Modified reconstitution method used in patch-clamp studies of *Escherichia coli* ion channels

Anne H. Delcour,* Boris Martinac,‡ Julius Adler,* and Ching Kung‡
*Departments of Biochemistry and Genetics, ‡Laboratory of Molecular Biology and Department of Genetics, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT We have modified the procedure of Criado and Keller (1987) to study ion channels of *Escherichia coli* reconstituted in liposomes. The modifications include (*a*) excluding the use of any detergent and (*b*) inducing blisters from liposomes with Mg^{2+}. These blisters, which appear to be unilamellar, are stable for hours. They could be repeatedly sampled with different patch-clamp pipettes each achieving seal resistance >10 GOhms. Activities of three types of ion channels are often observed by use of this method, including two voltage-sensitive cation channels of different conductances. Even the mechanosensitive channel, previously recorded from live *E. coli* cells (Martinac et al., 1987), was also detected in these blisters. Apparently the channel protein and any accesory structures, postulated to be needed for mechanotransduction, can be reconstituted together by this method.

INTRODUCTION

The patch-clamp recording technique (Hamill et al., 1981) examines the electrical activity of small patches of membrane, but still requires that the ion channels be embedded in structures that are large enough compared with the pipette opening. To meet this requirement for the study of bacterial membranes, we generated giant *Escherichia coli* cells or spheroplasts by five different methods and discovered ion channels in live bacteria (Martinac et al., 1987; M. Buechner, A.H. Delcour, B. Martinac, J. Adler and C. Kung, manuscript in preparation). Reconstitution of purified ion channel proteins or of native membrane vesicles in bilayers is also an advantageous technique to gain access to membrane proteins of cells that are not readily amenable to direct electrophysiological measurements, for example because of size limitation (Miller, 1986).

Porins, channel proteins in the outer membrane of Gram-negative bacteria, were initially studied in planar lipid bilayers (Benz et al., 1978; Benz, 1988; Lakey, 1987). This method, however, suffers from the use of lipid solvents which might be deleterious to some proteins or affect their properties. Subsequently, Schindler and Rosenbusch (1978, 1981) applied the raised monolayer technique of Montal and Mueller (1972) to the study of bacterial porins. However, with both techniques, the large size and, therefore, the large time constant, of the bilayer under study may obscure the detection of ion channels of small conductance and/or short lifetime.

Coronado and Latorre (1983) made phospholipid bilayers at the tip of the patch-clamp pipette by moving it through a lipid monolayer at the air-water interface. They studied the ion channels present in these bilayers after fusion of native membrane vesicles with the monolayer. However, this technique does not permit a control of the protein-to-lipid ratio in the reconstituted bilayer. This disadvantage is circumvented when the patch-clamp pipette seals directly onto liposomes which have been made by mixing exogenous lipids and native membranes in a controlled fashion. This approach has become increasingly popular since the initial experiments of Tank et al. (1982), and a number of reports of electrical recordings in liposomes has recently appeared (Criado and Keller, 1987; Keller et al., 1988; Correa and Agnew, 1988; Saito et al., 1988).

We have investigated methods of reconstitution of bacterial membranes into artificial liposomes in parallel with methods of direct patch-clamp of live bacterial cells. In our patch-clamp study of reconstituted liposomes, we required (*a*) the absence of detergent during fusion of native membranes with exogenous lipids, (*b*) the freedom to adjust the protein-to-lipid ratio so as to increase the chances of encountering rare channels, and (*c*) the reproducible formation of unilamellar structures which would readily form stable high-resistance seals in the patch-clamp pipette. Because native bacterial membrane vesicles did not fuse well with exogenous lipids by a simple freeze-thaw procedure, we chose the method developed by Criado and Keller (1987) with some modifications to avoid the use of detergents. We describe here a possible improvement of this protocol, whereby magnesium chloride is used to induce formation of large, most likely unilamellar, blisters on the sides of the liposomes. We had greater success in obtaining gigaohm seals when the pipette seals onto these blisters rather than onto the

Address correspondence to Ching Kung.

