

Pressure-sensitive ion channel in *Escherichia coli*

(bacteria/spheroplast/patch-clamp technique/osmoregulation)

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Contributed by Julius Adler, December 15, 1986

ABSTRACT We have used the patch-clamp electrical recording technique on giant spheroplasts of *Escherichia coli* and have discovered pressure-activated ion channels. The channels have the following properties: (i) activation by slight positive or negative pressure; (ii) voltage dependence; (iii) large conductance; (iv) selectivity for anions over cations; (v) dependence of activity on the species of permeant ions. We believe that these channels may be involved in bacterial osmoregulation and osmotaxis.

Ion channels are gated protein pores found in biological membranes; these channels regulate many cellular interactions with the environment, including responses to hormonal, neuronal, and sensory stimuli (1). Ion channels have been studied in animals, plants, and microorganisms (2–4). In bacteria, *in vivo* channel activity has not been demonstrated, although the activity of isolated channel proteins has been measured in artificial membranes (5, 6).

The patch-clamp technique allows recording of current through individual ion channels in the native membrane by sucking the membrane onto a recording pipette to form a tight (gigaohm) electrical seal (7). This method has been used to study single channels *in vivo* in many eukaryotic cells, and it has demonstrated that the large currents measured across the membranes of a whole cell are really composed of many small currents passing through individual channels.

The lower limit to the diameter of the patch-pipette opening is about 1 μm (1); this precludes measurement of ion channels in bacteria directly. Cells of *Escherichia coli*, however, can become giant spheroplasts when grown in the presence of chemicals such as mecillinam to prevent cell wall (peptidoglycan) synthesis, and membrane potential has been measured in such spheroplasts by conventional electrophysiology (8). Giant spheroplasts can also be formed by growth of cells in the presence of cephalixin to prevent cell division and form filamentous “snakes”; these snakes can then be treated with lysozyme and EDTA to dissolve the cell wall (the spheroplasts can revert to normal form when returned to growth medium in the absence of these chemicals) (9). We used this latter method to make spheroplasts with a diameter of $\approx 6 \mu\text{m}$. We demonstrate here the application of *in vivo* patch-clamp recording to such giant spheroplasts. This method should be generally applicable to any bacterial species.

We discovered that a low positive or negative pressure (tens of millimeters of mercury; 1 mm Hg = 133 Pa) applied to the spheroplast membrane activates ion channels. This pressure could be caused by an osmotic difference of as little as a few milliosmolar across the membrane. We believe that these channels may allow *E. coli* to detect and to respond to small osmotic changes in the surrounding medium. The preliminary work has been reported in abstract form (10).

MATERIALS AND METHODS

Materials. Organic components of the growth medium were purchased from Difco. Tris was purchased from Boehringer Mannheim; other salts and chemicals for preparation of spheroplasts were from Sigma. KCl and NaCl for the patch-clamp buffers were Aldrich Gold Label.

Preparation of Giant Spheroplasts. Spheroplasts were prepared from *E. coli* strain AW405 in a manner similar to that described previously (9). A culture was grown overnight in 10 ml of modified Luria–Bertani medium (1% Bacto-tryptone/0.5% yeast extract/0.5% NaCl) in a 125-ml flask at 35°C with shaking (about 200 rpm on a New Brunswick model G76 gyrotory water-bath shaker), then diluted 1:100 in the same medium and grown in the same way to an OD₅₉₀ of 0.5–0.7. This culture (3 ml) was then diluted 1:10 into modified Luria–Bertani medium in a 250-ml flask, and cephalixin was added to 60 $\mu\text{g}/\text{ml}$. The culture was then shaken at 42°C for 2–2½ hr until single-cell filaments reached sufficient length (50–150 μm) for formation of giant spheroplasts (5–10 μm in diameter).

