Two Types of Mechanosensitive Channels in the *Escherichia coli* Cell Envelope: Solubilization and Functional Reconstitution

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ABSTRACT Mechanosensitive ion channels (MSCs) which could provide for fast osmoregulatory responses in bacteria, remain unidentified as molecular entities. MSCs from *Escherichia coli* (strain AW740) were examined using the patch-clamp technique, either (*a*) in giant spheroplasts, (*b*) after reconstitution by fusing native membrane vesicles with asolectin liposomes, or (*c*) by reassembly of octylglucoside-solubilized membrane extract into asolectin liposomes. MSC activities were similar in all three preparations, consisting of a large nonselective MSC of 3-nS conductance (in 200 mM KCl) that was activated by high negative pressures, and a small weakly anion-selective MSC of 1 nS activated by lower negative pressures. Both channels appeared more sensitive to suction in liposomes than in spheroplasts. After gel filtration of the solubilized membrane extract and reconstituting the fractions, both large MSC and small MSC activities were retrieved in liposomes. The positions of the peaks of channel activity in the column eluate, assayed by patch sampling of individual fractions reconstituted in liposomes, showed an apparent molecular mass under nondenaturing conditions of about 60–80 kDa for the large and 200–400 kDa for the small MSC. We conclude that (*a*) the large MSC and the small MSC are distinct molecular entities, (*b*) the fact that both MSCs were functional in liposomes following chromatography strongly suggests that these channels are gated by tension transduced via lipid bilayer, and (*c*) chromatographic fractionation of detergent-solubilized membrane proteins with subsequent patch sampling of reconstituted fractions can be used to identify and isolate these MS channel proteins.

INTRODUCTION

Mechanosensitive channels (MSCs) are found in a variety of organisms, from animals and plants, to fungi and bacteria (1–3). Their role in specialized cells, like hair cells, has already been established (4). There is also increasing evidence that they play roles in the responses of nonspecialized cells to stretch, contraction, or osmolarity changes (5, 6).

Osmotic force is a major mechanical force acting on membranes of plants, fungi, protozoa, and bacteria. For enterobacteria that are often subjected to hydration and dehydration, changes in osmotic force are of great importance. Defenses against osmotic changes have been well documented in *E. coli* (see Ref. 7 for a review). Upon a hypertonic challenge (dehydration) these bacteria immediately run their constitutive Trk K^+ pump, then turn on the operons to make Kdp K^+ pump and for synthesis of osmoprotectants such as proline and glycine betaine. Upon a hypotonic challenge (hydration), *E. coli* adjusts its OmpF/OmpC ratio and turns on the synthesis of membrane-derived oligosaccharides in the periplasm. Placing *E. coli* in distilled water causes the organism to immediately jettison ions and small molecules while maintaining its macromolecules and viability (8). The mechanism of this solute exit is not understood on the molecular level. However, recent studies (9, 10) implicate mechanosensitive channels in the osmotic-shock response.

Bacterial MS channels found in giant spheroplasts (11, 12) by patch-clamp technique (13), are the only MS channels to date that can be functionally reconstituted by fusion of native membrane vesicles into liposomes (14, 15). In the present work we describe several steps toward the isolation of bacterial MS channel proteins. We were able to retrieve two types of functional MS channels upon detergent solubilization of bacterial cell envelope followed by reconstitution of solubilized material into liposomes. We compared properties of reconstituted MS channels with the properties of those found in the native membrane. We also report a new approach for fractionation and screening of channel-containing membrane extracts based on patch sampling of fractions reconstituted into liposomes.

MATERIALS AND METHODS

All experiments were performed using *E. coli* strain AW740 (*ompC*$^-$, *ompF*$^-$) (16). This double mutant is especially suitable for patch-clamp recording of single MS channels since it lacks two major porins, which are present in large copy numbers in the wild-type strain and usually create undesirably high conductance of patch membrane.

Preparation of giant spheroplasts

Giant spheroplasts were prepared as described (11, 17) with some adjustment for the *ompC*$^-$ *ompF*$^-$ strain. Ten ml of culture were grown overnight in Vogel-Bonner (VB) minimal medium with 0.6% lactate, then diluted 1:100 in the same medium and grown to an OD_{590} of 0.4. This culture was then diluted 1:10 with T-broth containing 60 μg/ml of cephalexin (Sigma Chemical Co., St. Louis, MO) to block septation. The culture was then shaken for 4–5 h at 42°C until the filamentous cells reached 50–150 μm in length. Treating these filaments with EDTA and lysozyme (11) yielded giant spheroplasts (17) of sufficient size for patch-clamp recording.