0006-3495/89/08/631/06 $2.00

original liposomes. As examples, we present recordings of three types of ion channels in *E. coli* that have been observed by use of this method a mechanosensitive channel, previously described in live cells (Martinac et al., 1987) has been encountered four times, a small cation-selective channel has been recorded seven times, and a voltage-dependent channel, also studied in live cells (Buechner, Delcour, Martinac, Adler and Kung, manuscript in preparation), has also been observed more than one hundred times in reconstituted blisters. A preliminary report of this work has been presented (Delcour et al., 1989).

MATERIALS AND METHODS

Preparation of native membrane vesicles

Two liters of *E. coli* cells strain AW405 (Armstrong et al., 1967) were grown in tryptone broth (1% tryptone [Difco, St Louis, MO], 0.5% NaCl) to an OD_{590} of 0.5 to 0.6 at 35°C. Cells were harvested at 8,500 rpm in a GS3 rotor (Dupont, Wilmington, DE) for 15 min at 4°C. The pellets were washed once with 50 mM potassium phosphate buffer (pH 6.6) containing 5 mM $MgSO_4$ and 1 mM dithiothreitol, and then resuspended at 20% wet weight per volume in the same buffer. Membrane vesicles were made by passing the cells through a French press (American Instrument, Silver Spring, MD) either once at 4,500 lb/in^2 (method I) (Hertzberg and Hinkle, 1974) or twice at 14,000 to 16,000 lb/in^2 (method II) (Smit et al., 1975). The unbroken cells and cell debris were discarded by centrifugation at 30,000 *g* for 10 min (Beckman Instruments, Fullerton, CA). The supernatant was collected and the membrane vesicles pelleted at 165,000 *g* for 2 h at 4°C. The pellet was resuspended with a 23-gauge needle and glass-teflon homogenizer (Wheaton, Millville, NJ) in either 0.5 ml of 50 mM KCl, 1 mM ethylene glycol bis-(β-aminoethylether)*N,N,N',N'*-tetraacetic acid (EGTA), 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2 ethane sulfonic acid (Hepes), pH 7.2 (we call this the "whole membrane fraction"), or in 1 to 3 ml of 25% (wt/wt) sucrose in 5 mM ethlenediaminetraacetic acid (EDTA, potassium salt), pH 7.2. In the latter case, the inner and outer membrane fractions were subsequently separated through sucrose-gradient centrifugation (Osborn et al., 1972), and then resuspended in the same buffer as the whole membrane fraction. All membrane fractions were stored at −80°C. Protein concentrations were determined by bicinchoninic acid assay (Smith et al., 1985) (Pierce Chemical, Rockford, IL) in the presence of 1% sodium dodecyl sulfate with bovine serum albumin as a standard.