These filaments were harvested by centrifugation at 1500 $\times g$ for 4 min, and the pellet was rinsed without resuspension by gentle addition of 1 ml of 0.8 M sucrose and incubation at room temperature for 1 min. The supernatant was then removed with a Pasteur pipette and discarded, and the pellet was resuspended in 2.5 ml of 0.8 M sucrose. The following reagents were added in order: 150 μl of 1 M Tris Cl (pH 7.8); 120 μl of lysozyme (5 mg/ml); 30 μl of DNase I (5 mg/ml); 120 μl of 0.125 M Na EDTA (pH 8.0). This mixture was incubated at room temperature for 4–8 min to hydrolyze the peptidoglycan layer, and the progress of spheroplast formation was followed under the microscope. At the end of this incubation, 1 ml of a solution containing 20 mM MgCl₂ (to remove the EDTA and activate the DNase), 0.7 M sucrose, and 10 mM Tris Cl (pH 7.8) was added slowly (over 1 min) while stirring, and the mixture was incubated at room temperature for 4 min more. The mixture was then layered over two 7-ml solutions containing 10 mM MgCl₂, 0.8 M sucrose, and 10 mM Tris Cl (pH 7.2) in 13 \times 100-mm culture tubes kept on ice. The tubes were centrifuged for 2 min at 1000 $\times g$. The supernatant was removed with a Pasteur pipette, and the pellet of spheroplasts was resuspended in the remaining liquid (about 300 μl). The concentration of spheroplasts was estimated by microscopic examination, and the spheroplasts were then diluted in 10 mM MgCl₂/0.8 M sucrose/10 mM Tris Cl, pH 7.2, to bring the concentration to about 10⁸ per ml (OD₅₉₀ of 0.2).

Electrical Recording. The spheroplasts were destroyed if frozen rapidly by immersion into liquid N₂, but if frozen slowly by storage in a –20°C freezer, they could be used for several months. We did not find any difference between the channel properties of fresh and frozen preparations, and both were used for the experiments. The number of channels observed per patch did decrease gradually over a period of several months when frozen spheroplasts were used.

Spheroplasts (1.5–3 μl) were placed in a 0.5-ml bath containing, unless otherwise stated, 250 mM KCl, 90 mM

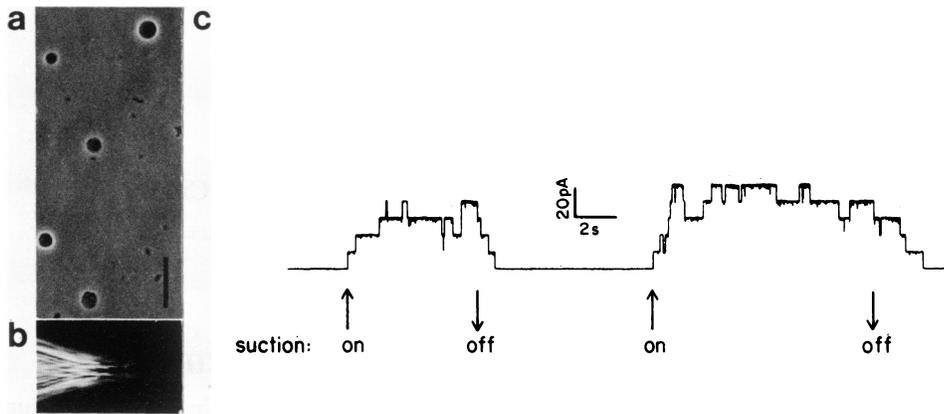


FIG. 1. (a) Phase-contrast micrograph of a sample of giant spheroplasts. Their average diameter was $6 \mu\text{m}$. (Bar = $20 \mu\text{m}$.) (b) A giant spheroplast on the tip of the recording pipette. (c) Channels from an on-cell patch of a giant spheroplast were activated when suction was applied to the recording pipette, and they closed after release of the suction (arrows); this caused large jumps in the measured conductance. The voltage imposed on the patch in this example was -10 mV .

