J. Membrane Biol. 150, 143–152 (1996)

The Journal of
Membrane
Biology

Effect of Mutation of Potassium-Efflux System, KefA, on Mechanosensitive Channels in the Cytoplasmic Membrane of *Escherichia coli*

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Received: 6 September 1995/Revised: 13 December 1995

Abstract. The effect of a *kefA* mutation on the mechanosensitive channels in the cytoplasmic membrane of *Escherichia coli* was established by introducing a mutation of the *kefA* gene into wild-type *E. coli* by P1 transduction. The mutation of the *kefA* gene not only made the cells sensitive to K^+ in the medium but also changed the mechanosensitive channel activity. The *kefA* mutation did not change the conductances of the two mechanosensitive channels in the cytoplasmic membrane of *E. coli*, but it prolonged the channel open time. Also, the *kefA* mutation made the cells more sensitive to pressure in comparison to wild-type cells. The high sensitivity to pressure of the *kefA* mutant was not modulated by betaine or by the potassium gradient across the membrane. The effect of the *kefA* mutation on mechanosensitive channels was not due to a membrane fluidity change. KefA might be a regulator for mechanosensitive channels.

Key words: *Escherichia coli* — Mechanosensitive channels — Cytoplasmic membrane — Potassium efflux system — KefA — Potassium sensitivity

Introduction

When *E. coli* cells are subjected to osmotic down shock, turgor pressure is increased [10]. To dissipate the excess pressure, *E. coli* extrude K^+ and other small molecules [10, 15, 22]. There are two known mechanisms that might be involved in this process: the potassium-efflux systems and mechanosensitive channels.

Among the three potassium-efflux systems in *E. coli*, KefB and KefC are important in potassium efflux produced by adding *N*-ethylmaleimide [8, 9]. The function of the third potassium-efflux system, KefA [8], has not been well characterized. Its existence became evident by observing that K^+ efflux elicited by betaine, diethanolamine, and uncoupler 2,4-dinitrophenol was not significantly different between a *kefB kefC* double mutant and a *kefB*$^+$*kefC*$^+$ strain [4]. The K^+ efflux produced by adding betaine results from the increased turgor pressure due to betaine accumulation. The *kefA* gene was identified by a mutation that caused *E. coli* cells to be sensitive to high K^+ concentration in the growth medium (Dr. Wolfgang Epstein, *personal communication*).

A variety of mechanosensitive channels has been observed in *E. coli* [5, 6, 16, 21, 31]. Although the function of each channel is not entirely known, a few of them have been shown to play roles during the process of efflux induced by osmotic-down shock [6, 16]. It has been found that osmotic down shock of *E. coli* cells could induce the release of internal ions and small metabolites, such as K^+, glutamate, ATP, and lactose, without lysis of the cells [6]. This process could be slowed down or blocked by gadolinium ions [6]. Gd^{3+} blocks several mechanosensitive channels in the cytoplasmic membrane of *E. coli* [6, 16]. Therefore it is possible that release of internal ions and small metabolites induced by osmotic down shock acts through these mechanosensitive channels; at least they might be one of the mechanisms involved in the releasing process. Also, the mechanosensitive channels observed by whole-cell patch-clamp recording of *E. coli* protoplasts could be activated directly by osmotic down-shock [16]. If both potassium efflux system and mechanosensitive channels are involved in the process of releasing turgor pressure, it is possible that a relationship exists between these two mechanisms.

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In our previous study [16], we observed two different kinds of mechanosensitive channels in the cytoplasmic membrane of *E. coli* by whole-cell patch-clamp recording of giant protoplasts. These channels could be activated either by osmotic down shock of the cells or by directly applying positive pressure into the cell through the recording pipette. We found that a mutation of the *kefA* gene affected the pressure-sensitivity of the channels, while a *kefB kefC* double mutation had no effect [16].

In this report, we further characterize the effect of the *kefA* mutation on the mechanosensitive channel properties and pressure sensitivity of the cells. From our experimental results we postulate a possible mechanism for the effect of the *kefA* mutation on the mechanosensitive channels.

Materials and Methods

Strains and Chemicals

The three strains used for starting this study are: AW405 [2], the wild-type strain; RQ2 [16], the *kefA* mutant; and DSC101 [30], the *kefA*$^+$ strain which has a *hupB1*::*kan*^{r} insertion, about 20% cotransduceable with *kefA* (Dr. Wolfgang Epstein, *personal communication*). According to Wolfgang Epstein (*unpublished results*), RQ2 is a recessive mutant of *kefA* which was selected by UV mutagenesis. Its growth was inhibited by 500 mM KCl + 1 mM betaine in tryptone broth. To introduce the *kefA* mutation into AW405, P1 transduction [23] was performed in two steps. First, a kanamycin resistance marker (*kan*^{r}) was introduced into RQ2 from DSC101 by P1 transduction and the transductant AW890 was selected by *kan*^{r} and sensitivity to K^+. AW891 is the RQ2 derivative which has lost the K^+ sensitivity after this P1 transduction. Second, *kefA* and *kan*^{r} were introduced together into AW405 by transduction with P1 lysate from AW890. The resulting *kefA* mutant, named AW892, was selected by scoring both *kan*^{r} and K^+ sensitivity. An RQ2 revertant, AW893, was isolated by its ability to grow up after 24 hr in tryptone broth containing 500 mM KCl + 1 mM betaine.

Cells were grown in tryptone broth, which contains 10 g/l tryptone and 5 g/l NaCl [3] or Luria-Bertani medium [3]. Tryptone and yeast extract were purchased from Difco Laboratory (Detroit, MI); the other chemicals came from Sigma Chemical (St. Louis, MO).

