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Ion channels from the *Bacillus subtilis* plasma membrane incorporated into planar lipid bilayers

Carmen Alcayaga[a], Rodrigo Venegas[a], Alicia Carrasco[b] and Daniel Wolff[a]

[a]*Laboratorio de Fisiología Celular, Departamento de Biología,* and [b]*Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile*

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Fusion of *Bacillus subtilis* plasma membrane vesicles with planar lipid bilayers induced the appearance of discrete current fluctuations characteristic of ion channels. These channels showed a wide range of conductances and kinetic behaviors. In 300 mM KCl, their conductances ranged from a few hundreds of pS to more than 1 nS, and most of them exhibited several sub-states. The channels poorly discriminated between small univalent anions and cations. Some of them showed voltage dependence and most of them presented a complex gating kinetics. The results are consistent with the hypothesis of the presence in the *B. subtilis* plasma membrane of pores composed of subunits that function cooperatively.

Ion channel; Plasma membrane; Lipid bilayer; Bacteria; *Bacillus subtilis*

1. INTRODUCTION

Several studies have demonstrated the presence in the outer membrane of Gram-negative bacteria of very high conductance channels, called porins [1,2]. Their functional properties have been extensively investigated by biochemical and electrophysiological techniques [1,3]. Less is known about the presence of ion channels in the bacterial cytoplasmic membrane. Although they have been postulated to exist, it has also been argued that their presence would be incompatible with the maintenance of proton and ion gradients. However, these gradients could be maintained if channel activity were under strict regulation. There are reasons to postulate the presence of channels in the plasma membrane of these prokaryotes. Since they are free-living cells that exchange ions and molecules with the environment, some physiological processes might be mediated by channels. For example, the involvement of channels has been postulated in processes such as the transport of vitamins [4], protein excretion [5] and the response to osmotic stress [6,7].

The development of the patch-clamp technique has allowed the recording of single channel currents directly in the plasma membrane of a variety of cells. Using this method, large conductance and stretch-activated channels have been identified in *Escherichia coli* spheroplasts [8–10] and in proteoliposomes from *E. coli* membrane fractions [11]. Since Gram-negative bacteria have two membrane envelopes, there is still controversy about the location of these channels. Recently, Simon and Blobel [12] reported the fusion of *E. coli* plasma membrane vesicles to planar lipid bilayers. Large aqueous channels opened when signal peptides were added to the cytoplasmic side of the membrane.

Gram-positive bacteria are more appropriate for investigating cytoplasmic membrane channels in prokaryotic organisms since they only possess one lipid bilayer. The only communications in the literature so far reporting ion channels in these bacteria are those of Zoratti and collaborators [13,14]. Using the patch-clamp technique they have reported the presence of high conductance, stretch-activated, multiple substate ion channels in giant protoplasts of *Streptococcus faecalis* [13] and *Bacillus subtilis* [14,15].

In this work, we report the detection of *B. subtilis* plasma membrane ion channels, reconstituted into planar lipid bilayers. These studies are relevant since they could help to elucidate bacterial physiology and might shed light into the knowledge of the evolutionary origin of these membrane proteins. Preliminary results have previously been reported in an abstract [16].

2. MATERIALS AND METHODS

2.1. *Preparation of plasma membrane vesicles*

Plasma membrane vesicles were prepared according to a protocol based on the methods described by Osborn et al. [17] and Cull and McHenry [18].

Cells of *B. subtilis* ORB 161 were grown with aeration at 30°C in Pennasay broth (Difco) to an absorbance of 0.6–0.7 at 600 nm. Cells were first de-flagelated by vigorous agitation for 30 s in a Waring blender and then harvested by centrifugation at 13,000 × *g* for 30 min, at 4.0°C. The pellet was washed and resuspended in 10 mM Tris-Cl buffer, pH 8.0 and spun down again at 16,000 × *g* for 20 min. The new pellet was washed and resuspended in a hyperosmotic buffer contain-

Correspondence address: D. Wolff, Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile. Fax: (56) (2) 271-2983.