MgCl_2 , 10 mM CaCl_2 , 0.1 mM EDTA , and 5 mM HEPES/KOH , pH 7.2. The patch pipettes (Boralex, Rochester Scientific, Rochester, NY) were coated with transparent nail enamel (Opulence, Cover Girl) before filling and were not fire-polished. The pipettes had a resistance of about $2.5 \text{ M}\Omega$ and were filled with a solution of 200 mM KCl , 40 mM MgCl_2 , 10 mM CaCl_2 , 0.1 mM EDTA , and 5 mM HEPES/KOH , pH 7.2. Suction was applied by mouth or syringe and gauged with a Hg manometer. All recordings were made by use of standard patch-clamp technique (7) at room temperature ($19\text{--}23^\circ\text{C}$) with a List-Medical EPC7 patch-clamp apparatus.

RESULTS

E. coli grows into filaments up to $150 \mu\text{m}$ long when treated with cephalaxin, which blocks septum formation (11); viable

giant spheroplasts ($\approx 6 \mu\text{m}$ in diameter, Fig. 1a) result from hydrolysis of the peptidoglycan layer of the filaments by treatment with lysozyme and EDTA (9, 12). Electrical recordings were made from single channels of these spheroplasts. We used strong suction ($\approx 100 \text{ mm Hg}$) for tens of seconds to draw a spheroplast onto the open tip of the pipette (Fig. 1b); this formed a tight ($1\text{--}2 \text{ G}\Omega$) seal, so that currents passing through channels at the tip of the pipette could be measured. After seal formation, suction was released. The formation of gigaohm seals on these spheroplasts required the presence of Mg^{2+} or Ca^{2+} in both the bath and the pipette solutions.

Channel openings were then recorded when mild pressure (tens of millimeters of mercury) was applied to the pipette by suction (Fig. 1c). Release of suction abruptly closed the