Preparation of Giant Protoplasts

The detailed procedure for preparing giant protoplasts was described previously by Cui et al. (16 and Fig. 1 reported there). *E. coli* cells were first grown into long filamentous cells (100 to 120 μm) by blocking cell division with cephalexin [26]. By use of lysozyme and EDTA, these long filamentous cells were then converted into giant spheroplasts (3–5 μm in diameter), which had both peptidoglycan and outer membrane. Osmotic shock and further lysozyme treatment were used to finally make these spheroplasts into giant protoplasts, which have only their cytoplasmic membrane intact.

Electrical Recording and Data Analysis

The giant protoplasts were made from giant spheroplasts in the recording chamber by means of the procedure just described. Suitable giant protoplasts, identified by microscope, had gray spherical images and usually had outer membrane blebs attached [7, 16]. After the recording pipette touched the cell (the nonbleb part), the seal between the recording pipette and the cytoplasmic membrane was increased by slight suction in the recording pipette. Following the establishment of a gigaohm seal (>10 GΩ), either whole-cell or outside-out excised patch clamp recording was performed by the procedure of Hamill et al. [20].

Unless otherwise stated, the electrode pipette solution contained (in mM): 400 KCl, 2.0 $CaCl_2$, 1.7 $MgCl_2$, 10 EGTA, 80 sorbitol and 10 HEPES, pH 7.2. After the recording configuration had been formed, the original chamber solution (450 sorbitol, 100 NaCl, 5 KCl, 20 $MgCl_2$, 5 $CaCl_2$, and 10 HEPES, pH 7.2) was changed to experimental solution (described in the legend of each figure) by superfusing with ten times the chamber volume. To activate the mechanosensitive channels positive pressure (a blow) was applied into the cell through the recording pipette. The pressure applied 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 data were filtered at 1 KHZ and stored directly in a computer. Axon DMA interface and pClamp software (Axon Instruments, Foster City, CA) were used for data acquisition and analysis.

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

To analyze the channel open time, outside-out excised patch configuration was used. Small pressure was applied into the recording pipette so that the channel openings were well spaced and multiple simultaneous openings were infrequent. Overlapping events were rejected during the analysis. The opening and closing of the channel were detected by applying a 50% threshold criterion [13]. Since it was difficult to collect a large number of single-channel open events in a single patch, especially for the *kefA* mutant AW892, the data from several independent membrane patches were pooled together and fitted by exponential distribution. The pooled data showed results similar to the data obtained from the single patch with a large number of openings.

To test the pressure sensitivity, the mean current was used to represent the steady state whole-cell current. Mean current is defined as integration of the current over the recording time and dividing this integration by the recording time: $I = (1/t) \int_0^t i(t)\, dt$, where I is the mean current and t is the recording time. In most cases, 40 sec of recording was used for calculating the mean current. No inactivation of the channel activity was observed during this period. Since no two protoplasts have the same size, we used current density, which is the mean current divided by the cell surface area, to represent the current in a unit area.

To test the pressure sensitivity, different amounts of pressure were applied into the cell through the recording pipette. The pressure applied (P) was converted to tension (T) by Laplace's law, $T = d \cdot P / 4$ (d is the diameter of the cell). Current density was plotted against tension to characterize the pressure sensitivity of the cell.

Isolation of Cytoplasmic and Outer Membranes

E. coli cells were grown in tryptone broth and harvested as described [17]. The cell pellet was washed once (mM): 10 Tris buffer, pH 7.4, and resuspended in 5 ml of 400 sucrose, 10 EGTA, and 10 Tris, pH 8.2. Lysozyme was added to the cell suspension at a final concentration of 0.2 mg/ml. After a half hour on ice, the cell suspension was passed through a French-press (American Instrument, Silver Spring, MD) twice at 10,000 lb/in^2. DNase (200 μl of 5 mg/ml) and RNase (200 μl of 5 mg/ml) were added before centrifuging away the unbroken cells [17]. The supernatant fraction was loaded directly on the top of a

sucrose gradient [27] and centrifuged for 22 hr at 25,000 rpm. The separated cytoplasmic and outer membrane fractions were collected and each was resuspended in 1 ml of 10 mM Tris, pH 7.4.

Measurement of Phospholipids

The phospholipid contents of 0.1 ml cytoplasmic and outer membrane fractions were determined by modifying the method of Ames and Dubin [1]. The phospholipid content was calculated by assuming an average molecular weight of 700 [25].

Measurement of Membrane Fluidity

The membrane fluidity of wild-type AW405 and the *kefA* mutant AW892 was measured by the fluorescence polarization method [29]. The procedure used was the same as that described previously [28]. The fluorescence probe DPH (1,6-diphenyl 1, 3, 5-hexatriene) was used. It was excited at 375 nm and the emission was detected at 430 nm. The fluorescence polarization parameter, *P*, was directly obtained by using a SLM 800 Photon Counting Spectrofluorometer (SLM Instruments, Urbana-Champaign, IL). The membrane microviscosity (η) was calculated by the equation $\eta = 2P/(0.46 - P)$ [29]. Membrane fluidity is the reciprocal of microviscosity.