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ing 30 mM Tris-Cl, 20% sucrose, pH 8.0. Lysozyme was immediately added to a final concentration of 500 μg/ml, to digest the cell wall. Cells were then incubated under gentle agitation at 30°C. Protoplast formation was monitored by phase contrast microscopy and was generally greater than 90% after 45 min. Protoplasts were collected by centrifugation at 75,000 × g for 30 min, then resuspended and lysed in 400 ml of hypo-osmotic medium containing 10 mM EDTA-K, 15 mM $MgSO_4$, 5 mM K_3PO_4, pH 6.8. After 45 min of continuous agitation the lysate was spun down for 30 min at 13,000 × g to remove intact cells. The supernatant containing the plasma membrane vesicles, was then centrifuged at 105,000 × g for 1 h.

The pellet was resuspended in a small volume of a buffer containing 300 mM sucrose, 5 mM EDTA-K, pH 7.5 and centrifuged in a discontinuous sucrose gradient (layers of 20, 30 and 50%, containing 5.0 mM EDTA, pH 7.5) for 12 h at 100,000 × g. Plasma membrane vesicles were collected from the 30–50% band interface and resuspended (1.5–2.5 mg protein/ml) in 300 mM sucrose, 10 mM Tris-Cl, pH 8.0 buffer and characterized by transmission electron microscopy and succinate dehydrogenase activity. From the protoplast resuspension step all buffers contained also 1 mM PMSF in order to inhibit protease activity. The concentrated supernatant from the protoplast centrifugation did not show channel activity when tested in planar bilayers.

Sonication of protoplasts for 3 min (6 periods of 30 s) in an iso-osmotic medium, instead of the osmotic shock, was sometimes used as an alternative method for preparing membrane vesicles.

2.2. *Planar bilayers and channel incorporation*

Bilayers were formed according to the method of Mueller et al. [19] in a 300 μm diameter hole, made in a 25 μm thick Teflon partition, separating two aqueous compartments. The phospholipid solution used was brain phosphatidylethanolamine in decane (12.5 mg/ml). Bilayer resistances were always >50 GΩ and their capacitances were in the range of 150–300 pF. Planar bilayers were formed at different saline concentrations between 100–300 mM KCl. All solutions were buffered at pH 7.0 with MOPS NaOH or KOH. To favor fusion of plasma membrane vesicles to planar lipid bilayers, a concentration gradient was made by increasing the saline concentration in the compartment (*cis*) where the vesicles were added.

Channel insertion occurred spontaneously after addition of the plasma membrane vesicles to a final concentration of about 5 μg/ml. Appearance of channels was detected as rapid current fluctuations when a constant voltage was applied across the membrane.

Current through the channels was measured with a two-electrode voltage clamp, as described by Alvarez et al. [20]. One chamber compartment (*cis*) was connected to a voltage pulse generator and the opposite side (*trans*) to a low-noise current–voltage converter through Ag/AgCl electrodes. Electrodes were connected to the solution via agar bridges made in 1 M KCl. The *trans* side was connected to virtual ground. The current was filtered with an eight-pole Bessel low-pass filter (Frequency Devices 900), amplified and recorded either on FM or on video tape after digital encoding by an Instrutec VR-10 PCM. Data analysis was done by using a LabMaster data acquisition interface and the pClamp 5.5 analysis program. All experiments were carried out at 22 ± 2°C.

3. RESULTS

The fusion of *B. subtilis* plasma membrane vesicles with planar bilayers promoted the incorporation of conductive units that displayed discrete current fluctuations typical of ion channels. These conductive units presented a great variability in conductance and kinetic behavior.