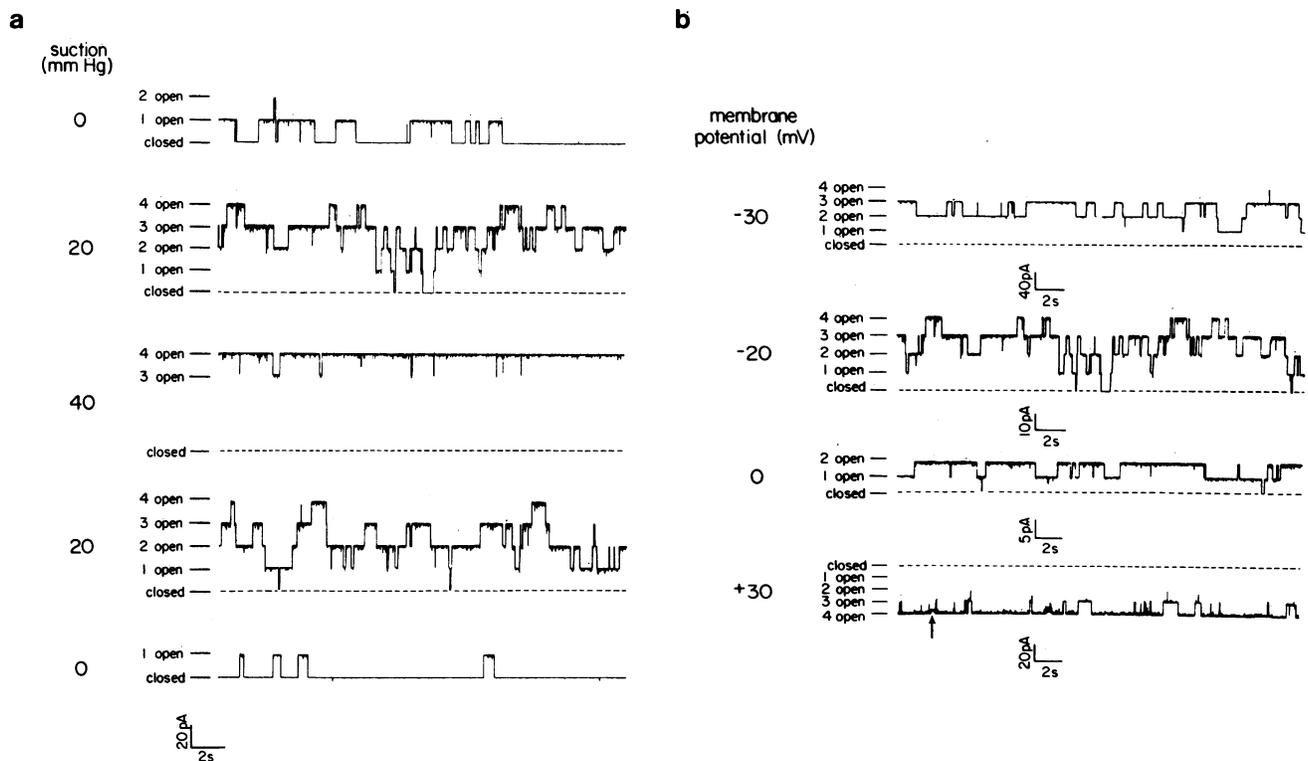


FIG. 2. Sensitivity of channels to pressure and voltage. (a) An on-cell patch shows the channel activity at different negative pressures (suction). The imposed membrane voltage was held constant (-20 mV), so the size of the current steps is the same at all pressures. (b) The channel activity of the same on-cell patch as in a is shown at different voltages. The negative pressure (suction) was held constant (20 mm Hg). For unknown reasons, the number of active channels (of a maximum of 4) was not the same at every voltage. There appeared to be no correlation between voltage imposed on the membrane and number of active channels. The size of the current steps varied with voltage imposed on the membrane, in accordance with Ohm's law. Note in b (arrow) a change of conductance smaller than the typical changes for this channel. Such substates, although infrequently observed, were present in almost every patch. They may indicate that the pressure-sensitive channel, like porin (13), is composed of a multimer of subunits that can occasionally open, close, or be blocked independently of each other.

channels. This activation of channels by pressure could be repeated an indefinite number of times, and it was seen in recordings both from whole spheroplasts ("on-cell" patches) and from excised patches of spheroplast membrane. Such channels have been encountered in almost every one of over 50 patches examined so far. The channels were also opened by positive pressure (blowing), but as this tended to break the seal, we present data obtained by suction only.

Effects of Pressure and Voltage. The reversible effect of suction (negative pressure) on the channel openings (at -20 mV) is shown in Fig. 2*a*. A maximum of 4 channels could be opened in this particular patch under these conditions. The number of channels per patch varied from 0 to 40, with about 5 being typical. Once activated, the channels often remained open for seconds. For different patches at the same voltage, a difference in pressure of as much as 20 mm Hg was required to obtain the same probability of opening; this variation might be caused by different geometry of the different patches, including size of pipette opening, amount of membrane inside the pipette, and variability between spheroplasts.

For the same patch as was used in Fig. 2*a*, the effect of varying the voltage on the channel openings (at suction of 20 mm Hg) is shown in Fig. 2*b*. The channel opening probability increased as the membrane was depolarized; this was also reversible (data not shown). Under these conditions, a maximum of 4 channels were open in this patch.

The data of Fig. 2 were put together with other data from the same patch to give Fig. 3, which shows the dependence of single-channel opening probability on the negative pressure (degree of suction) and voltage. The experimental points could be fitted to a series of theoretical curves described by the Boltzmann distribution. This distribution relates the probability that a channel is in the open state or in the closed state to the energy available to the channel. In Fig. 3*a*, the curves show the opening probability as a function of pressure at several different voltages. Note the parallel nature of these theoretical curves. At each voltage tested, when pressure was altered, the opening probability of the channels changed in accordance with theory. The channels show an e -fold change in activity (ratio of probability of being open to probability of being closed) for every 8-mm-Hg change in pressure. When the spheroplast was hyperpolarized, the opening probability of the channels decreased, and greater suction was required to open the channels. As the pressure needed to obtain the same opening probability varied from one patch to the next, data from only one patch were used for Fig. 3*a*; the data from two other patches showed similar dependencies of opening probability on pressure.

