J. Membrane Biol. 144, 31–42 (1995)

The Journal of
Membrane
Biology

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Characterization of Mechanosensitive Channels in *Escherichia coli* Cytoplasmic Membrane by Whole-Cell Patch Clamp Recording

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Received: 15 August 1994/Revised: 19 October 1994

Abstract. Whole-cell patch clamp recordings were done on giant protoplasts of *Escherichia coli.* The pressure sensitivity of the protoplasts was studied. Two different unit conductance mechanosensitive channels, 1100 ± 25 pS and 350 ± 14 pS in 400 mM symmetric KCl solution, were observed upon either applying positive pressure to the interior of the cells or down shocking the cells osmotically. The 1100 pS conductance channel discriminated poorly among the monovalent ions tested and it was permeable to Ca^{2+} and glutamate$^-$. Both of the two channels were sensitive to the osmotic gradient across the membrane; the unit conductances of the channels remained constant while the mean current of the cell was increased by increasing the osmotic gradient. Both of the channels were voltage sensitive. Voltage-ramp results showed that the pressure sensitivity of protoplasts was voltage dependent: there were more channels active upon depolarization than hyperpolarization. The mechanosensitive channels were reversibly blocked by gadolinium ion. Also they could reversibly be inhibited by protons. Mutations in two of the potassium efflux systems, KefB and KefC, did not affect the channel activity, while a null mutation in the gene for KefA changed the channel activity significantly. This indicates a potential modulation of these channels by KefA.

Key words: *Escherichia coli* — Protoplast — Mechanosensitive channels — Whole-cell patch clamp recording — Osmotic pressure — K^+ efflux system

Introduction

Mechanosensitive channels have been found in a variety of organisms including animals, plants, fungi, and bacteria (for a review, *see* Sachs, 1988). It has been suggested that these channels play roles in hearing, touch, osmotic regulation, and cell-volume regulation (Sachs, 1988; Morris, 1990).

Mechanosensitive channels in *E. coli* have been observed by single channel recording of giant spheroplasts (Martinac et al., 1987; Buechner et al., 1990; Szabo et al., 1990; Martinac, Adler & Kung, 1990; Kubalski et al., 1993) and also of liposomes into which the outer and/or cytoplasmic membranes were incorporated (Berrier et al., 1989; Delcour et al., 1989; Berrier et al., 1992; Sukharev et al., 1993; Sukharev et al., 1994). These channels open upon applying pressure through the recording pipette. Although it has been suggested that one possible physiological role of the mechanosensitive channels in *E. coli* is the response to osmotic down shock (Berrier et al., 1992), no direct study has shown that these mechanosensitive channels indeed open when the cell is subjected to the osmotic down shock.

Turgor pressure is increased when *E. coli* cells are subjected to osmotic down shock. To reduce this increased pressure, *E. coli* extrudes K^+ and other small molecules (Britten & McClure, 1962; Meury, Robin & Monnier-Champeix, 1985; Berrier et al., 1992). There are three known K^+ efflux systems in *E. coli,* KefA, KefB, and KefC (Booth et al., 1992). KefB and KefC play significant roles in K^+ efflux elicited by addition of N-ethylmaleimide (Bakker et al., 1987; Booth et al., 1992). The third K^+ efflux system, KefA, which has not yet been fully characterized, may be the important path for K^+ efflux produced by osmotic down shock (Bakker et al., 1987).

So far studies of ion channels in *E. coli* have been done by patch clamp single channel recording of liposomes, spheroplasts, and to a small extent protoplasts Berrier et al., 1994. To explore further the biological functions of the channels in *E. coli*, we made the follow-

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ing innovations. First, we prepared the giant protoplasts from 30° C-grown rather than 42° C-grown cells. Instead of using the usual 42° C (Martinac et al., 1987; Buechner et al., 1990; Martinac, Adler & Kung, 1990; Kubalski et al., 1993; Sukharev et al., 1994) we used 30° C because the sensory apparatus and flagella are missing in 42° C-grown cells (Adler & Templeton, 1967). Thus the use of 30° C-grown cells makes it possible for an electrophysiological study of sensory behavior, such as perhaps sensing changes in osmolarity. Second, since many of the biological functions of *E. coli* are carried out on the cytoplasmic membrane, we studied the intact cytoplasmic membrane by using giant protoplasts which lack intact outer membrane and peptidoglycan. Third, we did whole-cell patch clamp recording of the *E. coli* protoplasts, which has never been documented before. This technique allowed us to study the functions of ion channels, particularly those which may be very difficult to study by single channel recording.

In the present research, the pressure sensitivity of *E. coli* giant protoplasts, which have only the cytoplasmic membrane intact, was examined by using whole-cell patch clamp recording. This method allowed us to directly record the electrophysiological response of the cell to pressure applied to the interior of the cell as well as to osmotic down shock. In this way we discovered mechanosensitive channels on the cytoplasmic membrane of *E. coli* that were sensitive to the osmotic gradient across the membrane. These channels were found to be related to the KefA K^+ efflux system. Our results provide direct evidence for the involvement of mechanosensitive channels in the osmotic regulation of *E. coli.*

Materials and Methods

Strains and Chemicals

The strains used in this study, all derivatives of *E. coli* K12, are HCB437 (Δ(*tsr*)7021 Δ(*trg*)100 Δ(*cheA-cheZ*)2209) (Wolfe et al., 1987), MJF276 (*kefB*$^-$ *kefC*$^-$) (Elmore et al., 1990), RQ2 (*kefA*$^-$) and Frag-1 (parental strain of MJF276 and RQ2, Epstein & Davies, 1970). HCB437 was used except where otherwise indicated. We used HCB437 to avoid any possible effects of chemotaxis on electrophysiological responses, but in fact chemotactically wild-type strains gave the same results as HCB437. Tryptone and yeast extract came from Difco Laboratories (Detroit, Michigan) and gadolinium chloride from Aldrich Chemical (Milwaukee, WI). All the other chemicals came from Sigma Chemical (St. Louis, MO).

Preparation of Giant Protoplasts

Giant protoplasts were prepared from giant spheroplasts by modifying the procedure of Kubalski et al. (1992). Instead of using the *lpp*$^-$ *ompA*$^-$ double mutant, which is a giant spherical cell, we started with normal sized, normal shaped *E. coli* (HCB437, MJF276, RQ2, and Frag-1). These *E. coli* were first made into giant spheroplasts, and then the spheroplasts were converted into protoplasts (Fig. 1).

The procedure for making giant spheroplasts was basically similar to that described by Martinac et al. (1987) except for the following modifications: An overnight culture of *E. coli* was diluted into tryptone broth (1% tryptone and 0.5% NaCl) and grown at 35° C to OD_{590} of 0.6–0.7. Then 2 ml of this culture was diluted into 20 ml of Luria-Bertani medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). Cephalexin was added to a concentration of 60 μg/ml. The cells were grown at 30° C for about 5–5.5 hours until they were long enough (100–120 μm) for making giant spheroplasts by the procedure described previously (Martinac et al., 1987). These giant spheroplasts were suspended in (mM) 800 sucrose, 10 $MgCl_2$, and 10 Tris Cl^-, pH 7.2, and then stored at −80° C.

Just before the experiments, giant spheroplasts were converted into protoplasts in the recording chamber. An aliquot of the thawed spheroplasts (10–25 μl, depending on the concentration) was added into 1 ml of bath solution: (mM) 450 sorbitol, 100 NaCl, 5 KCl, 10 HEPES, pH 7.2, and 200 μM lysozyme. After 3 minutes, (mM) 20 $MgCl_2$ and 5 $CaCl_2$ were added to stabilize the protoplasts.