Preparation of native membrane vesicles

Membrane vesicles of *E. coli* cell envelope were prepared as described earlier (14) with some modifications. Four liters of cell culture were grown

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in VB-lactate medium to an OD_{590} of 0.8–0.9. Cells were French-pressed twice at 16,000 p.s.i. in 50 mM phosphate buffer, pH 7.0, containing 5 mM $MgSO_4$ and 1 mM dithiothreitol (DTT) supplemented with 1 mM phenylmethylsulfonyl fluoride and 1 mM *O*-phenantroline (all from Sigma). Membrane vesicles were collected by differential centrifugation and resuspended with a glass homogenizer in 1–2 ml of storage buffer (50 mM KCl, 1 mM EGTA, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.2) with 30 vol% glycerol. Protein content was determined by bicinchoninic acid assay (Pierce Chemical, Rockford, IL) in the presence of 1% dodecyl sulfate, with bovine serum albumin as a standard. The membrane suspension was aliquoted and stored at −80°C.

Preparation of liposomes and solubilized lipids

Prior to use, asolectin (phospatidylcholine, Type II-s; Sigma) was dissolved in chloroform (100 mg/ml) and purified according to Kagawa and Racker (18). To stabilize membrane patches, cholesterol was added to asolectin at a molar ratio of 1:10. The chloroform solution of lipids was stored under nitrogen at −20°C. Liposomes were prepared as follows: 200–400 μl of lipid solution was placed in a 15-ml glass tube and dried with a jet of nitrogen for 20–25 min. The tube was initially rotated gently so as to form a uniform thin lipid film on the wall. The lipid film was rehydrated in 2–4 ml of 1 mM DTT, 5 mM Tris-Cl buffer, pH 7.2, for 30 min, vortexed, and sonicated to clarity (model W185 sonifier; Heat Systems-Ultrasonics, Plainvew, NY). To prepare a detergent-stabilized lipid solution, the thin film containing 20–40 mg of lipid, dried as above, was hydrated in 2–4 ml of extraction buffer (5 mM EDTA, 5 mM EGTA, 1 mM DTT, 50 mM Tris-HCl, pH 7.4, containing 2% octylglycoside (OG, octyl-β-D-glucopyranoside; Calbiochem, San Diego, CA)) for 15 min with subsequent vortexing and sonication to clarity.

Reconstitution of membrane vesicles by fusing with liposomes

Giant multilamellar liposomes were fused with native membrane vesicles essentially as described (14), with the exception of freeze-thawing. Aliquots of membrane vesicles were mixed with 1 ml of liposomes (containing 10 mg of lipid) at the desired protein-to-lipid ratio and the mixture was pelleted at 200,000 *g* for 1 h. The pellet was then resuspended in 40 μl of 10 mM 4-morpholinepropanesulfonic acid (MOPS)-buffer (pH 7.2) containing 5% ethylene glycol, and 20-μl droplets were subjected to dehydration-rehydration cycle on glass slides (14).

Protein extraction and reconstitution of detergent-solubilized membranes

An aliquot (50–100 μl) of membrane vesicles was mixed with 1 ml of extraction buffer (see above) containing 3% OG, and incubated at room temperature for 20 min. During extraction, the mixture was vortexed periodically and in some cases sonicated mildly for 1–2 min (model B-12 bath sonicator; Branson Co., Shelton, CT). Insoluble particles were pelleted at 105,000 *g* for 1 h, and the protein concentration in the supernatant was assayed. Aliquots of the extract were mixed with 1 ml of lipid-detergent solution to obtain a protein-to-lipid ratio of 1:200. The mixture was dialyzed for 24 h against 2 liters of buffer containing 100 mM NaCl, 0.2 mM EDTA, 0.02% NaN_3, 5 mM Tris-HCl, pH 7.2, in the presence of Calbiosorb detergent-absorbing beads (Calbiochem, San Diego, CA), with two changes of buffer. Proteoliposomes were pelleted at 160,000 *g* for 1 h, resuspended in a 10 mM MOPS-buffer (pH 7.2) with 5% ethylene glycol and then subjected to the dehydration-rehydration cycle as described (14).