The same experimental points used in Fig. 3*a* are again used in Fig. 3*b* but are fitted to theoretical curves indicating the dependence of single-channel opening probability on voltage at several different negative pressures. Again the theoretical curves have a parallel nature. At each pressure tested, the relationship between opening probability and voltage obeys the Boltzmann distribution for a voltage-sensitive channel; the activity changes e -fold for every 15-mV change in membrane potential. Increasing the negative pressure shifts the curves toward activation at more negative pipette voltages.

Effects of Ion Concentration and Ion Species. When a spheroplast was lifted out of the bath solution and returned very quickly (<1 sec), the spheroplast was destroyed, leaving only a small patch of membrane across the opening of the pipette (excised patch). This allowed us to study the conductance and ion selectivity of the channels directly without having to take into account any difference in ion concentration between the bath and the inside of the spheroplast.

Fig. 4*a* shows the current-voltage relationship for a single channel of an excised patch in solutions that are symmetric (200 mM KCl on both sides of the membrane) or asymmetric

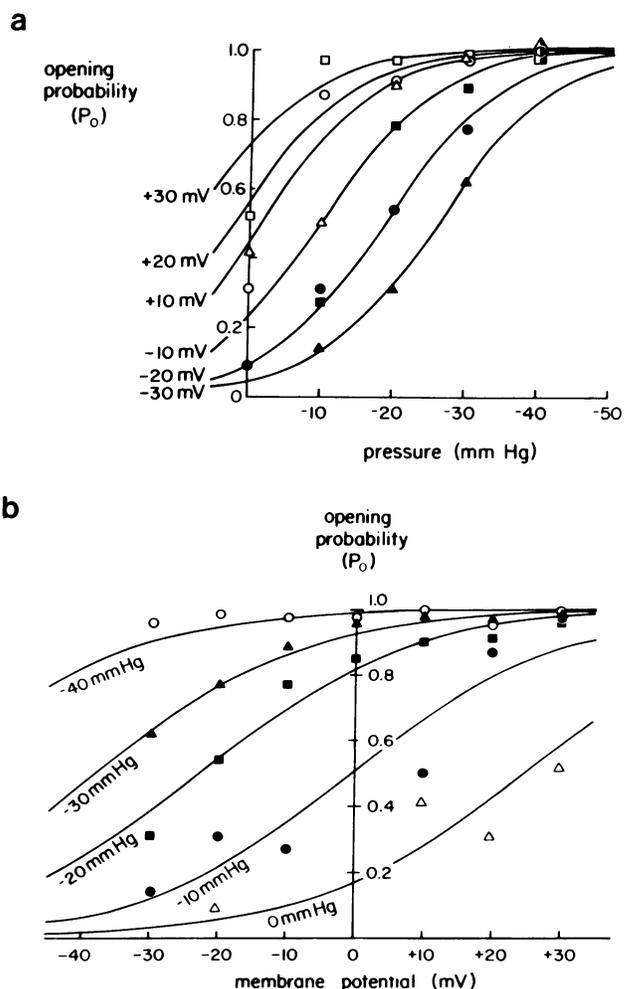


Fig. 3. Dependence of channel opening probability on pressure and voltage. (a) Data from Fig. 2 as well as additional data from the same on-cell patch are shown fitted to a specialized form of the Boltzmann distribution given by

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

where P_o is the opening probability, p is the negative pressure, $p_{1/2}$ is the pressure at which the channel is open half of the time ($P_o = 0.5$), and s_p is the slope of the plot of $\ln[P_o/(1 - P_o)]$ vs. pressure. The data were fitted to this logarithmic plot by linear regression. The opening probability of a single channel P_o was calculated by integrating the current passing through all channels during a recording period (min) and dividing this integral both by the number of channels active during that period and by the total current that would pass through a single channel open over the same period. This approach was used to exclude the effects of pressure and voltage on the number of active channels, so that solely the effects on gating properties of a single channel could be analyzed. \blacktriangle , \bullet , \blacksquare , \triangle , \circ , and \square represent -30 , -20 , -10 , $+10$, $+20$, and $+30$ mV, respectively. (b) Data from Fig. 2 as well as additional data from the same on-cell patch are shown fitted to a specialized form of the Boltzmann distribution given by