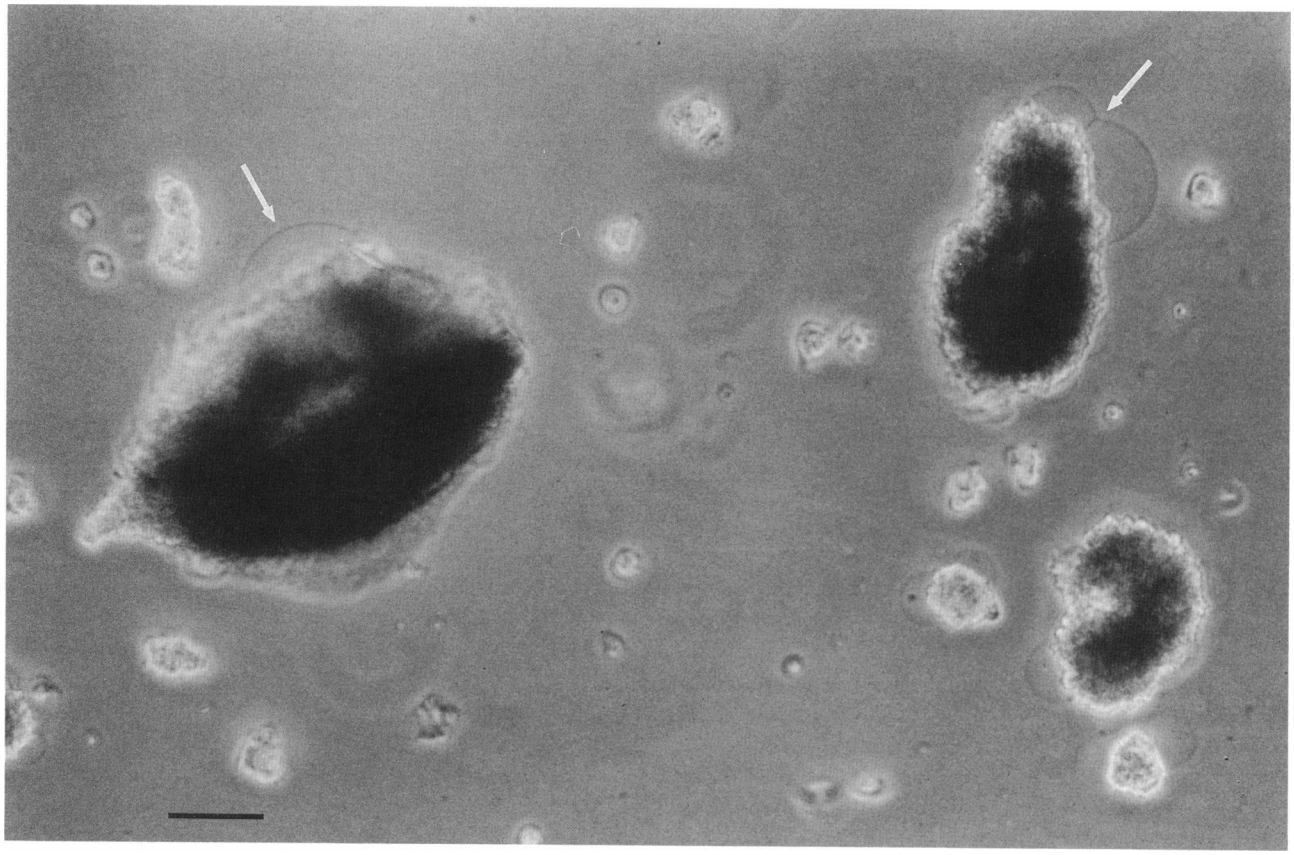

FIGURE 1 Light-microscope photograph of collapsed liposomes with blisters (*arrows*). Their transparency and their thin contour suggest that they are unilamellar. The blisters were made in Buffer B, as described in Materials and Methods. The bar represents 50 μm.

Preparation of liposomes fused with bacterial membranes

The fusion procedure was essentially as reported by Criado and Keller (1987), except for the following modification. Azolectin (Sigma Chemical Co., St Louis, MO) was made at 10 mg/ml in 5 mM Tris-Cl buffer (pH 7.2) by sonication to clarity (Heat-Systems Ultrasonics, Plainview, NY) followed by two freeze-thaw cycles in a dry-ice/acetone bath.

Aliquots of native membrane vesicles were mixed with 0.5 ml of freeze-thawed azolectin at the desired protein-to-lipid ratio (wt/wt) and the mixture pelleted for 1 h at 95,000 *g* at 4°C. Large membrane vesicles, such as those obtained by a freeze-thaw procedure, were needed during this step to obtain a reproducible recovery of the lipid. The pellet was then resuspended in 10 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS) (pH 7.2) containing 5% ethylene glycol, and 20 to 40 μl of this suspension were placed on a clean glass slide (Fischer Scientific, Pittsburgh, PA). After dehydration for ~4 h in a large desiccator at 4°C, a small volume of sterile buffer A (150 mM KCl, 0.1 mM EDTA, 10^{-5} M $CaCl_2$, 5 mM Hepes, pH 7.2) was placed on the surface of each dried lipid film, such that the final lipid concentration was 90 mg/ml. We found that such a high lipid concentration was needed to produce giant liposomes. Rehydration occurred overnight at 4°C in a Petri dish containing a wet filter paper pad. Azolectin was sonicated and twice frozen and thawed freshly for each liposome preparation. The liposomes were used only on the day they were prepared.