Electrical Recording and Data Analysis

Whole-cell patch clamp recordings were performed with 2–3 MΩ micropipettes (measured in 400 mM KCl) according to the procedure described by Hamill et al. (1981). The sign convention used the outside of the cell as reference, so the pipette voltage represented the membrane potential. Unless otherwise stated, the pipette solution was (mM) 400 KCl, 2.0 $CaCl_2$, 1.7 $MgCl_2$, 10 EGTA, 80 sorbitol and 10 HEPES, pH 7.2. After forming the whole-cell recording configuration, the recording chamber solution was changed to experimental solutions by superfusing with ten times the chamber volume of the solution. In case of testing the effect of osmotic down shock the osmolarity of the solution was varied by including different concentrations of sorbitol. The osmolarity was measured by using a 5500 Vapor Pressure Osmometer (Wescor, Logan, UT). The pressure applied through the recording pipette was monitored by a pressure transmitter (Micro Switch, Omega Engineering, Stamford, CT). Channel activities were recorded with an APC-8 amplifier (Medical System, Greenvale, NY). All the data were filtered at 1 KHz. Axon DMA interface and pClamp software (Axon Instrument, Foster City, CA) were used for data acquisition and analysis.

To construct the current-voltage relationships, the unitary current steps corresponding to each conductance were measured directly on expanded traces. At least twenty measurements were averaged for determining the amplitude of the unitary current in each recording.

Mean current was used to represent the steady state whole-cell current. It is defined as integration of the current over the recording time and dividing this integration by the recording time: $I = (1/t) \times \int_0^t i(t)\, dt$, where I is the mean current and t is the recording time. In most of cases, 40 sec of recording was used for calculating the mean current. No inactivation of the channel activity was observed during this period.

To calculate open probability, the duration and amplitude of each conductance level were measured by the Fetchan program of pClamp. The open probability was defined as that described by Martinac et al. (1987). Overlapping events of two different channels were excluded when calculating the open probability of each channel.

Results

Formation of Whole-cell Patch Clamp Recording Configuration on the Protoplasts

After the giant spheroplasts were diluted into the bath solution (*see* Materials and Methods) and further treated

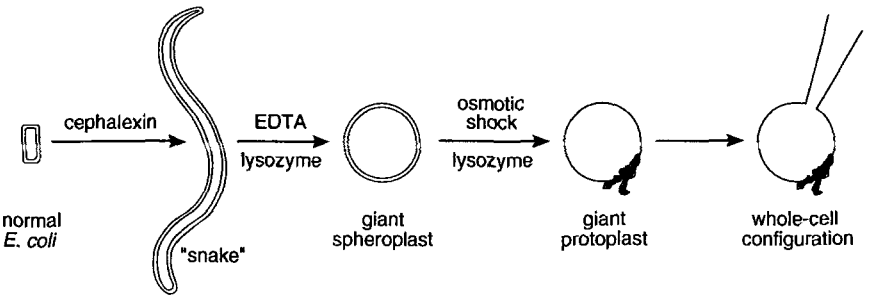

Fig. 1. Diagram of the preparation of giant protoplasts and the formation of the whole-cell patch clamp recording configuration.

with lysozyme, four different kinds of cell images were observed under the phase contrast microscope: shiny, black, transparent, and gray. With the shiny cells and the black cells, it was very difficult to form the whole-cell patch clamp configuration. Most likely outer membrane and peptidoglycan still surrounded the cytoplasmic membrane. It could be that the different degrees of the breakage of outer membrane resulted in the shiny and the black images, respectively. For the transparent cells, it was easier to break the membrane under the pipette tip after forming a gigaohm seal, but the cells were very leaky. We suspect the transparent cells were "ghost" cells of which the inner membranes had been broken and cytoplasmic materials had leaked out. Only the gray cells could by used to form a good whole-cell recording configuration under our conditions. This suggests that the gray cells have the cytoplasmic membrane exposed, and that made it easier to form the whole-cell patch clamp recording configuration. The gray cells usually have blebs attached to the cell membrane; we think the blebs are the ruptured outer membranes. We call the gray cells the giant protoplasts. They look just like the ordinary-sized protoplasts described by Birdsell and Cota-robles (1967). Channel activities of porins, which are located on the outer membrane, were not observed by whole-cell recording of the protoplasts. This indicates that the whole-cell patch clamp recording configuration was formed on the cytoplasmic membrane. Most of the protoplasts were 3–5 μm in diameter (measured by a micrometer under the microscope) after the whole-cell recording configuration had been formed. All the recordings presented here were done on such protoplasts.

Figure 1 is a diagram of the preparation of the protoplasts and the whole-cell recording configuration. Following the establishment of a gigaohm seal, the whole-cell recording configuration was formed either by applying suction to the recording pipette or by using transient voltage pulses (1.5 V for 2–10 msec). The rupture of the membrane spanning the pipette tip was indicated by a sudden increase in the capacitative current and the transition of the cell image from gray to a more shiny appearance, which was possibly due to exchange of the solutions between the cell and the recording pipette. The resting potentials of the protoplasts were measured by the current-clamp method. The average resting potential was −104 ± 5.3 mV (mean ± SEM, twenty experiments) when the seal was about 10 GΩ, with (mM) 165 K^+ glutamate$^-$, 250 KCl, 5 NaCl, 1.9 $CaCl_2$, 1.8 $MgCl_2$, 10 EGTA, and 10 HEPES, pH 7.2, in the recording pipette and 506 sorbitol, 100 NaCl, 5 KCl, 5 $CaCl_2$, 20 $MgCl_2$, and 10 HEPES, pH 7.2, in the bath solution. This is close to the resting potential measured by another method (Felle et al., 1980).

Channels Activated by Pressure and by Osmotic Down Shock

To test the pressure sensitivity of the giant protoplasts, we recorded the whole-cell current when either positive pressure was applied through the recording pipette (n = 50) or the protoplasts were superfused with low osmolarity solution (n = 11). Results in Fig. 2*A* show that when no pressure was applied there was little activity of the mechanosensitive channels. Applying positive pressure through the recording pipette to the interior of the cell made more channels active (Fig. 2*B*). Such channels could also be activated by superfusing the protoplasts with low osmolarity solution, i.e., by osmotic down shock (Fig. 2*C*). These results show that there are mechanosensitive channels in the cytoplasmic membrane of *E. coli* and indicate that the channels are involved in responding to osmotic down shock.

The Number of Mechanosensitive Channels

Two different unit conductance mechanosensitive channels were observed by whole-cell patch clamp recordings of giant protoplasts (Fig. 3*A*). The different conductances of these two channels could be clearly observed in an amplitude histogram (Fig. 3*B*). There were six peaks observed in this histogram. The mean amplitude (μ) of each peak after Gaussian fit is indicated. Peak 1 corresponds to the amplitude of the baseline. Peak 2 corresponds to the current amplitude of one small channel. Peak 3 corresponds to the current amplitude of one big channel. Peak 4 corresponds to the current amplitude of one small channel plus one big channel, because μ_4 is close to $\mu_2 + \mu_3$. For similar reasons, peak 5 corresponds to the current amplitude of two big channels and peak 6 corresponds to the current amplitude of three big chan-