Gel filtration

Membrane samples containing 4–8 mg of total protein were extracted in 4 ml of extraction buffer with 3% OG as described above, insoluble particles were pelleted (105,000 *g*, 1 h), and the supernatant was concentrated twice by Centricon 30 (Amicon, Beverly, MA) concentrator. Concentrated extracts were centrifuged at 10,000 *g* for 10 min, and 400 μl of extract was applied to the Superose 6 HR 10/30 fast protein liquid chromatography column (Pharmacia, Piscataway, NJ) equilibrated with the running buffer (2 mM EDTA, 2 mM EGTA, 20 mM Tris-HCl, pH 7.2, 1% OG). Prior to the experiment, the column was calibrated with the gel filtration standards (Bio-Rad, Richmond, CA), eluted with the same buffer, excluding OG. Gel filtration was performed at 0.3 ml/min flow rate and 1-ml fractions were collected. The protein content in the fraction containing the highest concentration of the total protein was determined. The volume of the aliquot was calculated so that the protein-to-lipid ratio after reconstitution of this fraction was 1:150. Aliquots of this volume from individual fractions were reconstituted into liposomes (typically 6 mg of lipid/sample) as described above.

Electrical recording and data analysis

Patch-clamp recording from giant spheroplasts was carried out as originally described (11). In some experiments residual cell wall on excised membrane patches was digested by adding 5 mg/ml lysozyme (Sigma) to the bath after patch excision and then incubating for 60–90 min. The giant multilamellar liposomes from either of the reconstitution procedures described above were placed in a recording chamber containing 200 mM KCl, 40 mM $MgCl_2$, 5 mM HEPES, pH 7.2. After 20–60-min incubation, transparent, unilamellar blisters were formed (13). Patch pipets (Boralex, Rochester, NY) of controlled diameter (2 μm) were filled with the same buffer as the chamber. Electrical recording was carried out using an EPC-7 amplifier (List Electronics, Darmstadt, Germany). Applied pressure was monitored with an electronic manometer (Micro Switch; Omega Engineering, Stamford, CT), and current and pressure traces were recorded on chart and/or on magnetic tape (Recorder 6500; Gould, Cleveland, OH). Data were digitized and analyzed with a program developed by Dr. Y. Saimi (University of Wisconsin) on a PDP-11 computer (Indec Systems, Sunnyvale, CA).

Data reproducibility

We describe four independent experiments on MS-channel recording on spheroplasts, 10 to 12 reconstitution experiments of each type and two gel filtration experiments. $I(V)$ curves were taken two times on independent preparations. Repetitive experiments of each kind gave consistent results.

RESULTS

Patch clamping of giant spheroplasts derived from the AW740 *ompC*$^-$ *ompF*$^-$ strain of *E. coli* revealed two types of mechanosensitive (MS) channels (Fig. 1 *a*): a "small" MS channel (SMSC) of 0.9 ± 0.1-nS unitary conductance (at positive pipet voltages, mean ± S.D., $n = 4$) and a "large" MS channel (LMSC) of 3.1 ± 0.3 nS ($n = 4$) conductance. These values were obtained under symmetric ionic conditions (see legend to Fig. 1). Both types of MS channel were often found in the same patch of spheroplast membrane (Fig. 1 *a*). After giga-seal formation and patch excision, repetitive suction reproducibly evoked SMSC and LMSC activity. Ramps of suction invariably activated the SMSC first, followed by the LMSC. Suction-step experiments showed that the SMSCs were activated at 30–70 mm Hg in different patches, whereas the LMSCs required 150–250 mm Hg to be applied to the patch pipette before they activated. The high pressures required for activating LMSCs in spheroplast patches led to patch rupture in many cases. The two types of MS channels also differed in their kinetics. The SMSC tended to open for long durations. The LMSC, however, showed a "spiky" behavior with the occasional appearance of subconducting states (see Fig. 1 *a*). Additional