Blister formation

Five to ten microliters of the rehydrated drop suspension were placed in the patch-clamp chamber which contained Buffer B (Buffer A plus 20

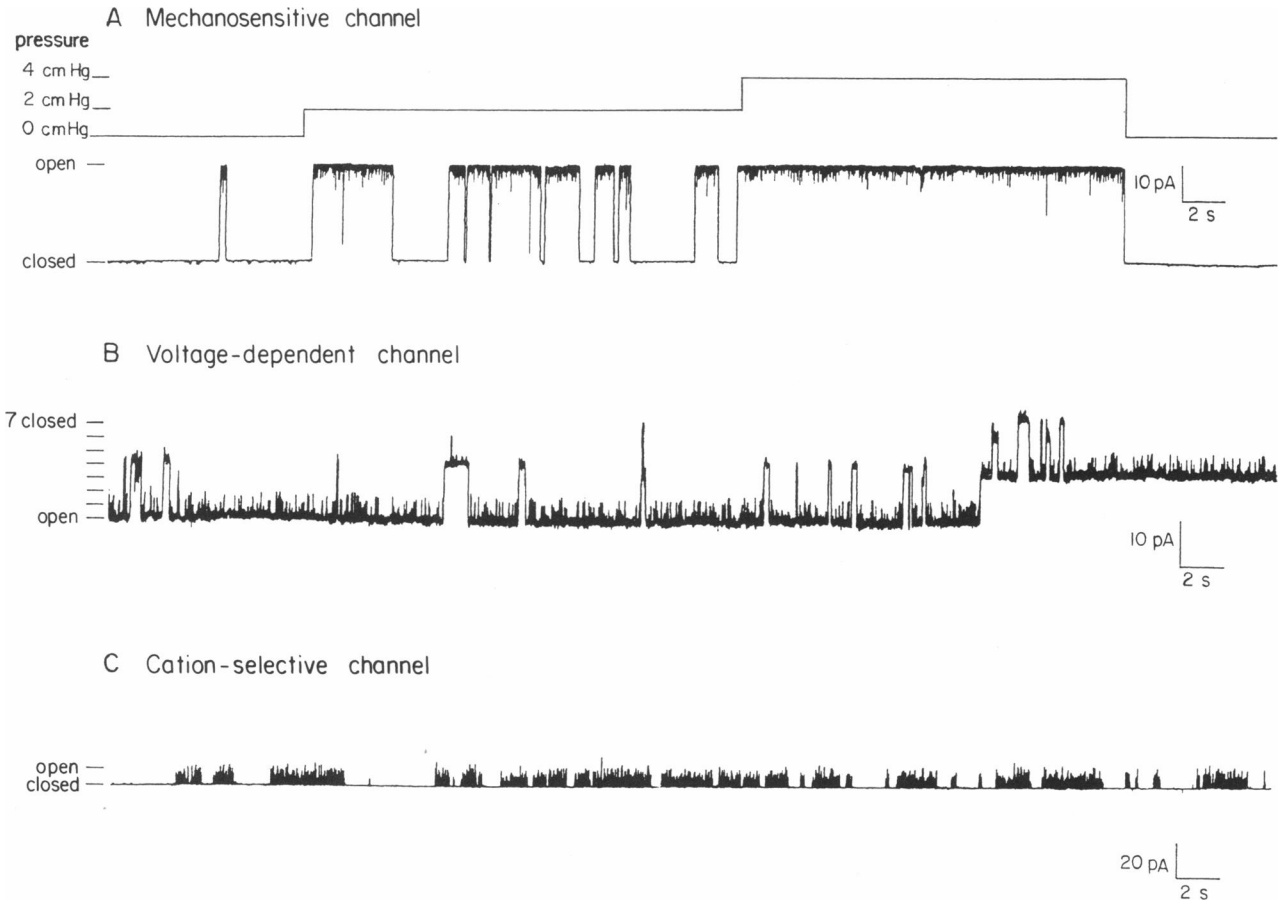

FIGURE 2 Selected traces of *E. coli* ion channels reconstituted into blisters by the method described. (*A*) A mechanosensitive ion channel. Channel activity is observed when negative pressure is applied to the patch (*top trace*). This channel happened to come from the "inner membrane" fraction, which was fused with azolectin at a protein-to-lipid ratio of 1:100. We attempted to separate the mixed inner and outer membrane vesicles by a standard method (Osborn et al., 1972), but the separation is not effective (see text). The pipette voltage was +40 mV. (*B*) A voltage-sensitive channel: outer membrane vesicles were fused with azolectin at a protein-to-lipid ratio of 1:600. The open level and maximally closed level are labeled. The lowest unlabeled tick marks the most frequent transitions. These transitions, seen mostly as spikes at this time scale, are clear flat-top transitions when displayed at higher time resolution and correspond to 90 pS. Other ticks indicate multiples of this conductance. Direct transitions to these levels are also shown. The pipette voltage was −50 mV. (*C*) A cation-selective channel of smaller conductance: whole membrane fraction was fused at a protein-to-lipid ratio of 1:30. The pipette voltage was +120 mV. All experiments (*A*, *B*, and *C*) were done in 150 mM KCl, 0.1 mM EDTA, 10^{-5} M $CaCl_2$, 5 mM Hepes, pH 7.2, in both the pipette and the patch-clamp chamber solutions.

mM $MgCl_2$) that caused the liposomes to collapse. Within 5 to 10 min, faint and most likely unilamellar blisters emerged from the sides of the collapsed liposomes (Fig. 1). These structures were very large (diameters ranged from 5 to over 100 μm) and stable for a few hours. Before each patch-clamp experiment, the bath was perfused with Buffer B to remove the smaller liposomes or debris that had not settled at the bottom of the chamber and might dirty the tip of the pipette. The liposomes were viewed with a 20× phase-contrast objective on an inverted microscope (Olympus, Tokyo, Japan).

Electrical recording

Single channel recordings were carried out according to standard patch-clamp techniques (Hamill et al., 1981). Pipettes were pulled (Sutter Instrument, San Rafael, CA) to a resistance of 5 to 10 Mohms in 150 mM KCl. They were not fire-polished and were coated with transparent nail enamel (Sally Hansen, Farmingdale, NY). A gentle touch of the electrode onto the blister, occasionally combined with gentle suction, consistently yielded instantaneous seals of 10 to 50 GOhms. The electrode was then pulled away from the blister through micromanipulation and the patch was excised by brief air exposure. The blister remained attached