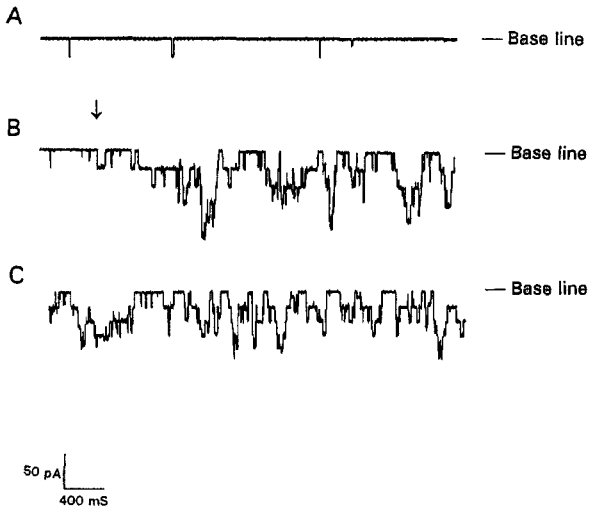

Fig. 2. The mechanosensitive channels were activated either by directly applying positive pressure through the recording pipette or by osmotic down shock. (*A*) Whole-cell recording with 913 mmol/Kg osmolarity solution inside the recording pipette and 908 mmol/Kg osmolarity solution outside the cell. (*B*) The recording condition was the same as in A, except that 15 mm Hg positive pressure was applied to the recording pipette at the point indicated by the arrow. (*C*) Recording with 913 mmol/Kg osmolarity solution inside the recording pipette and 823 mmol/Kg osmolarity solution outside the cell. All three recordings (*A, B, C*) were from the same cell. The recording pipette solution contained (mM) 250 KCl, 165 K^+ glutamate$^-$, 5 NaCl, 1.9 $CaCl_2$, 2.0 $MgCl_2$, 10 EGTA, 10 HEPES, pH 7.2, and 3 ATP. The bath solution contained (mM) 100 NaCl, 5 KCl, 20 $MgCl_2$, 5 $CaCl_2$, and 10 HEPES, pH 7.2, with different concentrations of sorbitol added to adjust the osmolarity. The membrane potential was held at −50 mV. The cell diameter was 4.7 μm.

nels together. The unitary conductances of the channels were determined by the slopes of I–V curves in 400 mM KCl symmetrical solution (Fig. 3*C*). The conductances are 1100 ± 25 pS and 350 ± 14 pS (mean ± SD), respectively. Because of the voltage sensitivity (*see* Voltage Sensitivity below) the conductance of the 350 pS channel was determined by the slopes of I–V curves at negative voltages. Depending on the amount of the pressure applied, the whole-cell current could comprise several units of these conductances. Both of the channels were observed either by applying positive pressure to the interior of the cell or by osmotic down shock.

The two mechanosensitive channels found in the protoplasts are different from one of the mechanosensitive channels (MscL) revealed by on-cell or excised-patch recording of spheroplasts (Sukharev et al., 1994): we have found that the two mechanosensitive channels of protoplasts still exist in knock-out mutants of *mscL* (*data not shown*).

Ion Selectivity

To determine the ion selectivity of the channels the unitary current corresponding to the 1100 pS conductance was plotted against the holding potential under different conditions. The permeability ratio of the ions was calculated by use of the Goldman-Hodgkin-Katz equation (Hille, 1992) and its modified form (Spangler, 1972).

In the 400 mM KCl symmetric solution, the reversal potential was close to zero. Replacing 400 mM KCl in the bath solution with 200 mM KCl + 400 mM sorbitol did not show significant change of the reversal potential (0.4 ± 1.2 mV, mean ± SEM, n = 4) (Fig. 4*A*, filled circles). Therefore, this channel shows no big preference between K^+ and Cl^-. The selectivities of the channel for different monovalent cations were determined by comparing the reversal potential with 400 mM KCl inside the recording pipette and an equal concentration of different monovalent cations (K^+, Na^+, Cs^+, Li^+) outside the cell (Fig. 4*B*). The reversal potential showed only small changes with different cation solutions. This result suggests that the channel poorly discriminates between these ions. This channel is also permeable to Ca^{2+}, with $P_{Ca^{2+}}/P_{K^+} = 0.96 \pm 0.01$ (mean ± SEM, n = 3) (Fig. 4*B*, open squares). The selectivity of this channel for Cl^- and glutamate$^-$ was compared (Fig. 4*B*, filled squares). Replacing 400 mM KCl with 372 mM K^+ glutamate$^-$ shifted the reversal potential 4.7 ± 0.4 mV (mean ± SEM, n = 3) to the right and the I–V curve was rectified at positive voltages. This could be due to the channel being less permeable to glutamate$^-$ than Cl^-. The permeability ratio of glutamate$^-$ over Cl^- is 0.71 ± 0.02 (mean ± SEM, n = 3). Because this channel is permeable to glutamate, it might be big enough to pass other amino acids and other small molecules. The ion selectivity of the 350 pS channel is not presented because the unit conductance of this channel was difficult to determine at positive voltages (*see* Voltage Sensitivity) below under our condition.

Osmotic-pressure Sensitivity

To quantify the osmotic-pressure sensitivity of the giant protoplasts, the protoplasts were osmotically down shocked by superfusing the cell with different low osmolarity solutions after forming the whole-cell patch clamp configuration. Figure 5*A* shows that more channels became active as the osmolarity difference across the membrane was made larger. The mean current of the cell was plotted against the osmolarity differences. The results from three different cells are shown in Fig. 5*B*. The mean current of none of the cells reached saturation since high osmolarities disrupt the whole-cell configuration. The data of Fig. 5*B* were also used to establish the current density-tension relationship (*data not shown*). However, the curves derived from the three cells did not correlate with each other very well. This could be due to the incomplete digestion of peptidoglycan. Depending on how much peptidoglycan was left, it could protect the cell to different degrees. The results of Fig. 5 suggest