In some cases the fusion of *B. subtilis* plasma membrane vesicles with the bilayer promoted the appearance of high conductance channels that decayed reversibly to

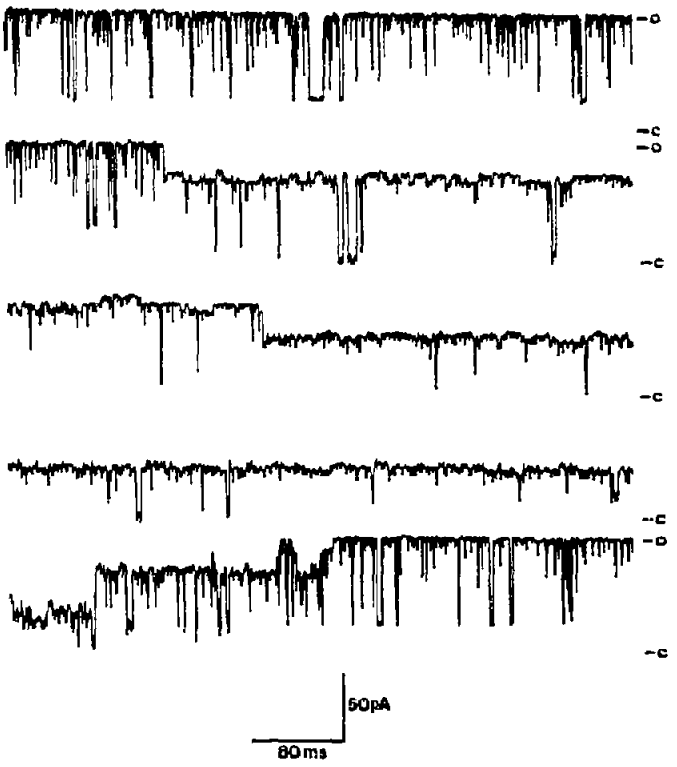

Fig. 1. Continuous current traces of a 1.4 nS conductance channel with sub-states and fast kinetics. The current was recorded at a constant voltage of 60 mV in a symmetric 300 mM KCl, 10 mM MOPS-K buffer, pH 7.0. Filter at 2 kHz.

intermediate conductance sub-states. An example of this behavior is shown in Fig. 1. The incorporation of a high conductance channel is shown. The channel is in the open state most of the time with very brief transitions to the closed state. After some time (59 s), the channel current decays to a lower value. Progressively the conductance decreases to further sub-states and the current becomes noisier. After a few seconds, the channel returns progressively to the highest conductance state. These conductances are difficult to characterize due to the diversity of sub-states and the complexity of their kinetics.

Another example of a high conductance channel with multiple sub-states is shown in Fig. 2. This channel fluctuates between intermediate and high conductance states with slower kinetics than the one shown in Fig. 1. Some complete closures, as observed in the third trace of Fig. 2A, indicate that the different conductance levels correspond to sub-states and not to different channels. The channel tends to dwell in the open state at negative potentials (Fig. 2B), indicating voltage dependence. Conductance amplitude histograms for this channel (Fig. 3) are complex and show the presence of several conductance levels.