to the collapsed liposome and resealed. The same blister could be sampled many times with fresh pipettes, provided that the solution was not exchanged for a magnesium-free buffer in which the collapsed liposomes reverted to smooth, most likely multilamellar, spheres. The experiments were carried out at room temperature with an EPC-7 amplifier (List-Electronic, Darmstadt, West Germany). The data were recorded on a chart recorder (Gould, Cleveland, OH) or on tape (Indec, Sunnyvale, CA) for further computer analysis, if needed.

RESULTS AND DISCUSSION

Here, we present patch-clamp data obtained with reconstituted bacterial membranes. The reconstituted liposomes were made according to the method of Criado and Keller (1987) with two major modifications that are described here. (*a*) Azolectin was prepared without the use of detergent by a rapid sonication to clarity followed by two freeze-thaw cycles. This preparation is quicker than detergent dialysis. We found that the success of obtaining gigaohm seals on liposomes or blisters was irreproducible when the azolectin was prepared with the detergent CHAPS (3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate). We speculated that an incomplete detergent removal might have been the source of this inconsistency. In our hands, the success of obtaining giant unilammellar liposomes and forming high resistance seals on such liposomes was not satisfactory. We therefore developed a protocol whereby unilamellar blisters are made from these liposomes. This method has the advantage that all the rehydrated drop suspension, and even pieces of the lipid film, can be used to produce blisters; the experimenter need not search for unilamellar giant liposomes. The blisters are robust and the recording chamber can be perfused easily without significant loss of the blisters, as long as magnesium chloride is kept in the buffer. However, once the patch has been excised, solutions can be exchanged freely, without concern for conservation of material, because blisters can be easily obtained from a fresh sample. These blisters formed independent of the presence or the concentration of membrane proteins. We were also able to obtain blisters from liposomes fused with ciliary membranes of *Paramecium tetraurelia* and with endoplasmic reticulum membranes of sea urchin eggs (Martinac, unpublished observations). Contrary to a previous report (Gögelein and Koepsell, 1984), we found that blisters made from pure synthetic lipids alone tended to yield artefactual channel-like currents. However, experiments done with azolectin (a mixture of lipids extracted from soybean) in the absence of fused native membranes, yielded high resistance seals and quiet background. Care must be taken to use very clean, sterile solutions, especially during the rehydration procedure, to avoid contamination by microbial membrane fragments.