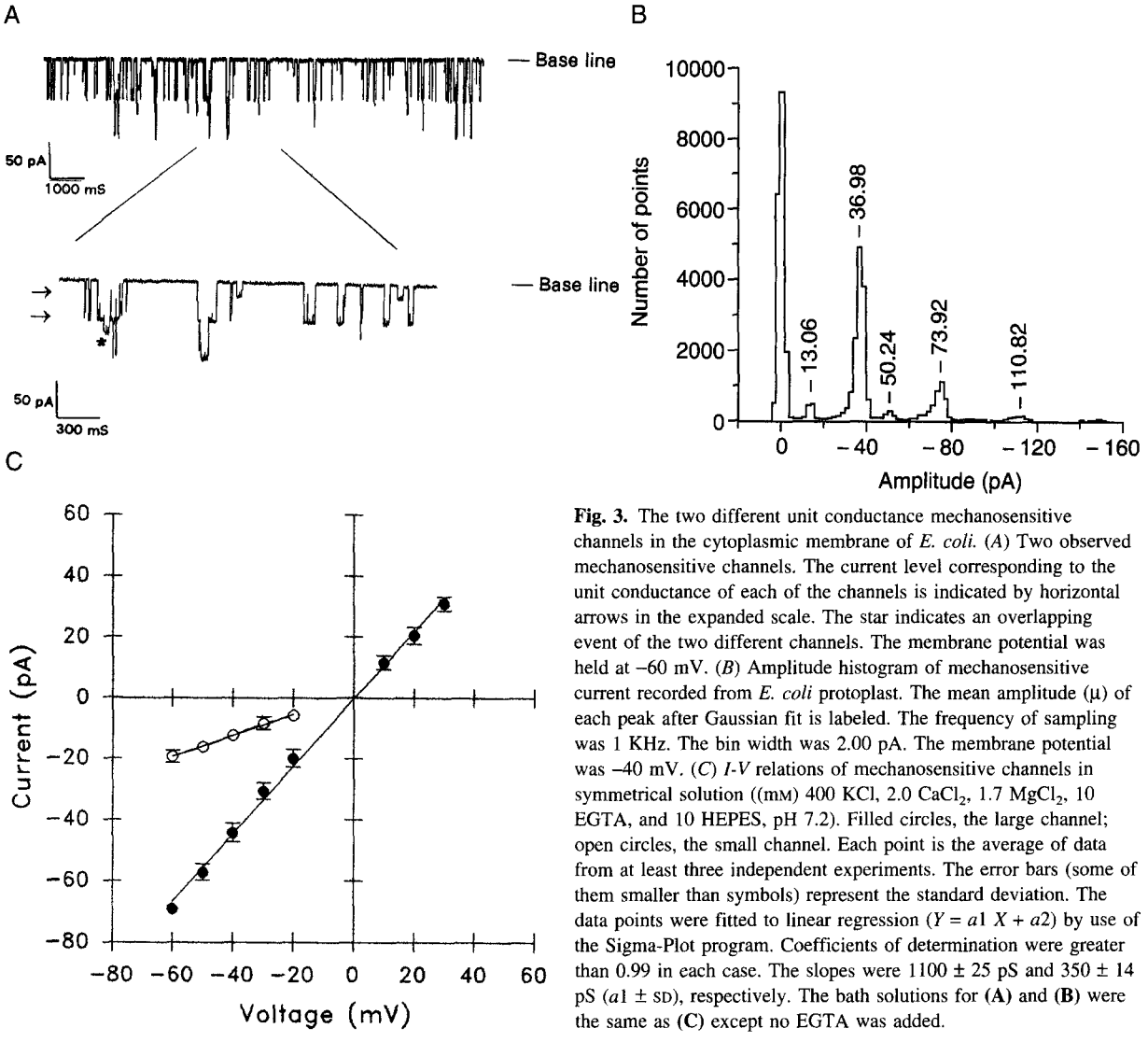

Fig. 3. The two different unit conductance mechanosensitive channels in the cytoplasmic membrane of *E. coli*. (*A*) Two observed mechanosensitive channels. The current level corresponding to the unit conductance of each of the channels is indicated by horizontal arrows in the expanded scale. The star indicates an overlapping event of the two different channels. The membrane potential was held at −60 mV. (*B*) Amplitude histogram of mechanosensitive current recorded from *E. coli* protoplast. The mean amplitude (μ) of each peak after Gaussian fit is labeled. The frequency of sampling was 1 KHz. The bin width was 2.00 pA. The membrane potential was −40 mV. (*C*) *I-V* relations of mechanosensitive channels in symmetrical solution ((mM) 400 KCl, 2.0 $CaCl_2$, 1.7 $MgCl_2$, 10 EGTA, and 10 HEPES, pH 7.2). Filled circles, the large channel; open circles, the small channel. Each point is the average of data from at least three independent experiments. The error bars (some of them smaller than symbols) represent the standard deviation. The data points were fitted to linear regression ($Y = a1\ X + a2$) by use of the Sigma-Plot program. Coefficients of determination were greater than 0.99 in each case. The slopes were 1100 ± 25 pS and 350 ± 14 pS ($a1$ ± SD), respectively. The bath solutions for **(A)** and **(B)** were the same as **(C)** except no EGTA was added.

that the protoplasts are sensitive to the osmotic gradient across the membrane by virtue of their mechanosensitive channels becoming active when the osmolarity outside the cell decreases.

Voltage Sensitivity

To test the effect of voltage on the pressure sensitivity of protoplasts, a voltage ramp from −80 to +60 mV (pipette voltage) was applied either with pressure or without pressure (Fig. 6*A* and *B*). The result shows that more mechanosensitive channels are active during depolarization than hyperpolarization (Fig. 6*A*). We can tell that the high channel activity during depolarization was not due to the activation of other voltage-sensitive channels because there was no large current difference between depolarization and hyperpolarization when no pressure was applied (Fig. 6*B*). This result indicates that the protoplasts are more sensitive to pressure upon depolarization than upon hyperpolarization. This phenomenon was observed in every cell tested.

The activities of both of the mechanosensitive channels are voltage dependent (Fig. 6*C*). When the membrane potential was less negative than −60 mV (e.g., −40 mV) the 1100 pS channel was predominant. However, when the membrane potential was more negative (e.g., −80 mV) the 350 pS channel became predominant. The open probability of each channel was calculated at different voltages. As shown in the Table, the open probability of the 1100 pS channel at −40 mV was considerably higher than at −80 mV (1.7×10^{-1} at −40 mV, 1.1×10^{-2} at −80 mV), while for the 350 pS channel the situation was the opposite (2.6×10^{-2} at −40 mV, 1.6×10^{-1} at −80 mV). These results indicate that the 1100 pS channel and the 350 pS channel have different voltage sensitivities.

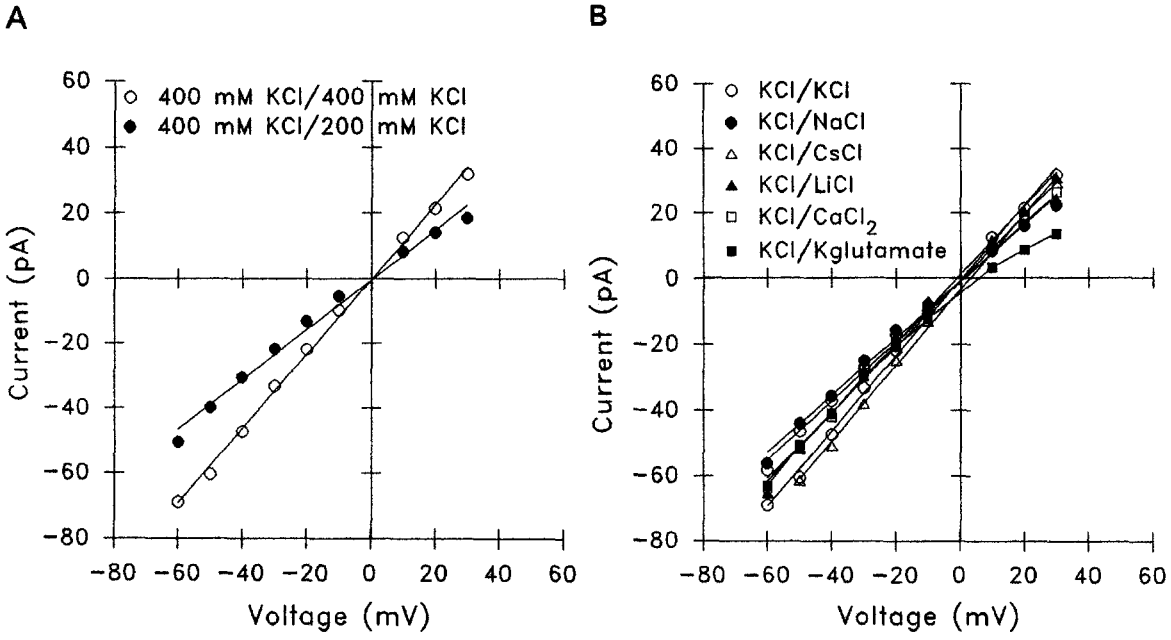

Fig. 4. Ion selectivity of the 1100 pS channel. (*A*) Current-voltage relationships. Open circles: the solutions in the recording pipette and bath both contained (mM) 400 KCl, 3 $MgCl_2$, and 10 HEPES, pH 7.2. Filled circles: pipette solution was the same as above and in the bath solution 400 mM KCl was replaced by 200 mM KCl + 400 mM sorbitol. (*B*) Current-voltage relationships with various bath solutions. The recording pipette solution was the same as in *A;* the bath solution contained (mM) 400 KCl (open circles), 400 NaCl (filled circles), 400 CsCl (open triangles), 400 LiCl (filled triangles), 267 $CaCl_2$ (open squares), or 372 K^+ glutamate$^-$ (filled squares). The reversal potential under each condition was determined by three independent experiments.