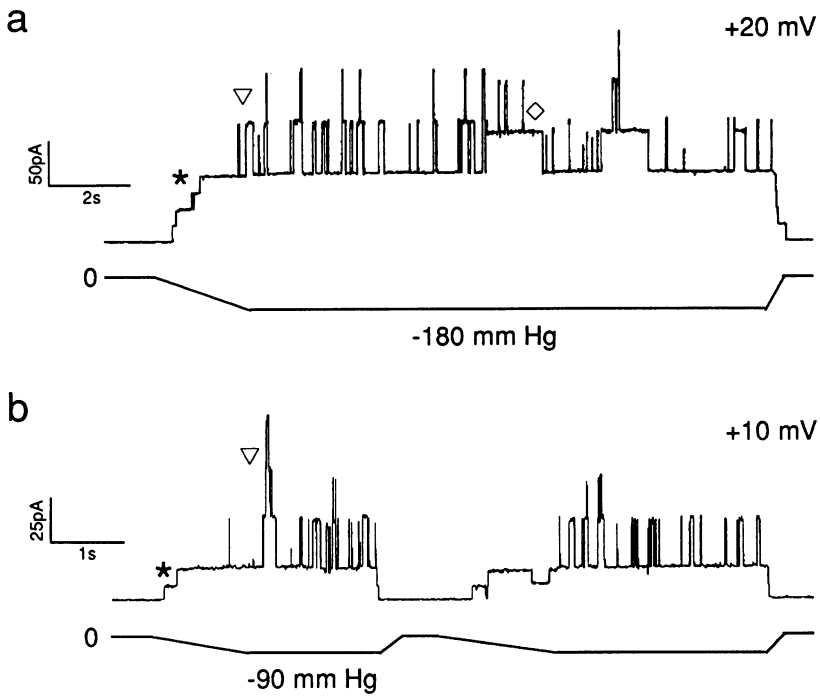

FIGURE 1 Activities of two types of mechanosensitive channels: the small (SMSC) and large, (LMSC) MS-channels from *E. coli* (strain AW740) cell envelope. (*a*) Currents recorded in a patch excised from a giant spheroplast showing activities of four SMS-channels (*) and LMS-channels (▽). The LMSCs often exhibit a subconducting state (◇). Pipet voltage was +20 mV. Bath and pipet buffer: 200 mM KCl, 90 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM HEPES, pH 7.2. (*b*) Activities of SMSC and LMSC recorded in a patch from asolectin liposomes fused with native membrane vesicles. Bath and pipet buffer: 200 mM KCl, 40 mM $MgCl_2$, 5 mM HEPES, pH 7.2. Pipet voltage was +10 mV. Lower trace in both cases (*a* and *b*) indicates the approximate profile of suction applied to the patch, while the exact value of steady-state pressure is given by numbers below. Note that the amount of pressure required for activation of both the two SMSCs (*) and the three LMSCs (▽) in liposomes (*b*) is significantly lower than in spheroplasts (*a*).

lysozyme digestion of excised patches decreased the amount of suction necessary to activate each type of channel by about 50% (data not shown), which is consistent with earlier observations on wild-type SMSCs (3). High negative pressures sometimes also led to large, discrete, and irreversible increase in the mechanosensitivity of LMSCs. The origin of this increase is unclear but it may reflect changes in patch geometry (e.g., an increase in patch area accompanied by a decrease in patch curvature) or in the viscoelastic properties of the patch following rupture of residual cell wall.

Fig. 1 *b* shows the activities of SMSCs and LMSCs reconstituted by fusing native membrane vesicles with liposomes. The unitary conductances for both channels were the same as those in native membrane (3.0 ± 0.2 nS, $n = 10$, for the LMSC and 1.0 ± 0.1 nS, $n = 5$, for the SMSC). However the amount of negative pressure required to activate the MS channels was much lower than described above (see Fig. 1 *b*). Similar activities of SMSCs and LMSCs were recorded after reassembly of OG membrane extract into liposomes by dialysis at a protein-to-lipid ratio of 1:150 (trace not shown). Incubation of OG extract with chymotrypsin (2 mg/ml) for 15 min at room temperature prior to reconstitution was accompanied by the complete loss of MS channel activity in 15 patches (some patches exhibited irregular, pressure-insensitive noise), whereas each of eight patches contained both types of MS channels in control experiment without chymotrypsin treatment.

LMSCs in all reconstituted systems showed spiky kinetics that was similar to that observed in spheroplasts. The solubilized and reconstituted SMSCs had slower kinetics, with openings lasting for seconds as compared to their properties in native membrane (data not shown). Besides the increase in apparent mechanosensitivity, there were additional changes in MS channel behavior after reconstitution. In spheroplast patches, channels of both types appeared immediately after applying appropriate suction. In liposome patches with reconstituted OG extracts, only about 20% of the experiments gave LMSCs that immediately responded to suction. In the remaining 80% of experiments suction had to be sustained for 1–3 min before the appearance of the LMSC activities. Once the LMSCs were activated in the patch, they responded promptly to subsequent pulses of suction. The SMSCs responded to suction immediately in all preparations. In both reconstituted systems we also observed irreversible inactivation of SMSCs by negative pressures above 100 mm Hg.