Typical activities of three frequently observed channels in the reconstituted blisters are represented in Fig. 2. The mechanosensitive channel (Fig. 2 *A*) and the voltage-dependent channel (Fig. 2 *B*) appear to be outer membrane components, since they display their activity in outer membrane vesicles and in patches taken directly from the surface of live cells (Martinac et al., 1987; Buechner, Delcour, Martinac, Adler and Kung, in preparation). They also show voltage dependences similar to those observed on live cells (Martinac et al., 1987; Buechner, Delcour, Martinac, Adler and Kung, in preparation). This indicates that these two proteins have retained their native orientation in the blisters. They are also observed in liposomes that have been fused with fractions enriched with inner membrane vesicles. Outer membrane vesicles are denser than those formed from the inner membrane because the outer membrane is made up of lipopolysaccharide in addition to phospholipid. The sucrose gradient, however, is unable to achieve a perfect separation of the inner and the outer membrane fractions (Osborn et al., 1972). This may be due to the existence of lipidic junctions between the two membranes (Bayer, 1979). A trace contamination of the inner membrane fraction by outer membrane channels can be very easily detected by patch-clamp, since these channels have large and slow conductances. This contamination can be reduced if smaller membrane vesicles are made by using much higher pressures in the French press (Method II, see Materials and Methods). We have found that, indeed, when such vesicles are made, the occurrence of the voltage-dependent channel of Fig. 2 *B* in the inner membrane fraction is much reduced (data not shown).

The reconstituted mechanosensitive channel displays qualitatively the same properties as in cells (Martinac et al., 1987): large conductance, activation by negative pressure in the range of 1 to 10 cm of mercury and activation by depolarizing voltages. The outer membrane

of *E. coli* is linked to the underlying peptidoglycan layer (cell wall) by covalent and noncovalent attachments. Patch-clamp studies of spheroplasts suggest that the peptidoglycan layer acts as a parallel elastic element, setting up the tension to which the mechanosensitive channel is sensitive (Martinac et al., 1989). It is conceivable that the channel together with pieces of the cell wall have been reconstituted together in the blisters. In excised patches from spheroplasts, the channel has been shown to become more sensitive to tension upon further digestion of the cell wall by added lysozyme (Martinac et al., 1989; Martinac, Buechner, Delcour, Adler and Kung, in preparation). Similar experiments could be performed to determine whether the cell wall is present in the patch excised from the blisters. Such experiments would indicate whether pressure activation can be conferred to an ion channel in the absence of underlying cytoskeletal-like elements.

The voltage-dependent channel depicted in Fig. 2 *B* is open most of the time and displays frequent, but brief, transitions to closed levels. The most frequent transition has a unit conductance of 90 pS and is cation-selective. Increasing depolarizing voltages induce transitions corresponding to the simultaneous closures of many identical ion conducting units. Fig. 2 *B* shows closures of 1 unit as well as 2, 3, and 4 units simultaneously. A detailed description of this channel will be presented elsewhere (Delcour, Martinac, Adler and Kung, in preparation).

Preliminary evidence indicates that the conductance depicted in Fig. 2 *C* is cation-selective and is of ~30 pS in symmetric 150 mM KCl solutions. As this channel has not yet been studied in more refined fractions, we cannot yet assess whether it belongs to the inner or the outer membrane.

The study of ion channels in bacteria is of interest from a biochemical, genetic, and evolutionary standpoint. We have previously described patch-clamp measurements on spheroplasts and giant cells of *E. coli* (Martinac et al., 1987; Buechner, Delcour, Martinac, Adler and Kung, in preparation). The application of the patch-clamp technique to fused bacterial membranes broadens our field of investigation because it allows us to study ion channel proteins in both the inner and the outer membranes of various bacterial species, including those for which formation of giant spheroplasts or protoplasts might be a problem. For example, we have also begun experiments on the single membrane of a Gram-positive bacterium, *Bacillus subtilis* (Delcour et al., 1989). With this technique, we hope to survey the different types of ion channels of *E. coli* membranes, as well as of other bacteria that might be of special interest. The combination of patch-clamp technique and bacterial genetics may prove to be quite powerful for the study of responses of organisms to their environment, as well as for the more biophysical description of structure-function relationships of ion channel proteins.