Blockage of Mechanosensitive Channels by Gadolinium Ions

Gadolinium is a trivalent lanthanide with an ionic radius close to that of Na^+ and Ca^{2+} (Yang & Sachs, 1988). Gd^{3+} has been shown to block mechanosensitive channels in *Xenopus* oocytes (Yang & Sachs, 1988, 1989), yeast (Gustin et al., 1988; Zhou & Kung, 1992), *Bacillus subtilis, Streptococcus faecalis,* and *E. coli* (Berrier et al., 1992). In *E. coli* Berrier et al. (1992) have shown that submillimolar concentrations of Gd^{3+} were sufficient to inhibit the osmotic-down-shock-induced release of metabolites such as lactose and ATP. Also, Gd^{3+} inhibited the mechanosensitive channels of the inner membrane which had been reconstituted into liposomes (Berrier et al., 1992).

We tested the effect of Gd^{3+} on the mechanosensitive channels of *E. coli* protoplasts. As shown in Fig. 7*A*, Gd^{3+} could reversibly block the mechanosensitive channels. The dose-response curves for Gd^{3+} were established with different pressure applied into the cell (Fig. 7*B*). The results showed that low concentrations of Gd^{3+} stimulated the channel activity, while high concentrations of Gd^{3+} blocked the channel activity. This effect was seen in the eight cells tested. The stimulation effect of Gd^{3+} was more obvious when smaller pressure was applied to the interior of the cell (Fig. 7*B*, filled triangles). Both of the two mechanosensitive channels could be blocked by Gd^{3+}.

Inhibition of the Mechanosensitive Channels by Protons

The mechanosensitive channels of *E. coli* giant protoplasts were sensitive to pH of the solution outside the cell. The channel activities could be reversibly inhibited by low pH (Fig. 8*A*). To test the sensitivity of protoplasts to different pHs, a pH range from 5.1 to 8.6 was tested on different cells. Fig. 8*B* shows that only the acidic pH had a big effect on relative activity, while the basic pH did not have a dramatic effect. The acidic pH effect was channel-activity dependent. For the cell with higher mean current at pH 7.2 (Fig. 8*B*, filled circles), more acidic pH was needed for blocking the channel compared to the cell with lower mean current at pH 7.2 (open circles). pH also affects the pressure sensitivity of the channels. As shown in Fig. 8*C*, higher tension was needed to produce the same amount of mean current at pH 6.0 and pH 5.0 compared to pH 7.2. However, the tension needed for an e-fold change in mean current was not dramatically affected by different pHs: 0.14 mN/m at pH 7.2, 0.12 mN/m at pH 6.0, and 0.15 mN/m at pH 5.0. These results suggest that it is the threshold for

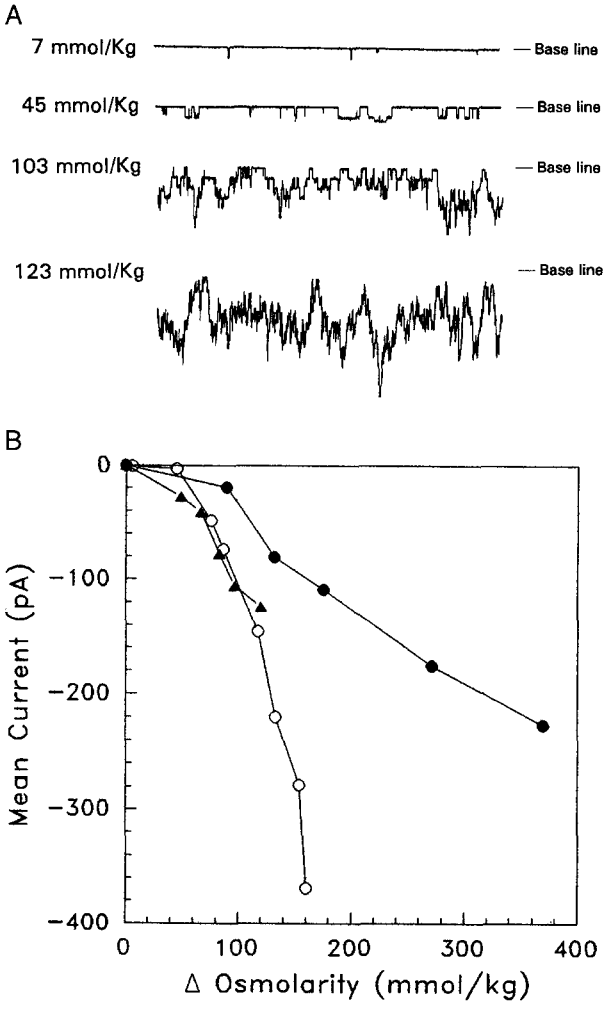

Fig. 5. The osmotic-pressure sensitivity of the protoplasts. (*A*) The cell was sensitive to the osmotic-pressure difference across the cytoplasmic membrane: the bigger the osmotic gradient the more the mechanosensitive channels opened. The osmotic gradient (inside − outside) is indicated for each recording. (*B*) The relationship of the mean current and the osmotic gradient across the membrane. The three curves represent the results derived from three different cells. The cell diameters were: open circles, 4.7 μm; filled circles, 2.3 μm; filled triangles, 3.4 μm. The pipette and bath solutions were the same as in Fig. 2. The membrane potential was −50 mV.

activation of the mechanosensitive channels that is affected by low pH.

To determine whether the effect of low pH was due to the acidified form of the buffer, solutions with different buffers were tested: HEPES (N-[2-hydroxyethyl]-piperazine-N′-[2-ethanesulfonic acid]), MOPS (3-[N-morpholino]propane-sulfonic acid), TES (N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid), and Bis-tris-propane (1,3-bis[tris(hydroxymethyl)methylamino)]-propane). All these solutions inhibited the channel activity at acidic pH similarly. Solutions without any buffer added could also block the channels at low pH. Therefore the inhibitory effect of low pH is not due to HEPES itself but it is due to protons.

Relation Between Mechanosensitive Channels and K^+ Efflux Systems

E. coli has three known K^+ efflux systems: KefA, KefB, and KefC (Booth et al., 1992) (*see* Introduction). To determine whether the mechanosensitive channels of *E. coli* protoplasts are related to any of these efflux systems, *kefA*$^-$ and *kefB*$^-$ *kefC*$^-$, double mutants were tested. As shown in Fig. 9*A*, MJF276 (*kefB*$^-$ *kefC*$^-$ double mutant) has the same mechanosensitive channels as its parent Frag-1, which has the wild-type *kefA*, *kefB*, and *kefC*. However the mechanosensitive channels of RQ2, which is a mutant of *kefA*, are quite different from those in the parental strain Frag-1 and in MJF276. Both of the channels of RQ2 tend to stay in the open state longer than channels in Frag-1 or MJF276. Also the protoplasts of RQ2 were more sensitive to pressure than protoplasts of Frag-1 and MJF276. The pressure sensitivities of RQ2 and Frag-1 were tested by applying different pressures into the cell. The results from three different RQ2 cells and three different Frag-1 cells are presented in Fig. 9*B*. The results indicate that RQ2 has a lower tension threshold for activating the mechanosensitive channels than Frag-1 does. The detailed relationship between K^+ efflux and mechanosensitive channels will be studied further.

The above results suggest that the *kefB*$^-$ *kefC*$^-$ double mutation does not affect the mechanosensitive channels in the cytoplasmic membrane of *E. coli*, but the mutation in *kefA* not only changes the channel properties but also affects the pressure sensitivity of the cell. The possible relationship between KefA and mechanosensitive channels is discussed below.