The unitary conductance of the SMSC is roughly one third of that of the LMSC. To test whether the large and small MSCs represent the same or distinct molecular entities, we fractionated membrane extracts by sizing on a Superose 6-HR fast protein liquid chromatography column. Fractions were collected, and each aliquot was individually reconstituted with equal amounts of lipid as described under Materials and Methods. Fractions 5 and 6 yielded an average of two SMSC per patch, but none of the 20 patches surveyed contained an LMSC (Fig. 2 *a*). On the other hand, fractions 8, 9, and 10 contained LMSC alone (Fig. 2 *b*). The profiles of SMSC and LMSC activities in the fractions eluted from the column are shown in Fig. 2 *c*. Each bar corresponds to the mean number of channels encountered in 10–20 patches. It should be noted that the reconstitution conditions (composition of dialysis buffer) affected the channel statistics in liposome patches: in the presence of 1 mM $MgCl_2$ LMSCs tended to form large clusters (of 7–20 channels), that made simple averaging meaningless. In the absence of divalent cations (0.2 mM EDTA) channel density in patches followed

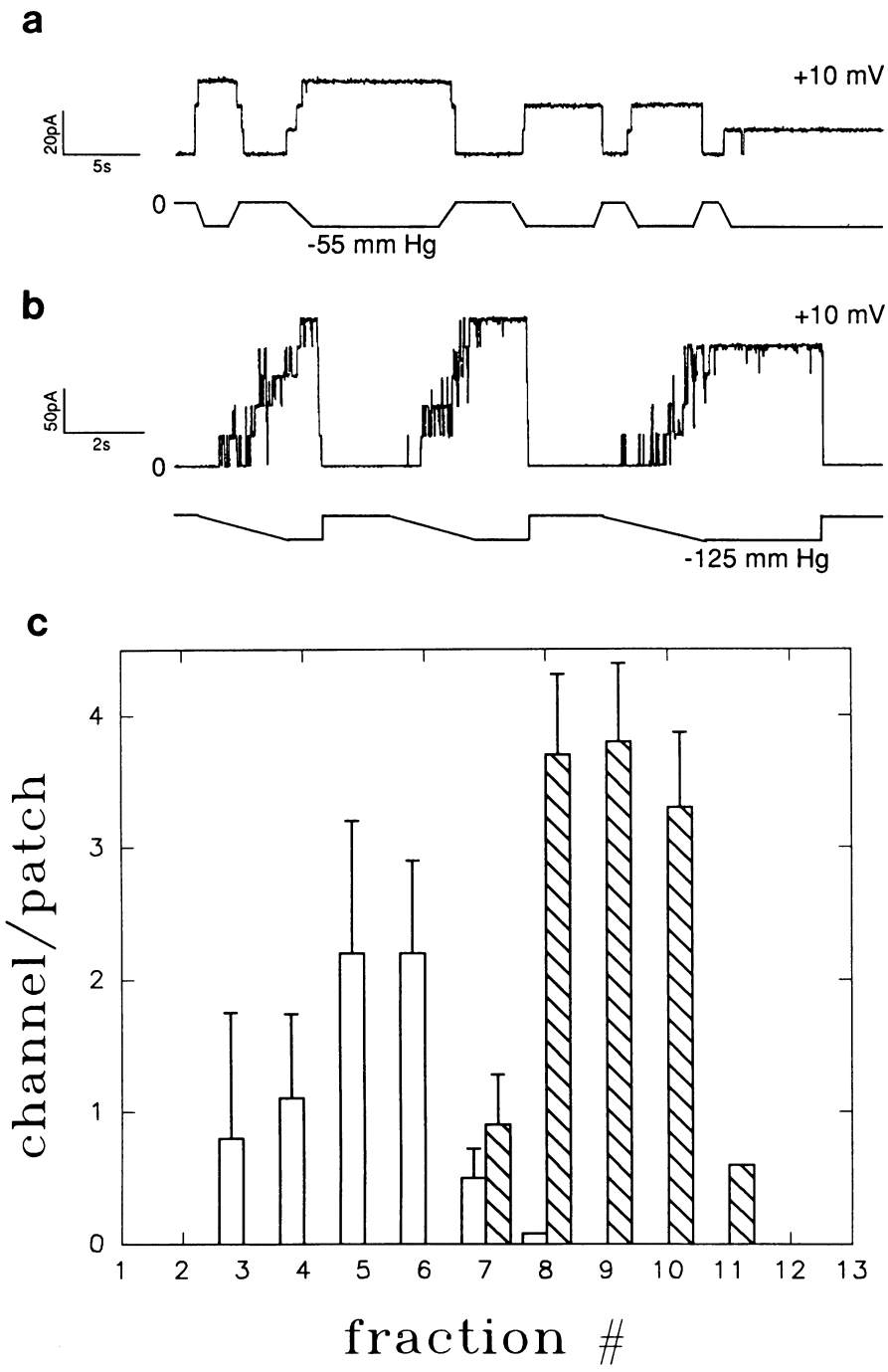

FIGURE 2 Activities of mechanosensitive channels after fractionation of whole-membrane OG extract on a Superose 6-HR gel filtration column and reconstitution of the fractions into asolectin liposomes. (*a*) SMSCs from reconstituted fraction 5 (see *c*). The SMSCs usually experienced irreversible inactivation upon repetitive suctions. (*b*) LMSCs from fraction 9 (see *c*). (*c*) Separation of SMSCs and LMSCs by gel filtration on a Superose 6-HR column. Fractions were collected, and the aliquots were individually reconstituted into asolectin liposomes under standard conditions. 10 to 20 patches were sampled from each fraction, and the numbers of channels of each type encountered per patch were averaged. Error bars show the standard error of the means (n = 10–20). The histogram represents the occurrences of the SMSC (*open bars*) and LMSC (*hatched bars*) in fractions eluted from the column.