$$P_o = \{\exp[(V - V_{1/2})/s_v]\} / \{1 + \exp[(V - V_{1/2})/s_v]\},$$

where P_o is the opening probability, V is the voltage imposed across the membrane, $V_{1/2}$ is the voltage at which the channel is open half of the time ($P_o = 0.5$), and s_v is the slope of the plot of $\ln[P_o/(1 - P_o)]$ vs. voltage. The data were fitted to this logarithmic plot by linear regression. The opening probability of a single channel P_o was calculated in the same manner as in a. \triangle , \bullet , \blacksquare , \blacktriangle , and \circ represent 0, -10 , -20 , -30 , and -40 mm Hg, respectively.

(200 mM KCl in the pipette and 400 mM KCl in the bath). The conductance of the channel can be calculated from the slope, which was different at hyperpolarizing and depolarizing

voltages. In a symmetric solution the channel conductance at hyperpolarizing voltages was 970 pS; at depolarizing voltages the conductance decreased to 650 pS. After the concentration of KCl in the bath was increased to 400 mM, the voltage needed to balance the concentration gradient of ions (reversal potential) shifted from 0 to +8 mV; this change in reversal potential indicates a preference for anions over cations under these buffer conditions. When various ions were substituted for K^+ or for Cl^- (Fig. 4b), the reversal potentials varied from each other by only 6 mV or less; this suggests that the channel has only a low selectivity for the ions tested.

The activity of the channels was altered by the presence of different ions in the bath solution. When potassium acetate was substituted for KCl (Fig. 5a), the activity of the channels increased. A similar change was observed when potassium glutamate was substituted for KCl (data not shown). When NaCl replaced KCl (Fig. 5b), the pressure sensitivity and opening probability of the channels decreased. Different ions may interact with the channel to different extents, and this may affect the kinetics of the channel gating and its pressure sensitivity. The effect of different permeant ion species on gating kinetics has been documented for more selective ion channels (14, 15). Different anions can also destabilize water structure, thereby reducing nonpolar attractions in aqueous media and perturbing membrane structure to different extents; this effect could also be responsible for changes in channel activity (16).

DISCUSSION

In this paper we report the discovery of a pressure-activated ion channel in giant spheroplasts of *E. coli* by use of the patch-clamp technique.

The envelope of Gram-negative bacteria such as *E. coli* consists of an outer membrane and a cytoplasmic membrane separated by a peptidoglycan layer (which had been dissolved by lysozyme in the spheroplasts used here). At this point we do not know from which of these two membranes we are recording.

The channel that we are studying here has a large conductance (970 pS), is activated by pressure and by voltage, remains open for seconds once activated, and is present in almost every patch. Some of these properties are similar to those of porins, major outer membrane proteins that have been extensively studied by reconstitution into liposomes (17, 18) and into artificial planar lipid bilayers (5, 6). This suggests that the pressure-sensitive channel is from the outer membrane of *E. coli* and may indeed be a porin. This channel most resembles the PhoE phosphoporin and NmpC porin, which are selective for anions over cations when reconstituted into planar lipid bilayers (6, 19, 20). The voltage dependence of the pressure-sensitive channel differs from that of phosphoporin, however. The phosphoporin pores are open at voltages between 100 mV and -100 mV (19), whereas the pressure-sensitive channel we have described here is likely to be closed at voltages between +40 mV and -40 mV when the suction is less than 40 mm Hg (Fig. 3b). Phosphoporin can be blocked by millimolar concentrations of ATP or by micromolar concentrations of polyphosphates (19), whereas the pressure-sensitive channel shows no such blockage (unpublished data). When examined by patch-clamp, surrounded by the natural *E. coli* membrane, however, the protein might retain its original properties to a greater extent than in reconstituted membranes. Activation of porin conductances in artificial membranes indeed required the presence of some lipopolysaccharide and was greatly affected by the composition of membrane used (21). The relationship between the pressure-sensitive channel and the NmpC porin is at present unknown.

