence showed that this MS channel is located in the outer membrane instead of the inner membrane of *E. coli*. First, giant spheroplasts had an outer membrane as seen in electron microscopy and immunofluorescent microscopy (Ruthe & Adler, 1985; Buechner, 1990). The pipette that forms a seal with the spheroplast is not likely to be able to pass through the outer and still seal onto the inner membrane. Second, MS channels in excised inside-out patches became more active upon digestion with lysozyme added to the bath (Buechner et al., 1990; Martinac et al., 1992). The lysozyme substrate, i.e., the peptidoglycan that restrains the channel-containing membrane from stretching, should therefore face the bath. Should the seal be formed on the inner membrane, the peptidoglycan should have been facing the pipette interior and not been accessible to lysozyme. Third, the present study showed that deleting outer-membrane components (lipoprotein and OmpA protein) profoundly affects the activities of the MS channel.

Although the major lipoprotein is missing in the *lpp* mutant (Sonntag et al., 1978) (Fig. 1), the mutant membrane still showed the activities of the MS channel, although in abnormal forms. We believe that the abnormal behavior reflects aberration in the mechanical properties of the mutant membranes (*see below*). Lipoprotein and the OmpA proteins are major membrane proteins and form the major covalent links between outer membrane and the peptidoglycan; *lpp ompA* double mutants tend to round up into large spheres. These spheres were observed to shed some of their outer membranes (Sonntag et al., 1978). These morphological changes suggest that the mechanical properties of the cell envelope are indeed altered in the mutants (Lugtenberg & van Alphen, 1983).

Mechanical Properties of the Outer Membranes

The outer membrane is normally linked covalently to a rigid peptidoglycan layer. Digesting this layer with lysozyme (Buechner et al., 1990; Martinac et al., 1992) shifts the Boltzmann curve of MS-channel mechanosensitivity to the left by 20 mm Hg, suggesting that peptidoglycan normally resists stretch. The shape of the Boltzmann curve remains unchanged by digestion, however, indicating no change in the channel or the element that transmits the stretch force. The simplest interpretation of these results is that stretch is transmitted through the outer-membrane bilayer and this force is normally resisted by the peptidoglycan.

Lipoprotein is one of the major links between the outer membrane and the peptidoglycan. We were unable to determine whether the Boltzmann curve of the *lpp* mutant has been shifted and in which direction on the pressure axis. The inflection point of this curve ranges over 60 mm Hg from patch to patch. Thus, it would not be valid to compare the positioning of the Boltzmann curves of membrane patches of the wild type and those of the mutant. On the other hand, the shape of this curve varies little. The $1/S_p$ ranges within 1.1 mm Hg in the patches from the wild type and within 2.7 mm Hg in the patches from the *lpp* mutant. We take this to mean that the force-transmitting outer membrane differs little among patches from the same bacterial strain. The Boltzmann curves of *lpp* are clearly much shallower than the wild-type curves (Fig. 3). The lack

of lipoprotein, therefore, seems to make the outer membrane less effective in transmitting the gating force to the MS channel. According to one model, MS channel embedded in a bilayer responds to the relative but simultaneous expansion and compression of the two monolayers (Martinac, Adler & Kung, 1990; Markin & Martinac, 1991). Therefore, one interpretation of our results could be that the inner monolayer of the *lpp* outer membrane is less densely packed, due to the lack of lipoproteins, and the reduced material density of the inner monolayer makes it more able to absorb compression and less able to transmit the compression to the MS-channel protein. This interpretation is consistent with the observation that small amphipaths, at concentrations known to activate the MS channel in the wild type membrane, were not able to activate the channel in the *lpp* membrane (Fig. 4). Although the small amphipaths were unable to compensate for the loss of material density in the inner monolayer, lysolecithin, a sizable phosphoglyceride, applied at a high concentration (1 mM), could restore some of the lost ability to transmit the necessary force to activate the MS channel without further suction (Fig. 5*A*) and to steepen the Boltzmann curve (Fig. 5*B*). Moreover, small amphipaths became effective when lysolecithin was inserted into the membrane. In one experiment, the MS channel in a *lpp* patch had an $1/S_p$ of 13.5 that decreased to 10.1 after the application of lysolecithin (Fig. 5). Additional application of trinitrophenol to this membrane now activated the MS channel at minimal suction and yielded an $1/S_p$ of 4.2 (Kubalski, *unpublished results*). After washout of both trinitrophenol and lysolecithin, the $1/S_p$ was 12.6 mm Hg, close to the starting value.