normal distribution. As seen from Fig. 2 *c*, each type of channel came off the column as a separate peak of activity, which fell approximately between fractions 5 and 6 for the SMSC and in the fraction 9 for the LMSC. These peak positions correspond to molecular weights of approximately 60,000–80,000 for LMSC and 200,000–400,000 for SMSC. Therefore, under nondenaturing solubilizing conditions, SMSC activity appeared associated with heavier particles than the larger conductance MSC.

The ionic preference of the two MS channels, that had been separated by gel filtration and reconstituted into liposomes, was studied. The $I(V)$ plots for the LMSC and SMSC obtained in symmetric solutions, as well in the presence of a three-fold gradient of KCl, are shown in Fig. 3. The $I(V)$ curve for the LMSC (Fig. 3 *a*) remained symmetrical upon gradient application, suggesting that the LMSC is nonselective. The $I(V)$ relationship for SMSC (Fig. 3 *b*) in symmetrical 100 mM KCl solution showed different slopes: at negative pipet voltages channel conductance was about two thirds of that recorded at positive voltages. Perfusing the bath with 300 mM KCl shifted the curve by about 5 mV toward the chloride reversal potential. This shift indicates that the

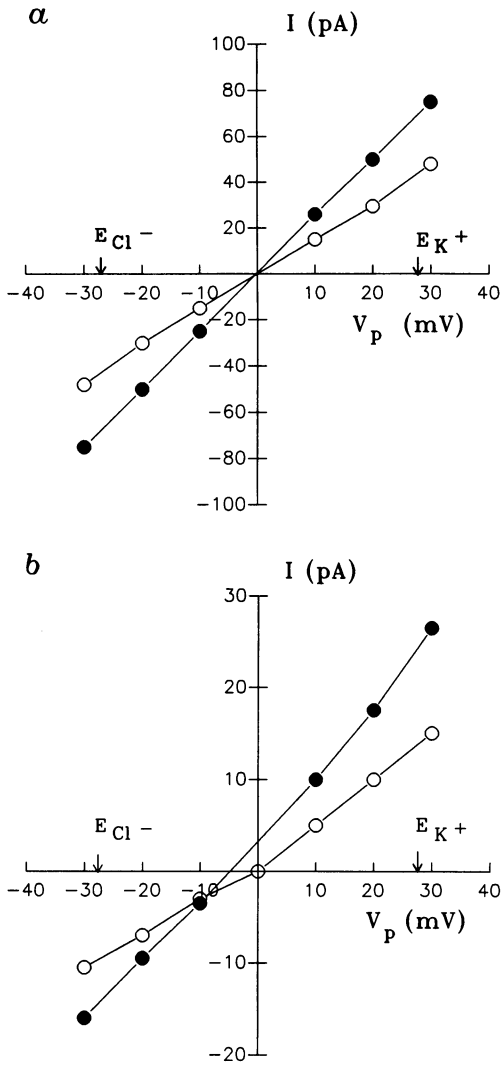

FIGURE 3 Current-to-voltage relationships for the LMSC (*a*) and the SMSC (*b*) obtained in symmetrical conditions (0.1 M KCl_{pipet}/0.1 M KCl_{bath} (○)) or in the presence of a three-fold gradient (0.1 M KCl_{pipet}/0.3 M KCl_{bath} (●)). Calculated reversal potentials for K^+ (+27.7 mV) and for Cl^- (−27.7 mV) are shown by arrows. Pipet and bath buffers contained, besides KCl, 5 mM $MgCl_2$, and 5 mM HEPES, pH 7.2.