Results

Potassium Sensitivity of *kefA* Mutants

RQ2, the strain containing *kefA*, is sensitive to high concentrations of K^+ in the growth medium (Wolfgang Epstein, *unpublished results*). As RQ2 was selected by mutation with UV light, it is possible that there are several mutations in it, and so the K^+ sensitivity could result from mutations in one or several genes. To study this, we introduced the *kefA* mutation into wild-type AW405 by P1 transduction, which resulted in the *kefA* mutant AW892, and then we tested for K^+ sensitivity. The growth curves in tryptone broth with 500 mM KCl and 1 mM betaine were measured. Figure 1*A* shows that the growth of AW892 and RQ2 were inhibited compared to AW405 and AW891, a *kefA*$^+$ derivative of RQ2; AW892 was actually more inhibited than RQ2. These results show that *kefA* makes the cells sensitive to K^+ in the growth medium, and that introducing *kefA*$^+$ into RQ2 eliminates the K^+ sensitivity of the cells. Therefore the sensitivity to high K^+ in the growth medium was attributed to the *kefA* mutation alone, not to any additional mutations. The kanamycin resistance marker in AW892 did not contribute to K^+ sensitivity since AW405 *kan*^{r}, like AW405, was not sensitive to high K^+ in the growth medium (*data not shown*). In the remaining experiments AW405 and AW892 were used for studying the effect of KefA on mechanosensitive channels in the cytoplasmic membrane of *E. coli.*

Unlike KCl, high concentrations of NaCl in the growth medium did not inhibit the growth of the *kefA* mutant AW892. As shown in Fig. 1*B*, AW892 was grown under three different conditions: tryptone broth, tryptone broth with (in mM): 400 NaCl + 1 betaine, and tryptone broth with 400 KCl + 1 betaine. Only 400 KCl

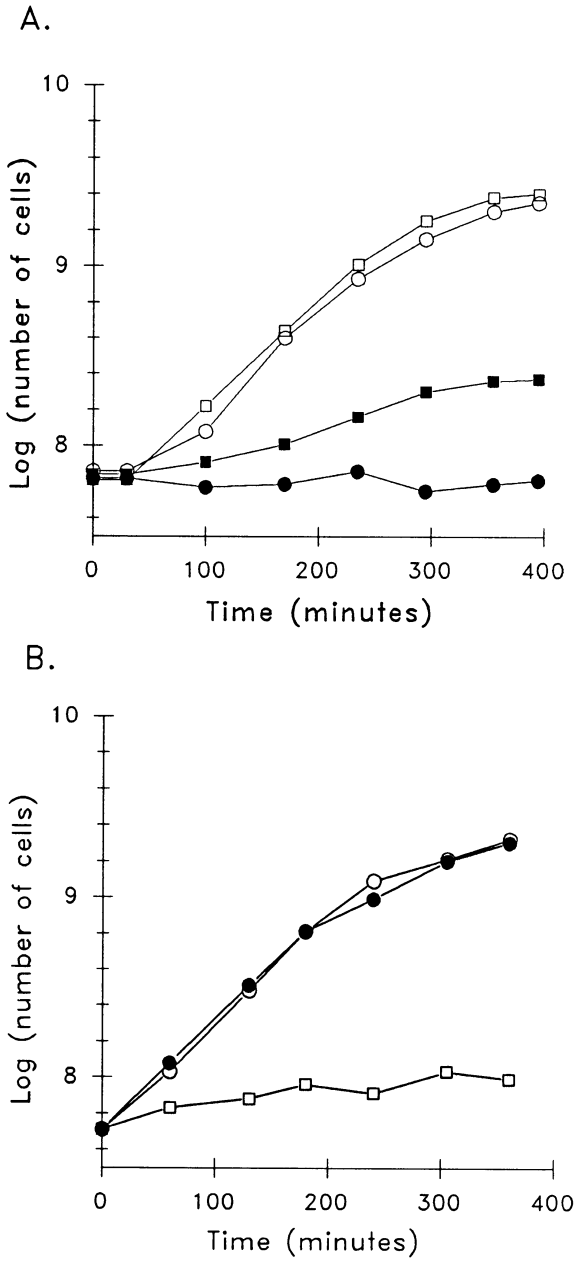

Fig. 1. Potassium sensitivity of growth. An overnight tryptone broth culture (100 μl) was inoculated into 10 ml of growth medium and shaken at 35°C. The growth of the cells was monitored by measuring the optical density at 590 nm. (*A*) Cells were grown in tryptone broth + 500 mM KCl + 1 mM betaine. ○: wild-type AW405; ●: *kefA* mutant AW892; □: AW891 (the RQ2 derivative which has lost its K^+ sensitivity); ■: *kefA* mutant RQ2. (*B*) AW892 cells were grown in three different media. ○: tryptone broth; ●: tryptone broth + 400 mM NaCl + 1 mM betaine; □: tryptone broth + 400 mM KCl + 1 mM betaine.