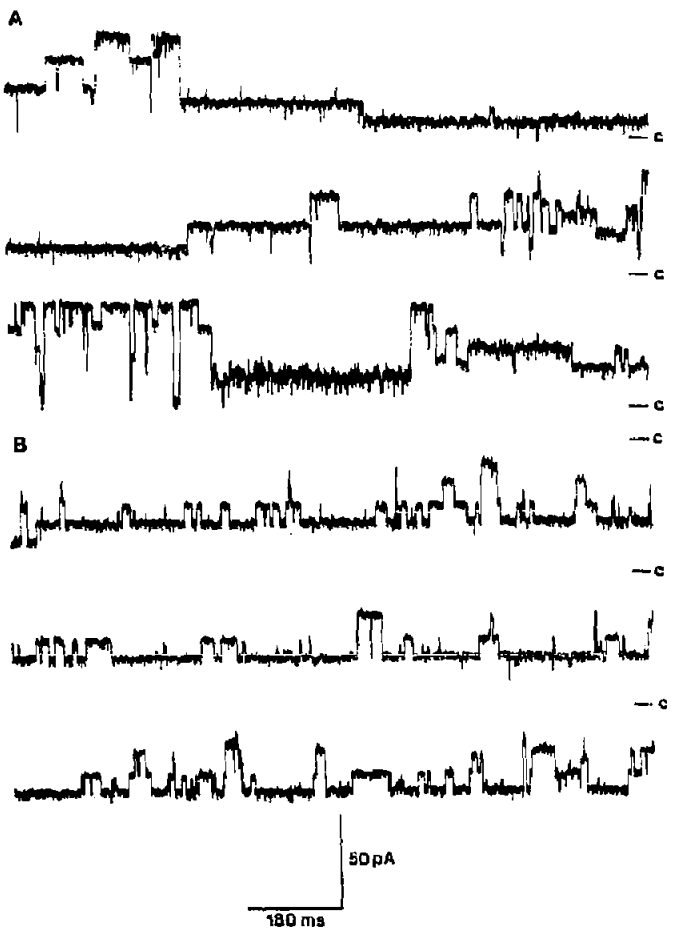

Fig. 2. Current records of a 1.5 nS conductance channel with substates and slow kinetics. The traces show continuous records obtained at 40 mV (A) and −40 mV (B), in symmetric 300 mM KCl, 10 mM MOPS-K buffer, pH 7.0. Filter at 2 kHz.

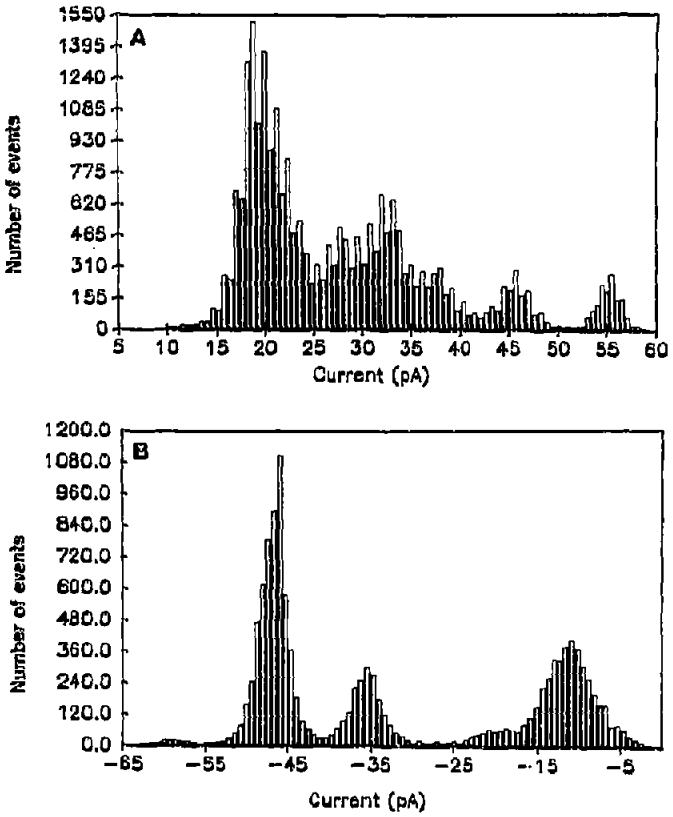

Fig. 3. Amplitude histogram of conductance values of the high conductance channel shown in Fig. 2, obtained at 40 mV (A) and −40 mV (B).

The channels illustrated in Figs. 1 and 2 presented close similarities with some *B. subtilis* high conductance channels showing spontaneous activity, in giant protoplasts (Fig. 4, [14]) and stretch-activated channels in proteoliposomes (Fig. 2B,C,D, [15]) recorded with the patch-clamp technique.

The incorporation of channels with lower conductance values was often seen. These lower conductance channels spontaneously incorporated to the bilayer after addition of the vesicles. They also appeared as a result of the irreversible temporal decay of some of the high conductance channels. They showed different gating kinetics and in some cases the open-closed transitions were very fast. They also presented substates of even smaller conductances that were in the range of 50–100 pS.

Fig. 4A illustrates a continuous recording of a 650 pS channel. At the beginning, the channel is in an open state and fluctuates very fast to a sub-state 50 pS lower in conductance. Occasionally the channel dwells in its closed state. After some time the channel decays to a 430 pS sub-state where it fluctuates between open and closed states. This channel was inhibited by lanthanides (Fig. 4B). The addition of Eu^{3+} when the channel was in 430 pS steady-state conductance, produced long closed states. Only occasionally fluctuations of short duration and a very low conductance (about 50 pS) were observed. This effect was partially reversible and might be due to either blockade or to an interaction of the ion with the channel gating mechanism.