One way to envision the outer membrane is to view the lipoproteins as a "natural amphipath" that inserts only into the inner monolayer of the outer membrane, as diagrammed in Fig. 6, top row. In its absence, the necessary compressive force (Fig. 6, arrows, top right) cannot be generated sufficiently to gate the channel (middle row). The situation can be remedied by the addition of an exogenous amphipath, preferably a large one at a high concentration, such as 1 mM lysolecithin (bottom row). If our interpretation of the data is valid, then it is interesting that *E. coli* does not seem to have a compensatory mechanism to counteract the loss of a natural amphipath. Although several high molecular-weight proteins seem to be overproduced in the *lpp* mutant (Fig. 1), they apparently had no effect or effect was insufficient to remedy the loss of force-transmitting effectiveness. According to Markin and Martinac (1991), the MS channel is gated by relative and simultaneous expansive and compressive forces in the two monolayers. The expansive force is not diagrammed in Fig. 6 for simplicity's sake. It is interesting that the further loss of the OmpA protein in the *lpp ompA* double mutant did not appear to change the membrane's ability to transmit the gating force by much, since the $1/S_p$ was not different between *lpp* and *lpp ompA* mutants. This is in agreement of the general idea stated above. The OmpA protein spans both monolayers and therefore does not act like an amphipath that inserts only into one monolayer. This observation of OmpA might be extended to other proteins that span both monolayers, including the large molecular-weight proteins overexpressed in the *lpp* mutant. Their presence or absence might not alter significantly the relative mechanical properties of the two monolayers.

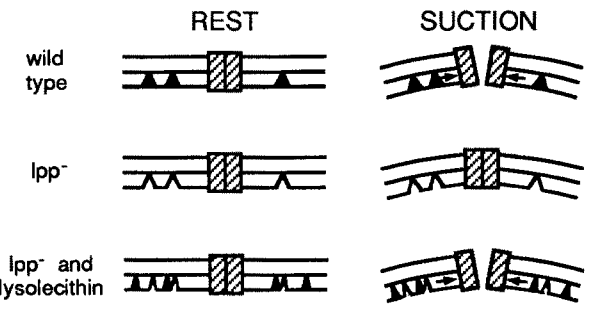

Fig. 6. A diagram illustrating the difference between the membranes of the wild type and the *lpp* mutant in their capabilities to transmit mechanical force to the MS channel. MS channel opens when a sufficient suction is applied to the wild-type membrane (top), because this membrane is efficient in transmitting the gating force (arrows). The same amount of suction does not activate the MS channel in a membrane lacking the major lipoprotein, a natural amphipath (middle), because the inner monolayer is more compressible than that of the wild type. Addition of lysolecithin, an exogenous amphipath, helps to restore partially the ability of the *lpp*-mutant membrane to activate its MS channel at the given applied suction (bottom).

The Substate Behavior of the MS Channel

That even the *lpp ompA* double mutant showed a suction-induced unit open state with a full conductance comparable to that of the wild type suggests that we are dealing with the activities of the same molecular entities. The prominent occurrence of channel substates in membrane patches of single or double mutant appears to reflect the mechanical properties of the membrane/cell-wall complex. For example, the loss of tight connections between the outer membrane and the cell wall in these mutants may allow the channels to exhibit these substates. Substate behavior has also been observed in excised inside-out membrane patches from wild-type spheroplasts treated with lysozyme from the outside (the

bath side) (Buechner et al., 1990; Martinac et al., 1992). The loss of material from the outer membrane itself, may also contribute to the origin of these substates. Channels in wild-type membranes excised from giant spheroplasts, without further lysozyme digestion, also exhibit substates, although much less frequently than in *lpp ompA* (Martinac et al., 1987, 1992). One way to envision the kinetic and substate behavior of these channels is that the channel protein naturally exists in different open conformations corresponding to the various substates and the full open state, although its normal dwell times in the substates are very short and usually beyond the resolution of our recording system. The mechanical properties of the outer-membrane/cell-wall complex do not change these conformations but bias the times in which the channels dwell in these various states. In any event, a satisfactory explanation for these complex substate and kinetic behaviors shall await the resolution of the molecular structure of the MS-channel protein itself.

Conclusion

This work is a study of the mechanical properties of the outer membrane of *E. coli*, using the MS channel as a reporter of bilayer expansion, and is also a study of the MS channel itself, on how it behaves in abnormal environments. We made use of what the *E. coli* system has to offer, i.e., we used mutants whose membrane parts have been deleted. As a study of the membrane and the MS channel *in situ*, it goes in parallel with our effort to isolate the MS channel and examine its behavior in artificial lipid bilayer. As expected, the natural membrane is complex and the behavior of the MS channel in this membrane reflects this complexity, parts of which cannot be explained given our current knowledge. Our observations led us to the following view.

(i) Lipoprotein behaves, in some respect, like an amphipath inserted into the inner monolayer. It forms a significant part of the material needed to transmit the gating force.

(ii) The loss of the major lipoprotein in mutants is apparently not sufficiently compensated for, in terms of its role as the natural amphipath.

(iii) This loss can be remedied by an addition of large exogenous amphipaths in high concentrations. The total compactness of the inner monolayer, and not necessarily the kinds of material that contribute to this compactness, determines the membrane's ability to transmit the gating force.

(iv) The MS channel exhibits different open states with different conductances. The dwell times in these different states appear to be related also to the mechanical properties of the membrane/cell-wall complex.

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