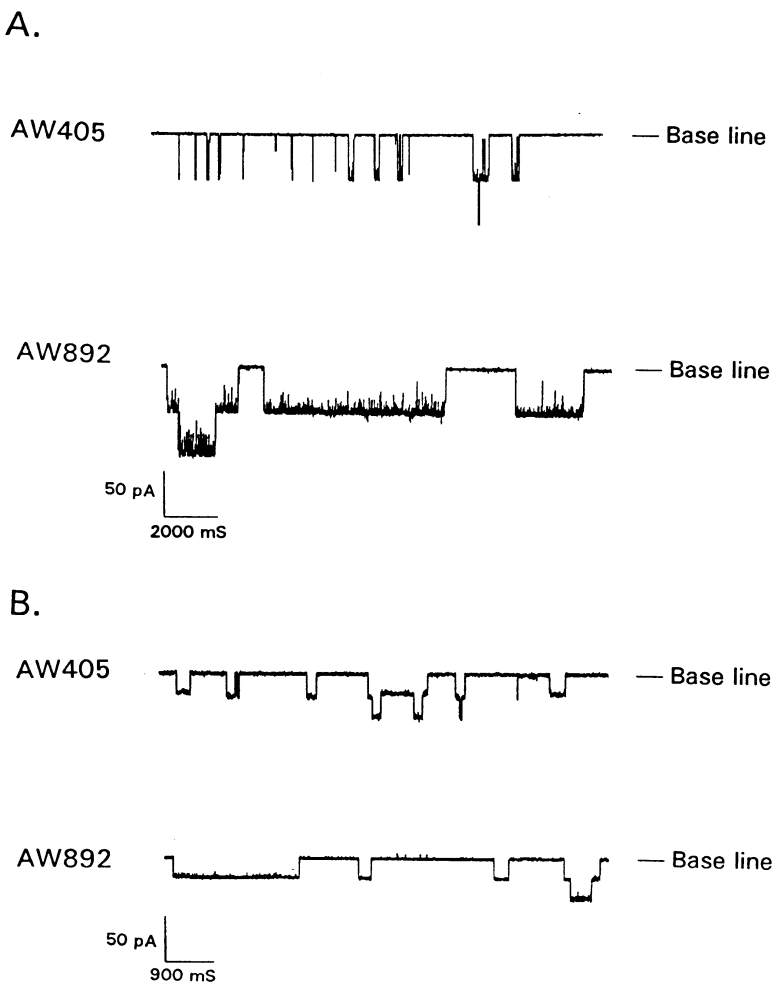

Fig. 2. Mechanosensitive channels in the cytoplasmic membrane of wild-type AW405 and *kefA* mutant AW892. (*A*) Outside-out excised patch recording of the big mechanosensitive channel in AW405 and AW892. The recording chamber solution contained (in mM): 520 sorbitol, 100 NaCl, 20 $MgCl_2$, 5 $CaCl_2$, and 10 HEPES, pH 7.2. (*B*) Outside-out excised patch recording of the small mechanosensitive channels in AW405 and AW892. Symmetrical 400 mM KCl solution was used. pH 5.2. The pipette voltage was –60 mV.

+ 1 betaine inhibited the cell growth, but not 400 NaCl + 1 betaine. This shows that inhibition of the growth of AW892 is not due to sensitivity to high-osmolarity growth conditions but rather to a high concentration of KCl in the growth medium. Actually, a KCl concentration in the tryptone broth as low as 50 mM with 1 mM betaine could inhibit the growth of AW892.

Mechanosensitive Channels in the Cytoplasmic Membrane of Wild-type and *kefA* Mutant

Two different mechanosensitive channels have been observed in the cytoplasmic membrane of *E. coli* by whole-cell patch-clamp recording of protoplasts [16]: one is an 1100 pS channel referred to here as the ''big channel,'' the other is a 350 pS channel called the ''small channel'' here. To find out how the *kefA* mutation affects these channels, whole-cell or outside-out excised patch-clamp recordings were performed on protoplasts of wild-type AW405 and the *kefA* mutant AW892. Figure 2 shows the outside-out excised patch recordings of the two mechanosensitive channels in the cytoplasmic membrane of AW405 and AW892. The mechanosensitive channels behaved differently in AW405 and AW892. Both of the two channels observed in the cytoplasmic membrane of AW405 were also present in AW892. However, the channels had longer open times in AW892: this is especially obvious for the big channel (Fig. 2*A*). To a lesser degree, the same was found with the small channel (Fig. 2*B*). The channel open time was further analyzed below (*see* Channel Open Time).

Channel Conductance

In order to find out whether the *kefA* mutation affects the conductances of the channels, the channel currents were recorded at various voltages and the *I-V* relationships were established (Fig. 3). The *I-V* relationship of the small channels was determined only at negative voltages because it was difficult to determine its unit current at positive voltages [16]. The conductances of the channels was determined by the slopes of the *I-V* relationships.

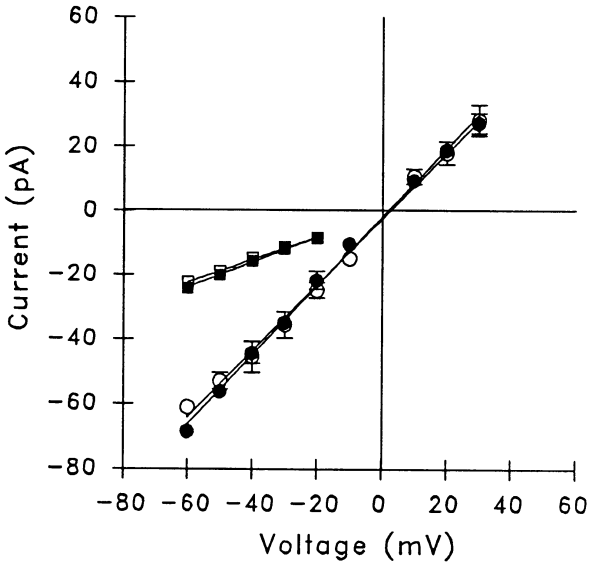

Fig. 3. Current-voltage relationship of mechanosensitive channels in symmetrical 400 mM KCl solution. ○: big channel of wild-type AW405; ●: big channel of *kefA* mutant AW892; □: small channel of wild-type AW405; ■: small channel of *kefA* mutant AW892. Each point is the average of data from at least three independent experiments. The error bars represent the standard deviation. The data points were fitted to linear regression ($y = a \cdot x + b$) by use of the Sigma-plot program.