We have examined the ionic selectivity in a few of the different channels we have recorded. In all cases the ion selectivity was also very low and channels did not discriminate between monovalent cations and anions. Fig. 5 shows a current–voltage relationship of the 650 pS channel in a KCl gradient. Using the Goldman–Hodgkin–Katz [21] equation a permeability ratio, P_K/P_{Cl} of 1.8 was calculated.

Fig. 6 illustrates records of a fast gating kinetics channel. This channel also present different sub-states and is not voltage dependent. Both records at −40 and 40 mV, have similar open probability.

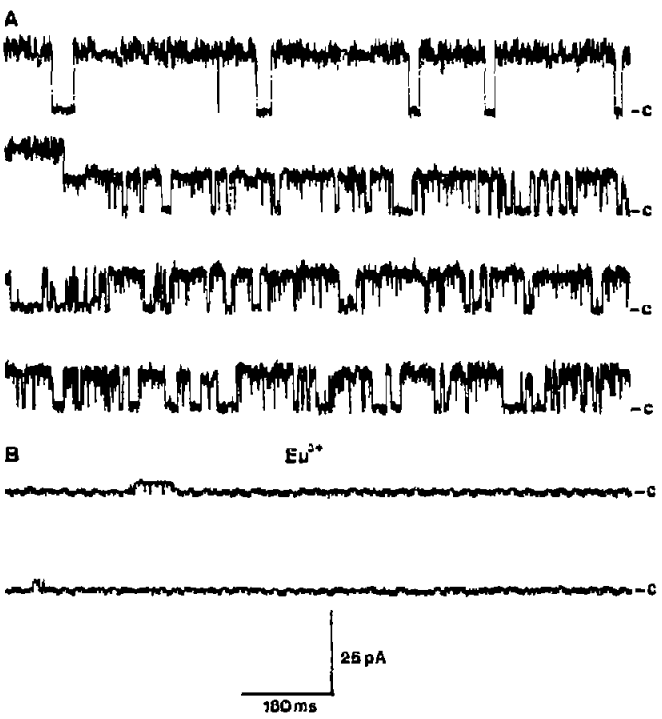

Fig. 4. (A) Time-course of the current decay of a channel with a main conductance of 650 pS. The recording was obtained at 40 mV in symmetric 300 mM KCl, 10 mM MOPS-K buffer, pH 7.0. (B) Channel activity was inhibited by 1 mM Eu^{3+}.

4. DISCUSSION

The results presented above show that the fusion of vesicles of *B. subtilis* plasma membrane fractions with planar lipid bilayers promoted the incorporation of ion channels. These channels exhibited a wide range of conductances and kinetic characteristics. Most of the recorded channels presented conductances between 300 and 700 pS and some of them, greater than 1 nS. Regardless of their conductance, these disparate channels

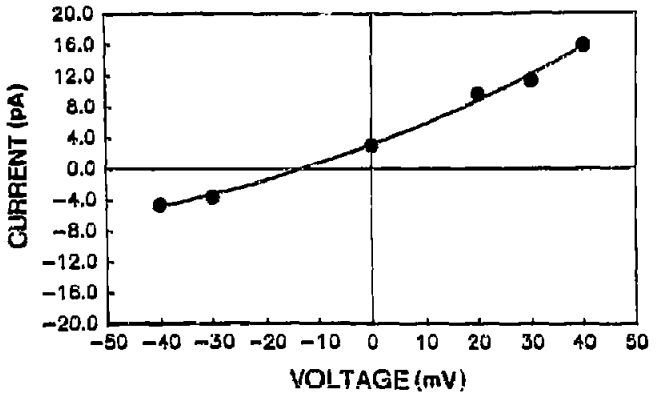

Fig. 5. Current–voltage relationship of the channel described in Fig. 4. in a 300/50 mM KCl, gradient. Under this condition the reversal potential obtained from the plot was −12 mV.

were rich in sub-states, they did not discriminate between small anions and cations and in general they presented heterogeneous and complex kinetics. These observations support the hypothesis of the existence of ionic pores in the plasma membrane of Eubacteria.