SMSC has a slight preference for anions. Similar properties of the SMSC from the wild-type strain of *E. coli* (AW405) have been reported previously (11).

The SMSC and LMSC, reconstituted following chromatographic separation, could be blocked by 0.3 mM Gd^{3+}, consistent with the results reported by Berrier et al. (10). However, it was often possible to reactivate SMSCs after blockage by Gd^{3+} by increasing suction, despite the continued presence of this cation in the bath (trace not shown).

We studied also the behavior of SMSCs at relatively high pipet voltages. The trace in Fig. 4 *a* shows the current across a spheroplast membrane patch containing several SMSCs, that had been opened by continuous suction. First application of a ±90 mV voltage pulse resulted in fast closing of SMSCs with clearly distinguishable single-channel steps. Application of subsequent pulses of the same voltage led to gradual closing of SMSCs via a series of multiple substates (Fig. 4 *a*, right). Analogous decaying currents obtained from SMSCs reconstituted from fraction 5 are shown in Fig. 4 *b*. Some patches from reconstituted fractions 5 and 6, besides SMSCs, also contained permanently open porin channels. PhoE-porin, for example, had a conductance of 700 pS (in 200 mM KCl), which is approximately 20% lower than that of the SMSC. PhoE had also stronger preference for anions and exhibited typical three-step closures (18, 19) at pipet voltages higher than +120 mV (data not shown). This set of experiments revealed that the SMSC, like porins (18, 19), also closes at high voltages. However SMSCs do not have predominant subconducting states as multiples of one third of the main conductance, but gate at different levels.

DISCUSSION

The present study demonstrates that *E. coli* has at least two distinct types of mechanosensitive channels. These channels appear to be similar to some of those reported by Berrier et al. (10), which seem to be components of an osmoregulatory system responsible for extruding ions and small metabolites from the *E. coli* cell upon osmotic downshock (9, 10). In contrast to voltage-dependent K^+, Na^+, or Ca^{2+} channels, which are well characterized at the molecular level (21, 22), there are no biochemical, genetic, or molecular-biological studies of these or other MS channels to date.

The LMSC and SMSC are apparently proteins. Although we cannot exclude the involvement of some tightly bound bacterial lipids as components of MS channel structure, these MS channels cannot be pure lipid pores since both the large and the small MSCs are sensitive to chymotrypsin. Our results show that the LMSC and the SMSC are distinct proteins (or multisubunit protein complexes) with approximate molecular weights of 60,000–80,000 and 200,000–400,000, respectively. Although the unitary conductance of the LMSC is about three times that of the SMSC, the former does not appear to be a trimer of SMSCs, because the latter has a higher molecular weight. Also, the SMSC is slightly selective for anions, while the LMSC is not. Since the unitary conductance of the SMSC is close to that of known porins and the SMSCs switch the conductance by two thirds upon reversal of voltage polarity (Ref. 11 and this study), which is a subconducting state typical for porins (20), and since SMSCs have a tendency to close at high voltages (Fig. 4), it is possible that the SMSCs are related to bacterial porins. It is presumably not OmpF or OmpC, since we used an *OmpF*$^-$ *OmpC*$^-$ strain. It is also not PhoE, which has been found in the same patches with SMSCs and showed distinct channel characteristics (see above).

The nature of the element transducing tension to the channel gate and the possible involvement of cell envelope components other than the lipid bilayer (cytoskeleton, or bacterial cell wall) in MS-channel gating has been discussed in the literature (reviewed in Ref. 23). The fact that bacterial MS