Neither of the two channels in AW892 had significantly different conductances than in AW405. The conductances (mean ± SD, $n \geqslant 3$) of the two channels in AW405 were 356 ± 34 pS and 1071 ± 26 pS; in AW892 they were 393 ± 21 pS and 1073 ± 77 pS. This shows that the *kefA* mutation did not affect the unit conductances of the mechanosensitive channels.

Channel Open Time

As shown in Fig. 2, the *kefA* mutation prolonged the open times of the mechanosensitive channels. To further analyze this character, the single-channel activities of each of the two mechanosensitive channels were recorded under conditions such that simultaneous opening of several channels was rare. The histogram of open time was fitted to the sum of exponential distributions: $N = \sum_{i=1}^{\eta} (W_i / r_i) \cdot \exp(-t / r_i)$. The weight of the i th term (W_i) and the time constant (r_i) are listed in Table 1.

For the big channel, the open-time distribution was greatly changed in AW892 compared to AW405 (Fig. 4*A* and *B*). Not only the channel open-time was prolonged but also more exponential functions were needed to best fit the open-time distribution. In AW405, channel open times observed were from 1 msec to 510 msec and the histogram of open-time distribution was best fitted by two exponential functions. For AW892, the channel open-time was prolonged: it was distributed from 1 msec to 9420 msec. Three exponential functions were needed to best fit the histogram of the open time.

For the small channel, the open-time was not dramatically changed, although it was also prolonged (Fig. 4*C* and *D*). The open-time distribution of the small channels in both AW405 and AW892 could be fitted by two exponential functions. The difference of the time constants of the small channels in AW405 and AW892 did not disappear by using different fitting methods (square *Y* or log *Y*, where *Y* is the number of events) or by different ways of dividing the curve.

The different effects of the *kefA* mutation on these two kinds of mechanosensitive channels could be explained if KefA is more closely related to the big mechanosensitive channel than the small one.

Pressure Sensitivity

To test the pressure sensitivity, whole-cell patch-clamp recordings were performed on protoplasts of wild-type AW405 and the *kefA* mutant AW892. Different amounts of pressure were applied into the protoplasts through the recording pipette. The corresponding mechanosensitive currents activated were recorded. The mean current (*see* Materials and Methods) was used to represent the steady state whole-cell mechanosensitive current. Because the mechanosensitive channels are activated by tension but not directly by pressure [19], the current density was plotted against tension, which was calculated on the basis of cell diameter and the applied pressure (*see* Materials and Methods).

Figure 5*A* shows the data obtained from three AW405 cells and three AW892 cells. The data mostly indicated that the membrane ruptures at membrane tensions just a little in excess of those that steeply activate the channels. The data were fitted to an exponential distribution $y = -a \cdot \exp(b \cdot x)$. The pressure sensitivity was evaluated by the tension threshold for channel activation and steepness of the curve, *b*. The curves for AW892 were not only shifted to lower tension but also changed in steepness: $b = 8.9 \pm 2.3$ (mean ± SD, $n = 5$) in AW892 and 3.9 ± 1.2 (mean ± SD, $n = 5$) in AW405. These results indicate that the channels in AW892 had a lower tension threshold than those in AW405 and also that the channels in AW892 were much more sensitive to pressure than those in AW405. The curves did not reach saturation at the pressure applied. With higher pressure the whole-cell configuration was disrupted before the curves got saturated.

To further study the effect of the *kefA* mutation on pressure sensitivity, we tested AW405 and AW892 under other conditions. Betaine has been known as an osmotic protectant of *E. coli* [12]. Betaine accumulates inside the

Table 1. The open-time fitting parameters of the mechanosensitive channels in wild-type AW405 and *kefA* mutant AW892

	AW405	AW892
Big channels	$W_1 = 5.4 \times 10^3$, $r_1 = 1.8 \times 10^1$	$W_1 = 1.9 \times 10^3$, $r_1 = 2.0 \times 10^1$
	$W_2 = 4.5 \times 10^3$, $r_2 = 7.0 \times 10^1$	$W_2 = 7.4 \times 10^3$, $r_2 = 2.7 \times 10^1$
		$W_3 = 1.1 \times 10^4$, $r_3 = 1.6 \times 10^3$
Small channels	$W_1 = 5.2 \times 10^3$, $r_1 = 2.5 \times 10^1$	$W_1 = 7.9 \times 10^3$, $r_1 = 2.7 \times 10^1$
	$W_2 = 1.8 \times 10^4$, $r_2 = 1.2 \times 10^2$	$W_2 = 1.3 \times 10^4$, $r_2 = 2.6 \times 10^2$

The open-time histograms (Fig. 4) were fitted by the sum of the exponential distributions, $\sum_{i=1}^{n} (W_i / r_i) \cdot exp(-t/r_i)$, where W_i is the weight of the i th term and r_i is the time constant.

cells when it is present in high-osmolarity growth medium [11, 14]. As we have shown above (*see* POTASSIUM SENSITIVITY), 400 mM KCl + 1 mM betaine inhibited the growth of AW892 while 400 mM NaCl + 1 mM betaine did not inhibit its growth. To find out whether the pressure sensitivity is different under these two different conditions, we mimicked these growth conditions by using 300 mM betaine + 250 mM KCl inside the recording pipette and either 400 mM KCl or 400 mM NaCl outside the cells. The results are shown in Fig. 5*B*. The pressure sensitivity of the cells with 400 mM KCl outside was no different from the pressure sensitivity with 400 mM NaCl outside. The AW892 cells under both of the conditions (Fig. 5*B*) were more sensitive to the pressure than AW405 (Fig. 5*A*). These results indicate that although 400 mM KCl inhibits cell growth of AW892 while 400 mM NaCl does not (Fig. 1*B*), they do not affect differently the mechanosensitive channels or pressure sensitivity (Fig. 5*B*).