Our results, obtained with the bilayer method, have important similarities with those obtained with the patch-clamp technique in Gram-positive and -negative bacteria. Using this method Zoratti et al. [13–15] have detected the presence of very high conductance channels in the membranes of *S. faecalis* and *B. subtilis* giant protoplasts that were activated by stretch and voltage. These large pores presented sub-states, different gating kinetics and temporal decay similar to that observed by us in some high conductance channels. Similarly, they showed lack of ionic selectivity and were inhibited by lanthanides [22].

The main difference between our results and those obtained with the patch-clamp method is that the channels observed most frequently in bilayers usually presented smaller conductances. The method we used did not allow us to test the stretch sensitivity of the large conductance channels. However, the similarities in other properties, such as the presence of sub-states, lack of ionic selectivity and inhibition by lanthanides, suggest that we are detecting the same molecular entities.

The patch-clamp technique has been extensively used to investigate ion channels in the Gram-negative bacterium, *E. coli*. The presence of channels which exhibit voltage and stretch activation and sub-state structure has been reported in spheroplasts [8–10] and proteoliposomes obtained from *E. coli* fractions [11,22]. The location of these channels is still under debate. While Martinac et al. [8] have provided evidence that they are located in the outer membrane, the results obtained by Berrier et al. [11] suggest that they are in the plasma membrane.

Our observations and those of some other groups showing high conductance channels which present sub-states, suggest that these pores in the bacterial plasma membrane are constituted of subunits gating with a high degree of cooperativity. In the intact bacterial cell, cooperative behavior is presumably regulated by some still unknown mechanism. A disruption of the control mechanism could induce the disaggregation of the subunits, allowing the association into ensembles with lower stoichiometries.

The method for bacterial membrane vesicle preparation is an exhaustive process and includes lysozyme wall digestion, hypo-osmotic lysis or the sonication of protoplasts. Any of these manipulations might disrupt the spatial configuration that allows the channel regulation mechanisms. Lysozyme digestion, for example, could alter some connections between the cell wall and the plasma membrane. This disruption could promote subunit separation which might explain the lower conductance values of *B. subtilis* channels reconstituted in bilay-

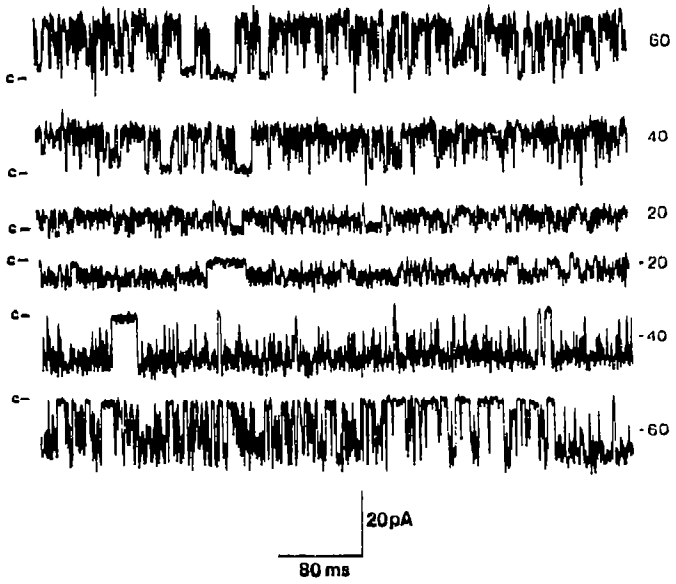

Fig. 6. Single channel records of a 320 pS main conductance channel showing sub-states and fast kinetics, at 6 different voltages. The experiment was done in symmetric 300 mM KCl, 10 mM MOPS-K buffer, pH 7.0. Filtered at 2 kHz.

ers compared with those obtained with the patch-clamp technique. Disruption of the native structure could also explain the wide diversity of channels we observed.