Discussion

In this report the pressure sensitivity of *E. coli* giant protoplasts was studied by whole-cell patch clamp recordings. The results provide direct evidence for the involvement of mechanosensitive channels in osmoregulation (Figs. 2 and 5). Two different conductance mechanosensitive channels (1100 ± 25 pS and 350 ± 14 pS in symmetrical 400 mM KCl solution) were observed either by applying positive pressure to the interior of the cells or by osmotic down shock of the cells. These two channels probably correspond to the 100–150 pS and 330 pS mechanosensitive channels (in 100 mM KCl solution) found by excised-patch recording of the inner-membrane-integrated liposomes (Berrier et al., 1992). We did not observe the mechanosensitive channels which were described by Sukharev et al. (1994). It is possible that

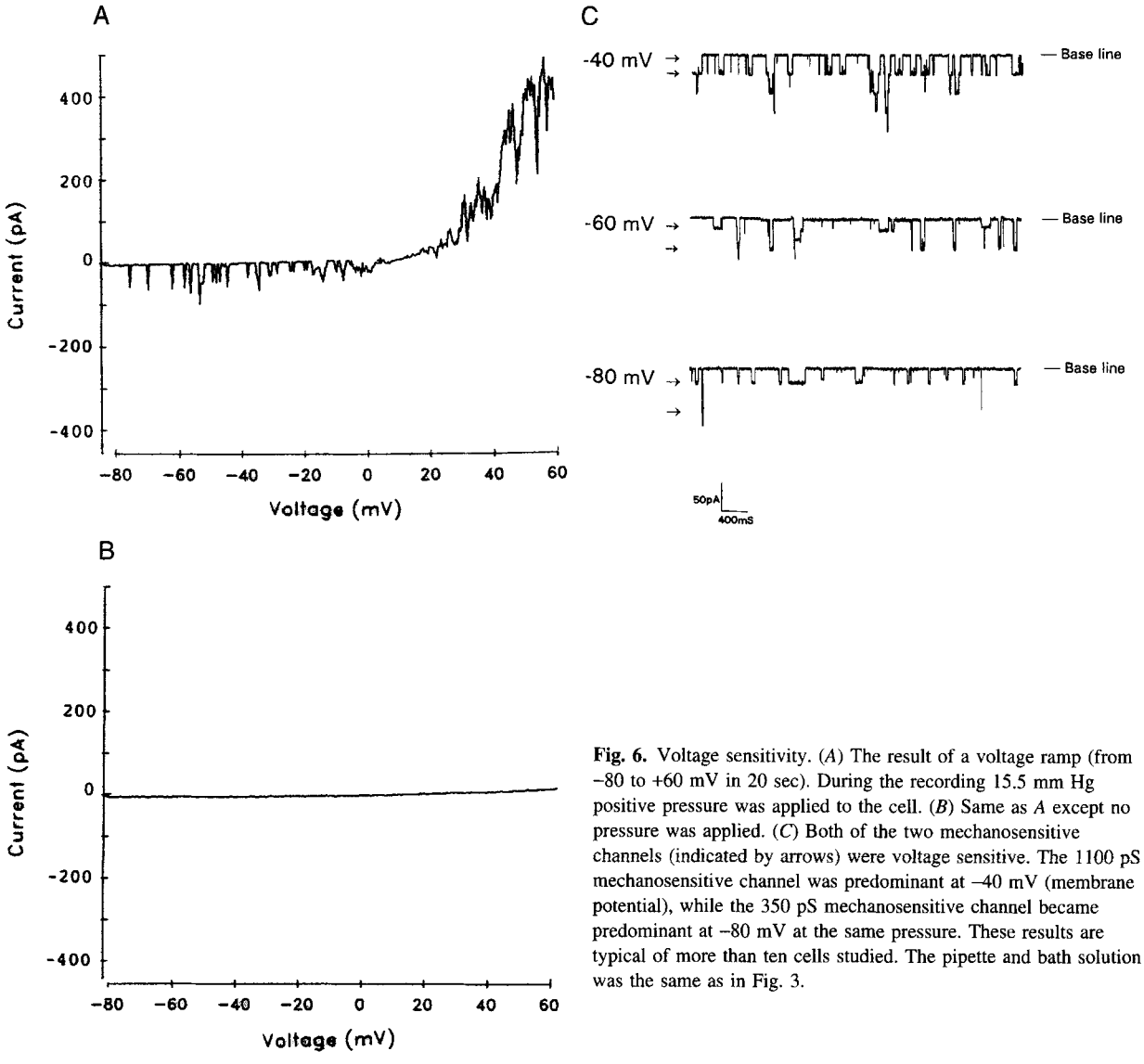

Fig. 6. Voltage sensitivity. (*A*) The result of a voltage ramp (from −80 to +60 mV in 20 sec). During the recording 15.5 mm Hg positive pressure was applied to the cell. (*B*) Same as *A* except no pressure was applied. (*C*) Both of the two mechanosensitive channels (indicated by arrows) were voltage sensitive. The 1100 pS mechanosensitive channel was predominant at −40 mV (membrane potential), while the 350 pS mechanosensitive channel became predominant at −80 mV at the same pressure. These results are typical of more than ten cells studied. The pipette and bath solution was the same as in Fig. 3.

those channels are located on the outer membrane of *E. coli* cells. Therefore it would not be possible to detect them by whole-cell patch clamp recording of protoplasts.

The activation of the mechanosensitive channels during osmotic down shock can be explained by the idea that the mechanosensitive channels are the receptors for membrane expansion (Martinac et al., 1992). When the cell is subjected to osmotic down shock the pressure across the membrane is increased. The increased pressure causes the stretch of the membrane, which presumably results in the activation of the mechanosensitive channels. The opening of the mechanosensitive channels allows the release of K^+, glutamate$^-$, Cl^-, and other small molecules. This helps the cell to release the excessive pressure and thus to adapt to the osmolarity change. It is possible that these mechanosensitive channels are regulated by osmotic regulators or sensors of *E. coli*.

The mechanosensitive channels of the protoplasts were also studied in excised patch recordings (*data not shown*). The two mechanosensitive channels observed in the whole-cell recording of the protoplasts could be found also in the excised patches (outside out). These channels could be activated either by positive pressure or negative pressure (blowing or sucking) which was applied through the pipette to the membrane.

The voltage ramp results (Fig. 6) showed that the pressure sensitivity of the protoplasts was voltage dependent: the cells were more sensitive to pressure upon depolarization (interior becoming more positive) than hyperpolarization (interior becoming more negative). This result may have significant biological meaning. When *E. coli* cells are subjected to osmotic down shock, there will be active mechanosensitive channels. This will cause a transient depolarization of the cell before K^+ is

Table. Comparison of the open probabilities of the 1100 pS channel and the 350 pS channel at different voltages

	−40 mV	−60 mV	−80 mV
1100 pS channel	1.7×10^{-1}	4.0×10^{-2}	1.1×10^{-2}
350 pS channel	2.6×10^{-2}	6.4×10^{-2}	1.6×10^{-1}

Open probability at each voltage was calculated from data of 40 sec-recordings. The open probability of the 1100 pS channel and the 350 pS channel were calculated separately. Overlapping events of the 1100 pS and the 350 pS channels were excluded when open time and amplitude were measured. The voltages indicated here represent the membrane potential of the cell.

totally released. The transient depolarization will make more mechanosensitive channels active because of voltage sensitivity. That in turn will speed up the release of solutes from the cell and prevent possible cell bursting caused by osmotic down shock.

The mechanosensitive channels of the protoplasts were blocked by Gd^{3+}. Gd^{3+} has been used for blocking pressure-sensitive channels in different kinds of cells (Gustin et al., 1988; Yang & Sachs, 1988; Yang & Sachs, 1989; Berrier et al., 1992). In *E. coli* 100 μM Gd^{3+} blocks several big conductance pressure-sensitive channels but not the small conductance pressure-sensitive channels (Berrier et al., 1992). In our study, Gd^{3+} was able to block both of the pressure-sensitive channels found. However, higher concentrations of Gd^{3+} were needed for total blockage. We found that depending on its concentration Gd^{3+} could either be an activator or an inhibitor of the mechanosensitive channels. Although the mechanism of blocking mechanosensitive channels by Gd^{3+} is not known, it is possible that Gd^{3+} could not only block the channels but also affect the gating of the channels in a way that increases the pressure sensitivity. At low concentrations of Gd^{3+}, the increased pressure sensitivity could overcome the blocking effect of Gd^{3+}. Therefore the overall effect of low concentrations of Gd^{3+} was to stimulate the channel activity.