MEMBRANE FLUIDITY

The mechanosensitive channels in the *kefA* mutant AW892 had a prolonged open time (Fig. 4) and low tension threshold for activation (Fig. 5). One possible mechanism underlying these phenomena could be that there is a general membrane property change in the *kefA* mutant. The *kefA* mutation could allow a small pressure across the membrane to achieve the activation of the channel.

Since membrane fluidity change will affect the mechanical properties of the membrane, such as elastic moduli [24], it was possible that changes in the mechanosensitive channel activity in the *kefA* mutant were due to a change in membrane fluidity. To test this possibility, we compared the membrane fluidity of wild-type AW405 and *kefA* mutant AW892 by using the fluorescence polarization method [29]. It is difficult to accurately measure the membrane fluidity of intact *E. coli* cells because the fluorescence probe DPH (1, 6-diphenyl 1, 3, 5-hexatriene) can not easily access the cytoplasmic membrane, even in the case of EDTA-treated cells [18]. Therefore we measured the membrane fluidity by first separating the cytoplasmic and outer membranes on a sucrose gradient. The phospholipid content of each membrane fraction was measured (*see* Materials and Methods). Membrane suspensions corresponding to 50 μg of phospholipids were used for fluorescence polarization measurements. The membrane microviscosity (η) for each membrane fraction are listed in the Table 2. Since membrane fluidity is the inverse of microviscosity, the higher the η value the lower the membrane fluidity. The results indicate there is no significant difference of η values between AW405 and AW892 (T-test, $\alpha = 0.05$). This means that both the cytoplasmic and outer membrane fluidity of the two strains are very similar. Therefore the different channel activities of AW892 and AW405 are not due to a membrane fluidity change.

MECHANOSENSITIVE CHANNELS IN A REVERTANT OF *kefA* MUTANT

To further test the relation between KefA and the mechanosensitive channels we also studied an RQ2 revertant, AW893, whose growth was not inhibited by 500 mM KCl (*see* Materials and Methods). Unlike in RQ2, we observed in AW893 mainly small mechanosensitive conductances (Fig. 6). Since both the K^+ sensitivity and the mechanosensitive channels in the *kefA* revertant were changed, these results could be explained in two ways: (1) There was a mutation in the big mechanosensitive channel gene in the revertant and this channel mutation decreased the K^+ sensitivity. (2) There was another mutation in the *kefA* gene which reduced the K^+ sensitivity, and at same time this mutation could cause the inactivation of the big mechanosensitive channel. Either way, the K^+ sensitivity is closely related to the mechanosensitive channels in the cytoplasmic membrane of *E. coli.*

Discussion

This work demonstrates that a *kefA* mutation, which affects the potassium sensitivity of the cells, can change