At present the function of these channels in the bacterial physiology is not known. Their physiological role has been questioned since it has been considered that their activity would be incompatible with the maintenance of the electrochemical proton gradient across the plasma membrane. However, this gradient could be maintained if, in the living bacterial cell, channel activity is under strict regulation. As mentioned in the introduction, one possibility is that these channels participate in volume regulation during osmotic stress. In isotonic conditions, the channels might be closed. If the cell is exposed to a hypo-osmotic medium they could be activated by membrane stretching, allowing the loss of K^+ ions or other osmolites [22].

It has also been suggested that high conductance channels could be involved in protein transport [5]. During polypeptide transfer through these channels there will be no ion conduction and the proton gradient will not collapse. The results of Simon and Blobel [12], showing that signal peptides promoted the opening of *E. coli* inner membrane ion channels incorporated into planar lipid bilayers, strongly support this hypothesis. Another possibility is that these channels are an abnormal expression of the proton channels which are part of the bacterial motility apparatus [24]. Studies to test some of these hypotheses are now being carried out.

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REFERENCES

[1] Nikaido, H. and Vaara, M. (1985) Microbiol. Rev. 49, 1–32.
[2] Benz, R. (1988) Annu. Rev. Microbiol. 42, 359–393.
[3] Benz, R. and Bauer, K. (1988) Eur. J. Biochem. 176, 1–19.
[4] Kadner, R.J. (1990) Mol. Microbiol. 4, 2027–2033.
[5] Wickner, W., Driessen, A.J.M. and Hartl, F.U. (1991) Annu. Rev. Biochem. 60, 101–124.
[6] Tsapis, A. and Kepes, A. (1977) Biochim. Biophys. Acta 469, 1–12.
[7] Meury, J., Robin, A. and Monier-Champeix, P. (1985) Eur. J. Biochem. 151, 613–619.
[8] Martinac, B., Buechner, M., Delcour, A.H., Adler, J. and Kung, C. (1987) Proc. Natl. Acad. Sci. USA 84, 2297–2301.
[9] Buechner, M., Delcour, A.H., Martinac, B., Adler, J. and Kung, C. (1990) Biochim. Biophys. Acta 1024, 111–121.
[10] Szabó, I., Petronilli, V., Guerra, L. and Zoratti, M. (1990) Biochem. Biophys. Res. Commun. 171, 280–286.
[11] Berrier, C., Coulombe, A., Houssin, C. and Ghazi, A. (1989) FEBS Lett. 259, 27–32.
[12] Simon, S.M. and Blobel, G. (1992) Cell 69, 677–684.
[13] Zoratti, M. and Petronilli, V. (1988) FEBS Lett. 240, 105–109.
[14] Zoratti, M., Petronilli, V. and Szabó, I. (1990) Biochem. Biophys. Res. Commun. 168, 443–450.
[15] Szabó, I., Petronilli, V. and Zoratti, M. (1992) Biochim. Biophys. Acta (in press).
[16] Alcayaga, C., Carrasco, A. and Wolff, D. (1990) Arch. Biol. Med. Exp. 23, R409.
[17] Osborn, M.J., Gandler, J.E., Parisi, E. and Carson, J. (1972) J. Biol. Chem., 247, 3962–3972.
[18] Cull, M. and McHenry, C.S. (1990) Methods Enzymol. 182, 147–154.
[19] Mueller, P., Rudin, D., Tien, T. and Westcott, W. (1963) J. Phys. Chem. 67, 534–535.
[20] Alvarez, O. and Latorre, R. (1978) Biophys. J. 21, 1–17.
[21] Hodgkin, A.L. and Katz, B. (1949) J. Physiol. 352, 37–77.
[22] Berrier, C., Coulombe, A., Szabó, I., Zoratti, M. and Ghazi, A. (1992) Eur. J. Biochem. (in press).
[23] Delcour, A., Martinac, B., Adler, J. and Kung, C. (1989) J. Membr. Biol. 112, 267–275.
[24] Blair, D.F. and Berg, H.C. (1990) Cell 60, 439–449.