Stretch-activated channels have been found in rat dorsal root ganglion (22), in chicken skeletal muscle (23), in snail heart (24), and recently in yeast (M. C. Gustin, X.-L. Zhou, B.M., and C. Kung, unpublished data). Some properties of the K^+ and Na^+ channels of squid giant axon also change when hydrostatic pressure is applied (25), but the pressure required is several atmospheres and almost certainly has no physiological relevance. The pressure-sensitive channel described here can theoretically be activated by an osmolarity difference across the membrane of as little as a few mil-

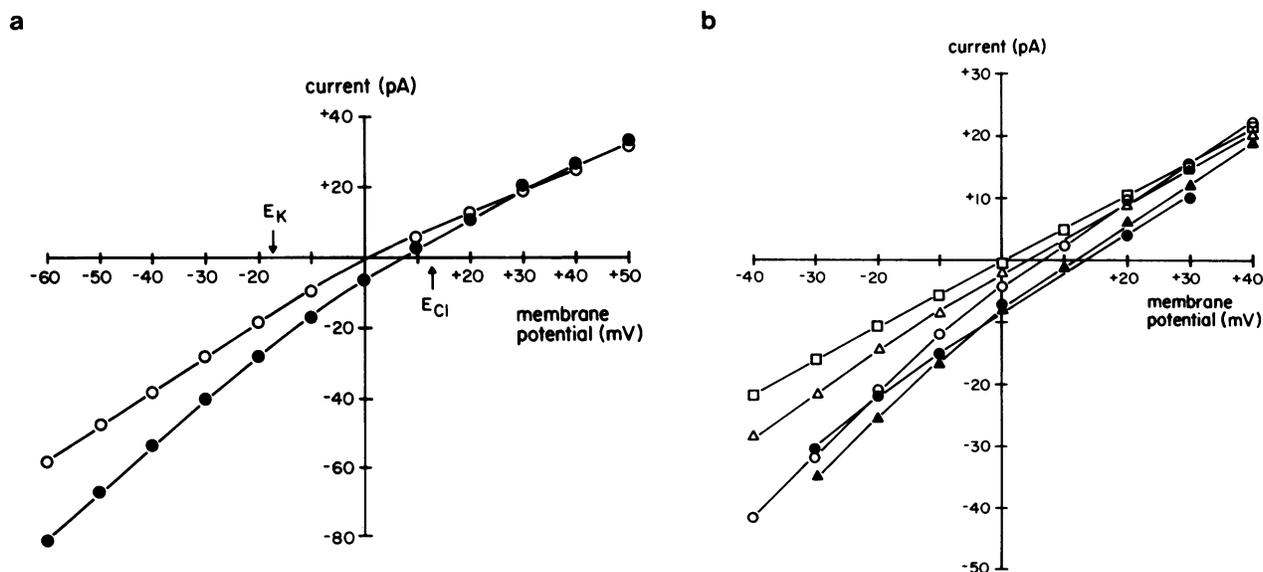


FIG. 4. Current-voltage plots for excised patches. The size of the current steps is plotted against the imposed membrane voltage. (a) The same bath and pipette solutions were used, consisting of 200 mM KCl, 40 mM $MgCl_2$, 10 mM $CaCl_2$, 0.1 mM EDTA, and 5 mM HEPES/KOH, pH 7.2 (\circ). Changing the KCl concentration in the bath to 400 mM shifted the reversal potential by about 8 mV in the positive direction (\bullet). In that case, the reversal potentials for Cl^- and K^+ (E_{Cl} and E_K) are, respectively, +12.9 mV and -17.5 mV and are indicated on the graph by arrows. (b) A different patch examined in the presence of various ions in the bath: 250 mM KCl (\circ); 250 mM glutamate (269 mM K^+) (\square); 250 mM potassium acetate (Δ); 250 mM KNO_3 (\blacktriangle); and 250 mM NaCl (\bullet). The pipette and bath solutions were the same as in a, except that 5 mM HEPES/NaOH, pH 7.2, was used as the buffer in the bath when Na^+ was tested.