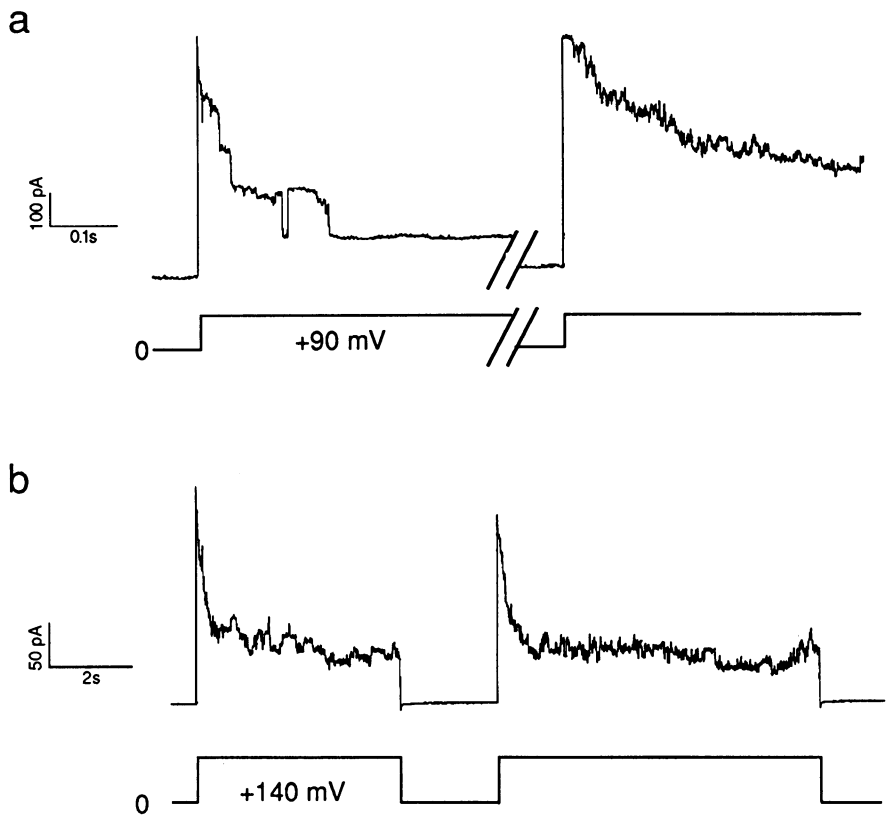

FIGURE 4 The closure of SMS-channels upon high voltage. (*a*) The SMSCs in spheroplast membrane patch were opened by continuous suction of 80 mm Hg and subjected to repetitive pulses of +90 mV. The current responses to the first and the third pulse, applied 23 s after the first one are shown. (*b*) The SMSCs from fraction 5 (see Fig. 2 *c*) were reconstituted into liposomes and activated by continuous suction of 40 mm Hg. Two SMSCs were present in this particular patch. Application of pipet voltage of +140 mV led to a gradual closing of both SMSCs to different subconducting levels.

channels remain fully functional in liposomes following chromatography (Fig. 2) suggests that the peptidoglycan layer (cell wall) is not necessary for gating. Moreover, similar increases in the apparent mechanosensitivity of both LMSCs and SMSCs after cell wall digestion, as well as upon reconstitution into artificial lipid bilayer, strongly suggests that tension gating the channels is transduced directly via the bilayer, whereas the cell wall restrains the membrane stretch (24, 25). Since the unitary conductance of both MS channels and their gating kinetics (at least for LMSC) remained unchanged after digestion of the cell wall, or reconstitution in artificial bilayer, we believe that these procedures do not affect MS-channel proteins themselves; rather, they change the mechanical properties of the medium that transduces stretch force.

The relatively nonspecific blockage of bacterial and eucaryotic MS channels (2) by Gd^{3+} may implicate not only the direct interaction of this ion with channel proteins, but also its binding to the lipid bilayer, thus affecting the viscoelastic properties of the stretch transducer. As for other ions, we have not observed spontaneous activity of the MS channels in reconstituted systems as originally reported for SMSCs recorded in giant spheroplasts in the presence of high concentrations of KCl (11). The discrepancy may come from differences in lipid composition of artificial versus native membrane in which channels are embedded. Since various ions affect nonpolar attractions between molecules in aqueous media (26) the specific ion-induced perturbations may cause a change in membrane tension or elasticity resulting in spontaneous activity of MS channels (11).

Specific blockers and antibodies were used in identification and isolation of several ion-channel proteins (27, 28). The fractionation of MS channels is more difficult because there are neither specific blockers (or ligands) nor antibodies to these proteins reported to date. Only amiloride and its analogues are considered as potential probes for tagging some types of eucaryotic MS channels (29). We attempted "functional" fractionation of bacterial MS channels by following their activities, as one would proceed in the purification of enzymes. As this study shows, it is possible to assay the MS-channel activities by reconstituting individual fractions into liposomes followed by patch-clamp sampling. Both LMSC and SMSC proteins, were stable enough to survive solubilization, sizing chromatography, and reconstitution procedures, and were active in foreign lipids. Averaging of the numbers of channels per patch at a given protein-to-lipid ratio allowed us to assess the specific activity of MS channels in each fraction. We have obtained consistent results in independent experiments on reconstitution of the same fraction under equal conditions. Fractionations toward identification of bacterial MS channels are in progress.

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