The opening of mechanosensitive channels of the protoplasts was inhibited by low pH. The channel activity decreased sharply with low pH, while high pH did not affect the channels as much. This inhibition by low pH was not due to the acidic form of the buffer but due to protons. Although the effect of protons on *E. coli* mechanosensitive channels has not been documented previously, it is not unusual that protons can affect channel activity. It has been reported that acidic pH could decrease OmpF and OmpC channel size and affect the channel activity in *E. coli* (Todt, Rocgue & McGroarty, 1992). Also, it has been shown that increasing the extracellular pH from 7.4 to 10 can modulate the stretch-activated channel in chick skeletal muscle (Guharay & Sachs, 1985). Both of the mechanosensitive channels reported here could be inhibited by low pH, although the 350 pS channel was less sensitive than the 1100 pS channel (*data not shown*). The low pH effect on the mechanosensitive channels could be due to protons increasing the tension threshold for activation of the channels. This could result from protonation of amino groups of the channel protein or pH-dependent conformational changes of the channel structure.

The mechanosensitive channels in the protoplasts of

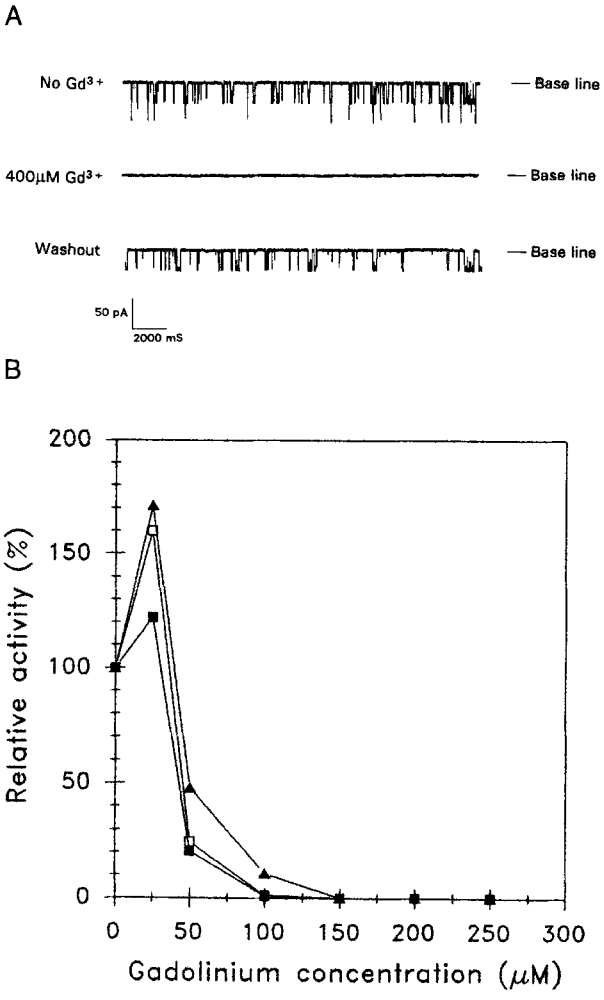

Fig. 7. Blockage of the mechanosensitive channels by Gd^{3+}. (*A*) The mechanosensitive channels were reversibly blocked by the addition of 400 μm $GdCl_3$ to the bath solution. 27 mm Hg pressure was constantly applied to the cell. The cell diameter was 2.3 μm. (*B*) Dose-response curves of Gd^{3+} at different pressures. The relative activity was defined as the mean current at each concentration of $GdCl_3$ divided by the mean current with no $GdCl_3$ in the bath solution. The constant pressure applied into the cell was (mm) 11 Hg (filled triangles), 13 Hg (open squares), and 15 Hg (filled squares). Cell diameter was 2.3 μm. The bath solution was (mM) 506 sorbitol, 100 NaCl, 5 KCl, 20 $MgCl_2$, 5 $CaCl_2$, and 10 HEPES, pH 7.2. $GdCl_3$ was added to the indicated concentrations. The membrane potential was −50 mV.