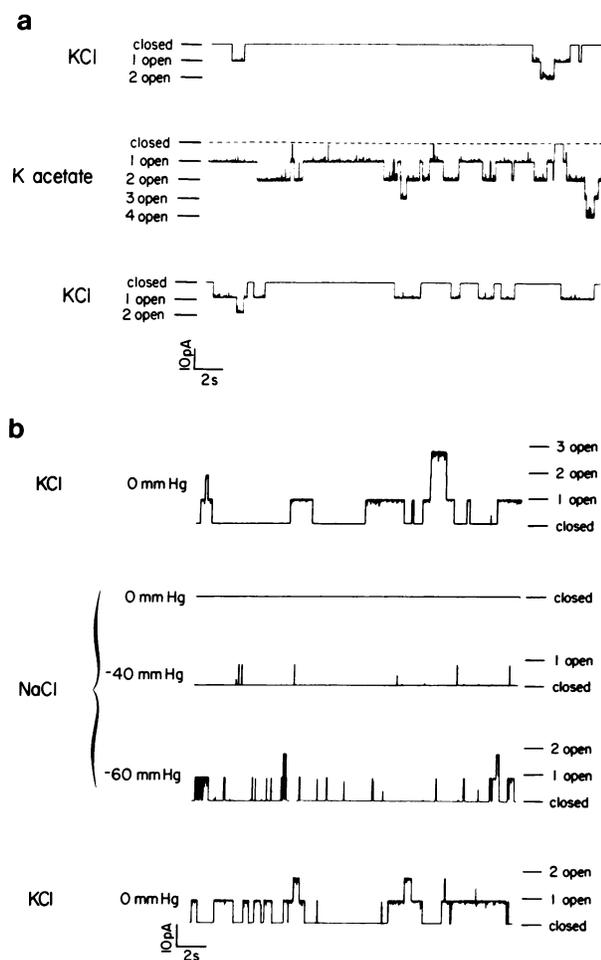


FIG. 5. The reversible effect of different ions on pressure sensitivity of channels is shown for a single excised patch. Pipette solution was the same as for Fig. 4. (a) The bath solution held 250 mM KCl or 250 mM potassium acetate; the latter caused an increase in the channel activity. The bath solution contained also 90 mM $MgCl_2$, 10 mM $CaCl_2$, 0.1 mM EDTA, and 5 mM Hepes/KOH, pH 7.2. The membrane potential was held at +20 mV. (b) The bath solution was made with 250 mM KCl or with 250 mM NaCl; the latter decreased the pressure sensitivity of the channel. The bath solution contained also 90 mM $MgCl_2$, 10 mM $CaCl_2$, 0.1 mM EDTA, and 5 mM Hepes/KOH, pH 7.2, when KCl was tested, or 5 mM Hepes/NaOH, pH 7.2, when NaCl was tested. The membrane potential was 0 mV.

liomolar. This extreme sensitivity indicates that the activation of this channel by pressure could be physiologically relevant, although the function remains to be established.

A pressure-sensitive ion channel could act as a sensor to detect osmotic changes in the external osmolarity through the change in turgor pressure on the membrane. Many responses of bacteria to changes in osmolarity have been studied. These include the production of osmoprotectant molecules in the cytoplasm (26) and in the periplasm (27), the regulation of synthesis of the OmpF and OmpC porins relative to each other (28), the regulation of the enzymes involved in K^+ transport (29) and maltose metabolism (30), and the release of low molecular weight compounds from the cytoplasm into the external medium (31), as well as a behavioral response

(osmotaxis) (refs. 32 and 33 and C.-Y. Li, C. Kung, and J. Adler, unpublished data). An ion channel could provide a common mechanism for these several responses. Elucidation of the mechanism of the pressure-sensitive channel, its physiological function, and its possible role in osmoregulation must await further study.

We thank Drs. Y. Saimi and M. C. Gustin for comments on the manuscript and Steve Limbach and Leslie Rabas for technical assistance. This work was supported by National Science Foundation Grant DMB-8402524, and by National Institutes of Health Grants AI08746, GM22714, and GM36386.

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