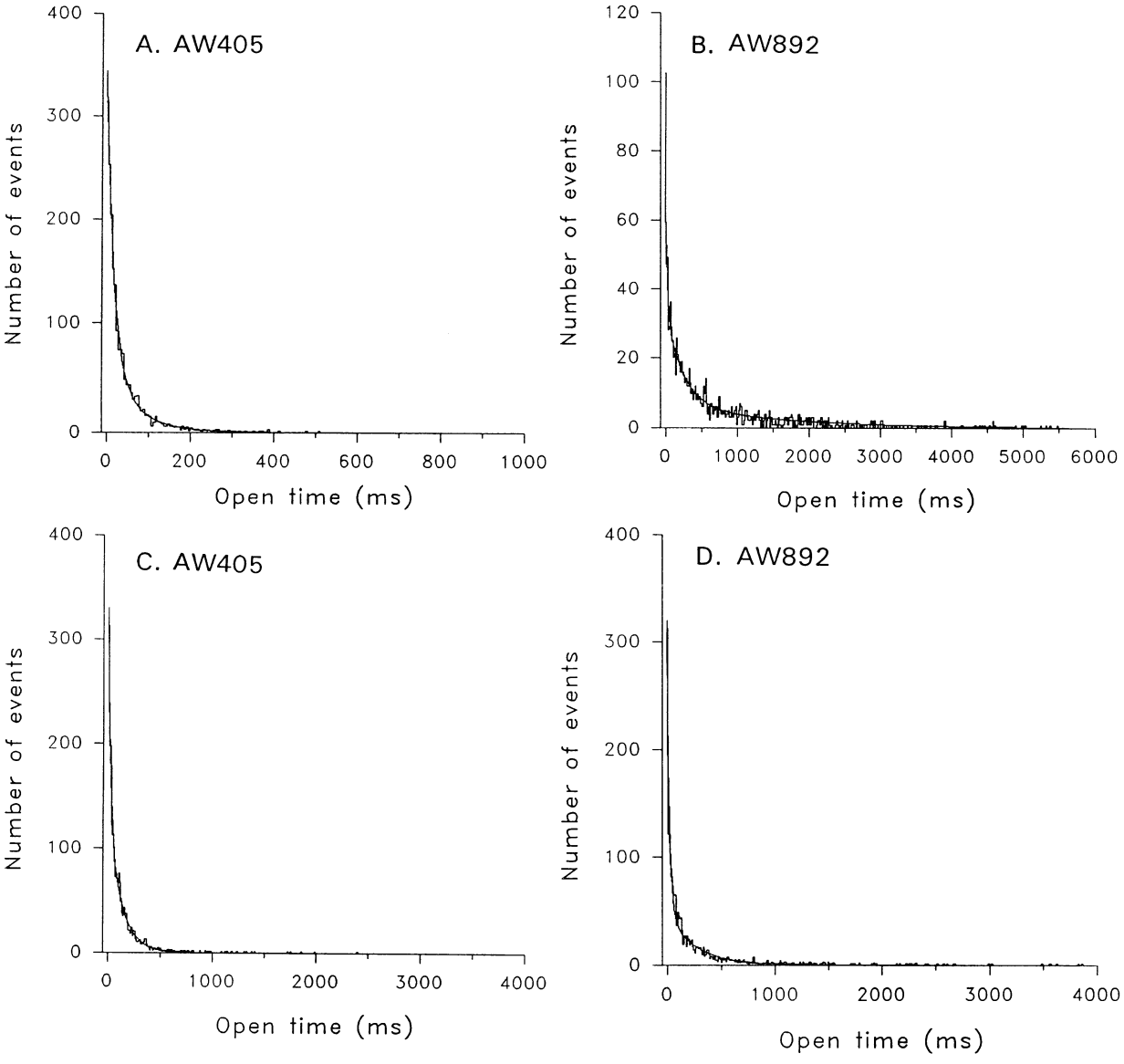

Fig. 4. Open-time distribution of mechanosensitive channels in wild-type AW405 and *kefA* mutant AW892. (*A*) Open-time distribution of the big mechanosensitive channel in AW405. The bin width was 5 msec. (*B*) Open-time distribution of the big mechanosensitive channel in AW892. The bin width was 15 msec. Note that the ordinate and abscissa are different for (*A*) and (*B*). (*C*) and (*D*) Open-time distribution of the small mechanosensitive channels in AW405 and AW892. The bin width of the data was 10 msec. Each histogram of open time was fitted to the sum of exponential distributions: $N = \sum_{i=1}^{\eta} (W_i / r_i) \cdot \exp(-t / r_i)$, where N is the number of events, W_i is the weight of the i th term, r_i is the time constant, and t is the open time. The goodness of fit of the open times in *A*, *B*, *C*, and *D*, was $\geqslant$2.1. The pipette voltage was –60 mV. The recording conditions were the same as in Fig. 2.

the activity of the mechanosensitive channels in the cytoplasmic membrane of *E. coli.* Although the *kefA* mutation did not alter the conductances of the two different kinds of mechanosensitive channels, it affected the channel open times and the pressure sensitivity of the cells.

These results suggest that the diameters of the channel pores were unaffected but the gating of the channel was altered by the *kefA* mutation. The prolonged channel open time of the big channel in the *kefA* mutant AW892 could be explained by incomplete closure once it was in the open state. However, it is also possible that the channel did close but the closing was so brief that the recording system could not detect such quick events, and so the adjacent two or several openings were detected as single openings which resulted in apparent longer open times.

The *kefA* mutation increased the open time of the big mechanosensitive channel while it had less effect on the

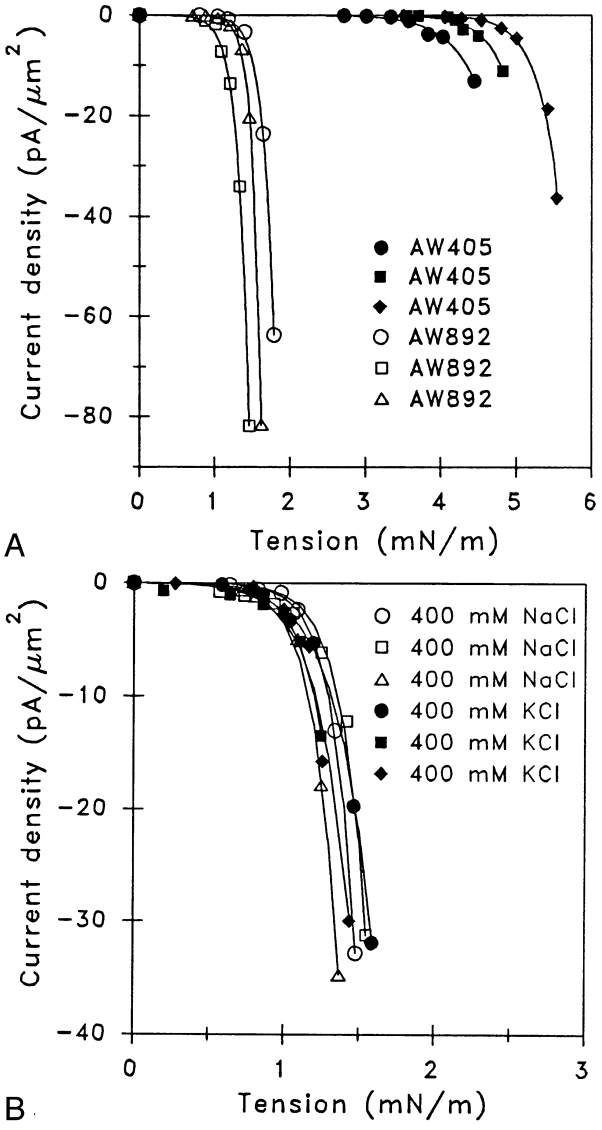

Fig. 5. Pressure sensitivity of wild-type AW405 and *kefA* mutant AW892. The current of the cell was recorded by the whole-cell patch-clamp recording configuration under different pressures. (*A*) ●, ■, ◆: AW405; ○, □, △: AW892. The recording pipette solution contained 400 mM NaCl instead of KCl. The recording chamber solution was the same as in Fig. 2*A*. (*B*) Pressure sensitivity of AW892 under different conditions: ○, □, △: three independent experiments with 400 mM NaCl solution outside of the cells; ●, ■, ◆: three independent experiments with 400 mM KCl solution outside the cells. The recording pipette solution contained 300 mM betaine and 250 mM KCl. Current density = mean current / cell surface area. Tension = cell diameter · pressure / 4.

open time of the small channel. These results support the idea that the big channel and the small channel are different entities, rather than that the small channel is a subunit of the big channel.