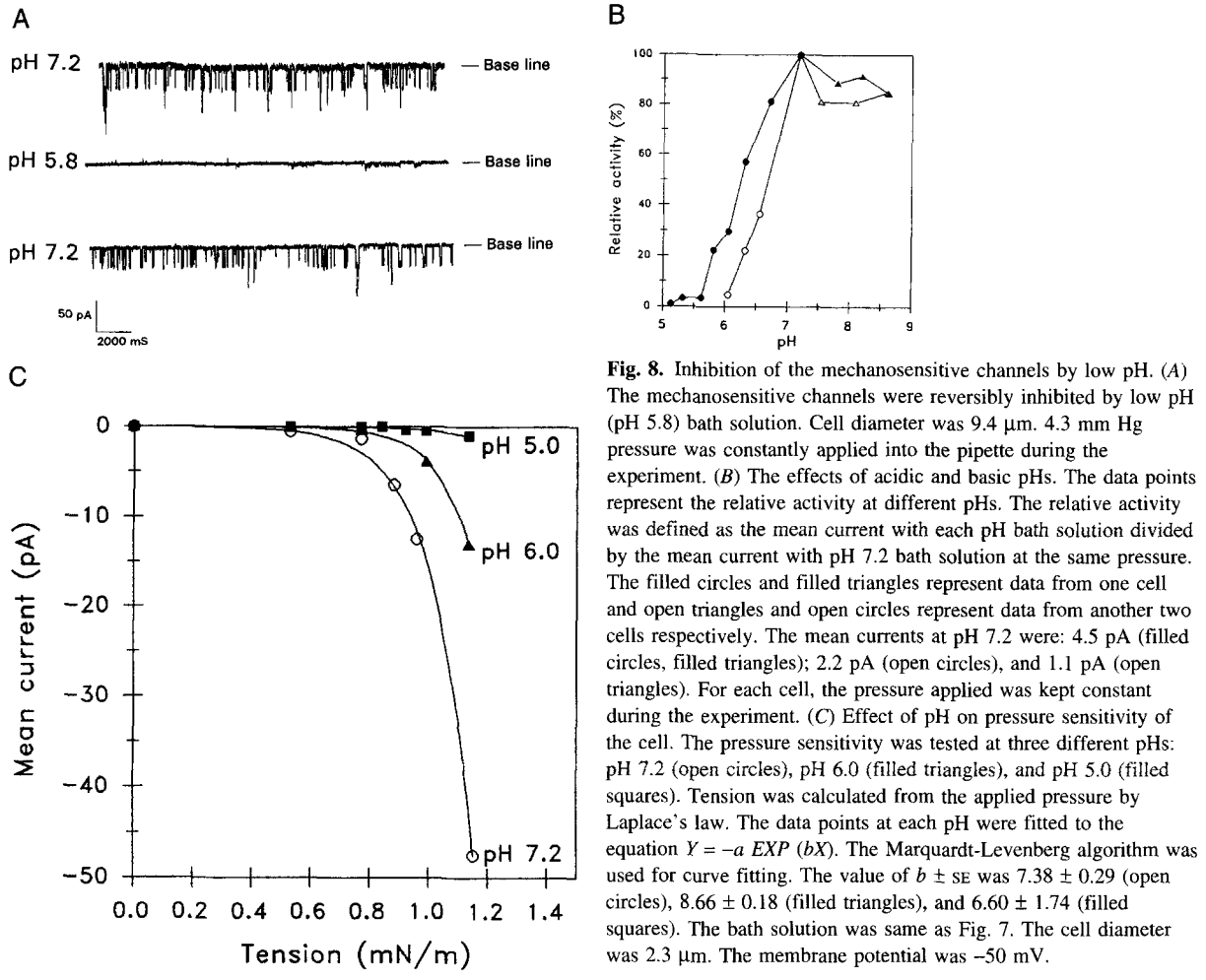

Fig. 8. Inhibition of the mechanosensitive channels by low pH. (*A*) The mechanosensitive channels were reversibly inhibited by low pH (pH 5.8) bath solution. Cell diameter was 9.4 μm. 4.3 mm Hg pressure was constantly applied into the pipette during the experiment. (*B*) The effects of acidic and basic pHs. The data points represent the relative activity at different pHs. The relative activity was defined as the mean current with each pH bath solution divided by the mean current with pH 7.2 bath solution at the same pressure. The filled circles and filled triangles represent data from one cell and open triangles and open circles represent data from another two cells respectively. The mean currents at pH 7.2 were: 4.5 pA (filled circles, filled triangles); 2.2 pA (open circles), and 1.1 pA (open triangles). For each cell, the pressure applied was kept constant during the experiment. (*C*) Effect of pH on pressure sensitivity of the cell. The pressure sensitivity was tested at three different pHs: pH 7.2 (open circles), pH 6.0 (filled triangles), and pH 5.0 (filled squares). Tension was calculated from the applied pressure by Laplace's law. The data points at each pH were fitted to the equation $Y = -a\ EXP\ (bX)$. The Marquardt-Levenberg algorithm was used for curve fitting. The value of $b \pm$ SE was 7.38 ± 0.29 (open circles), 8.66 ± 0.18 (filled triangles), and 6.60 ± 1.74 (filled squares). The bath solution was same as Fig. 7. The cell diameter was 2.3 μm. The membrane potential was −50 mV.

RQ2 (*kefA*$^-$) were different from those in Frag-1 (*kefA*$^+$ *kefB*$^+$ *kefC*$^+$) and MJF276 (*kefB*$^-$ *kefC*$^-$) (Fig. 9). The results indicate that the mutations in *kefB* and *kefC* do not affect the mechanosensitive channel activity, while the mutation in *kefA* does. This is consistent with what has been found for the possible roles of *KefA*, *KefB*, and *KefC* in K^+ efflux caused by different stimuli (Bakker et al., 1987; *see* Introduction). The phenotype of RQ2 is that the cells will not grow in a medium with high concentration of K^+ (e.g., tryptone broth + 500 mM KCl + 1 mM betaine) (W. Epstein, *unpublished results*). This is possibly due to the fact that the defect of *kefA* in RQ2 results in accumulation of K^+ inside the cells, which leads to inhibition of cell growth. The results presented here indicate that RQ2 is more sensitive to pressure than its parental strain Frag-1. One possible explanation could be that both KefA and mechanosensitive channels function during osmotic down shock; defective KefA could either directly or indirectly affect the mechanosensitive channels in a way that would possibly increase the pressure sensitivity so as to allow cell survival. Although the mechanosensitive channels are changed in RQ2, we do not think KefA itself is the mechanosensitive channel. This is because the mutation of *kefA* in RQ2 is a null mutation (W. Epstein, *personal communication*) and also because both the 1100 pS and the 350 pS channels still could be observed in RQ2. The detailed relationship between KefA and mechanosensitive channels needs to be studied further.

Aside from the mechanosensitive channels, another current which was often observed was a small outward current produced by depolarization of the cell from −60 to +40 mV (*data not shown*). No single channel activity of this current could be observed. Other currents could only be observed occasionally and they were unstable.

In this report, we successfully carried out whole-cell patch clamp recordings of *E. coli* protoplasts. The relatively quiet background of the protoplasts when pressure was not applied could be explained if most of the channels in the cytoplasmic membrane of *E. coli* are tightly

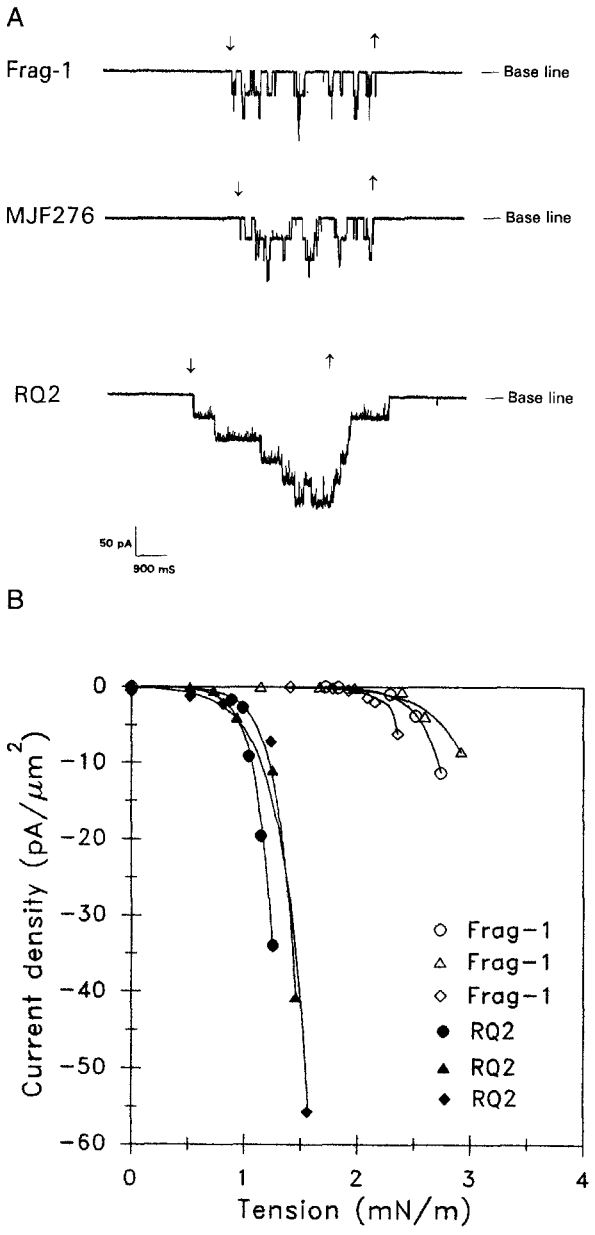

Fig. 9. The mechanosensitive channels in protoplasts of wild-type cells and K^+ transport-system mutants. (*A*) The mechanosensitive channels in Frag-1 (*kefA*$^+$ *kefB*$^+$ *kefC*$^+$), MJF276 (*kefA*$^+$ *kefB*$^-$ *kefC*$^-$) and in RQ2 (*kefA*$^-$ *kefB*$^+$ *kefC*$^+$). The bath solution was the same as in Fig. 7. The membrane potential was −50 mV. Pressure on and off is indicated by arrows. (*B*) The pressure sensitivities of Frag-1 and RQ2 were tested by applying different amounts of positive pressure into the cell. Current density was the mean current divided by the surface area of the cell. The cell diameters for Frag-1 cells were: open diamonds, 1.9 μm; open circles, 3.4 μm; open triangles, 6.3 μm. The cell diameters for RQ2 cells were: filled circles, 3.1 μm, filled diamonds, 3.9 μm; filled triangles, 6.3 μm.

regulated. They may open only upon presence of specific stimuli. Whole-cell recording provides a method to explore further the physiological role of ion channels in *E. coli*.

We thank Dr. A. Kubolski for his manuscript "Generation of giant protoplasts of *E. coli* and an inner-membrane selective conductance." We thank H.C. Berg, I.R. Booth, W. Epstein, and C. Kung for gifts of strains, and B. Martinac and A. Kubalski for the initial help in technique. This work was supported by a grant from the National Institutes of Health (AI08746).

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