The *kefA* mutation has dual effects. One is that it sensitizes the cells to high K^+ concentration, which results in inhibition of growth by high concentrations of K^+ in the growth medium (Fig. 1*A*); the other is that it changes the mechanosensitive channel activity and makes the cells more sensitive to pressure (Fig. 4 and Fig. 5). To investigate whether the factors that affect the growth of the *kefA* mutant also affect the pressure sensitivity we tested effects of betaine and K^+ on the channels. The results shown in Fig. 5*B* indicate that neither including the osmotic protectant betaine in the recording pipette nor changing the K^+ gradient across the membrane affected the pressure sensitivity of AW892 cells. Even with absence of K^+ in both the recording pipette and the recording chamber, the *kefA* mutant AW892 still had higher pressure sensitivity than the wild-type AW405 (Fig. 5*A*). These results suggest that the pressure sensitivity of AW892 was not directly modulated by betaine or K^+.

Table 2. Membrane microviscosity of wild-type AW405 and *kefA* mutant AW892

	AW405	AW892
	$\eta \pm$ SD (poise)	$\eta \pm$ SD (poise)
Cytoplasmic membrane	1.01 ± 0.06, $n = 5$	0.97 ± 0.08, $n = 4$
Outer membrane	1.43 ± 0.06, $n = 3$	1.45 ± 0.05, $n = 3$

Membrane fractions corresponding to 50 μg of phospholipids were used for fluorescence polarization measurements [29]. Each sample was mixed with the fluorescence probe DPH by vigorously vortexing. A sample without DPH was used as control. η is microviscosity.

A possible mechanism for high concentrations of K^+ inhibiting the growth of AW892 (Fig. 1*A*) could be that the cells are unable to maintain a normal intracellular K^+ concentration due to the *kefA* mutation. Because the cells grow well in low K^+ media but grow poorly in high K^+ media (Fig. 1*B*), it is possible that the mutated cells (AW892 and RQ2) could not extrude the excess K^+ in a high K^+ environment. This would lead to an increase of the pressure across the membrane, especially in the presence of betaine in the growth medium. The increased pressure would activate the mechanosensitive channels and cause the leakage of the cells. The prolonged channel opening would make the cells lose components necessary for growth.

The cytoplasmic membrane fluidity of AW892 is not significantly different from that of AW405. Therefore the channel activity change is not due to a membrane fluidity change. The different effects of the *kefA* mutation on the small and big channels also indicate that the effects are not a result of general membrane fluidity change.

A possible mechanism for the effect of KefA on mechanosensitive channels is that KefA might be a binding protein. It is possible that KefA binds to the mechanosensitive channel like a spring. A mutation of *kefA*

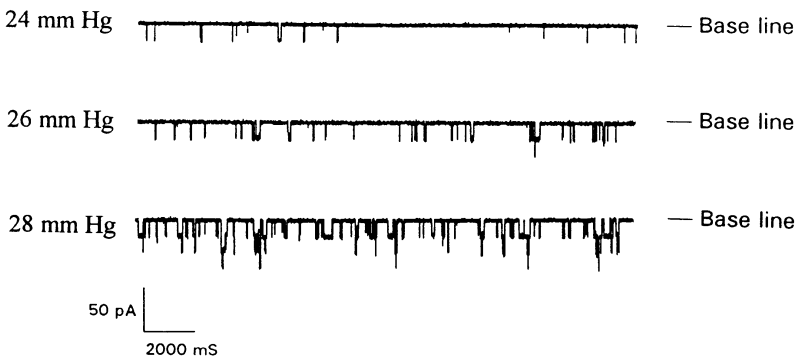

Fig. 6. Whole-cell patch clamp recordings of the mechanosensitive channels in the cytoplasmic membrane of AW893, the revertant of RQ2. The pressure applied into the recording pipette is indicated for each trace. The recording condition was the same as Fig. 2*A*. The pipette voltage was –60 mV.

would release the tension on the channel applied by this spring. Because of lack of the tension, only a relatively small pressure would be needed to open the mechanosensitive channel, which would make the *kefA* mutants, AW892 and RQ2, more sensitive to pressure in comparison to wild-type cells. Also, because there is no extra tension causing the open channel to close, it would remain in the open state longer. This could explain the prolonged open time (Fig. 4) and high pressure sensitivity (Fig. 5) of mechanosensitive channels in these *kefA* mutants. If this mechanism were correct, then KefA is likely a regulatory protein and is localized together with mechanosensitive channels. This theory could be further evaluated after obtaining the gene sequences for KefA and the mechanosensitive channels.

The results obtained with the *kefA* mutant and its revertant indicate that the mechanosensitive channels in the cytoplasmic membrane of *E. coli* are closely related to KefA. Up to now no mechanosensitive channels have been linked to any transport systems. The discovery that mechanosensitive channels in the cytoplasmic membrane of *E. coli* are regulated by the KefA potassium transport system can highlight the further study of the physiological role of these channels.

We thank Dr. Wolfgang Epstein for the gift of strains and Peter Chivers for technical help in fluorescence polarization measurements. We thank Dr. Kenneth McCormack for helpful discussion. This work was supported by National Institutes of Health grants AI08746 and GM52214. Changhai Cui received a Phillips Fellowship from the Department of Biochemistry, University of Wisconsin-Madison.

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