We thank Christine Hirscher for expert technical assistance, Leanne Olds for making the illustrations, and Matthew Buechner for useful discussions and comments on the manuscript.

This work was supported by grants to J. Adler and C. Kung from the United States Public Health Service (DK 39121) and the Lucille P. Markey Charitable Trust.

A. H. D. was supported by a postdoctoral fellowship from the Muscular Dystrophy Association.

Received for publication 20 March 1989 and in final form 30 May 1989.

REFERENCES

Armstrong, J. B., J. Adler, and M. M. Dahl. 1967. Nonchemotactic mutants of *Escherichia coli*. *J. Bacteriol.* 93:390–398.

Bayer, M. E. 1979. The fusion sites between outer membrane and cytoplasmic membrane of bacteria: their role in membrane assembly and virus infection. *In* Bacterial Outer Membranes. M. Inouye editor. John Wiley & Sons, Inc., New York.

Benz, R., K. Janko, W. Boos, and P. Läuger. 1978. Formation of large, ion-permeable membrane channels by the matrix protein (porin) of *Escherichia coli*. *Biochim. Biophys. Acta.* 511:305–319.

Benz, R. 1988. Structure and function of porins from Gram-negative bacteria. *Annu. Rev. Microbiol.* 42:359–393.

Coronado, R., and R. Latorre. 1983. Phospholipid bilayers made from monolayers on patch-clamp pipettes. *Biophys. J.* 43:231–236.

Correa, A. M., and W. S. Agnew. 1988. Fusion of native or reconstituted membranes to liposomes, optimized for single channel recording. *Biophys. J.* 54:569–575.

Criado, M., and B. U. Keller. 1987. A membrane fusion strategy for single-channel recordings of membranes usually non-accessible to patch-clamp pipette electrodes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 224:172–176.

Delcour, A. H., B. Martinac, J. Adler, and C. Kung. 1989. Patch-clamp studies of bacterial membranes in a reconstituted system. *Biophys. J.* 55:494a. (Abstr.)

Gögelein, H., and H. Koepsell. 1984. Channels in planar bilayers made from commercially available lipids. *Pfluegers Arch. Eur. J. Physiol.* 401:433–434.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recordings from cells and cell-free membrane patches. *Pfluegers Arch. Eur. J. Physiol.* 391:85–100.

Hertzberg, E. L., and P. C. Hinkle. 1974. Oxidative phosphorylation and proton translocation in membrane vesicles prepared from *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 58:178–184.

Keller, B. U., R. Hedrich, W. L. C. Vaz, and M. Criado. 1988. Single channel recordings of reconstituted ion channel proteins: an improved technique. *Pfluegers Arch. Eur. J. Physiol.* 411:94–100.

Lakey, J. H. 1987. Voltage-gating in porin channels. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 211:1–4.

Martinac, B., M. Buechner, A. H. Delcour, J. Adler, and C. Kung. 1987. Pressure-sensitive ion channel in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* 84:2297–2301.

Martinac, B., A. H. Delcour, M. Buechner, J. Adler, and C. Kung. 1989. Pressure-induced membrane tension activates a mechanosensitive ion channel in the outer membrane of the bacterium *Escherichia coli*. *Biophys. J.* 55:494a. (Abstr.)

Miller, C. 1986. *Ion Channel Reconstitution.* Plenum Publishing Corp., New York.

Montal, M., and P. Mueller. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. USA.* 69:3561–3566.

Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson. 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. *J. Biol. Chem.* 247:3962–3972.

Saito, Y., N. Hirashima, and Y. Kirino. 1988. Giant proteoliposomes prepared by freezing-thawing without the use of detergent: reconstitution of biomembranes usually inaccessible to patch-clamp pipette microelectrode. *Biochem. Biophys. Res. Commun.* 154:85–90.

Schindler, H., and J. P. Rosenbusch. 1978. Matrix protein from *Escherichia coli* outer membranes forms voltage-controlled channels in lipid bilayers. *Proc. Natl. Acad. Sci. USA.* 75:3751–3755.

Schindler, H., and J. P. Rosenbusch. 1981. Matrix protein in planar membranes: clusters of channels in a native environment and their functional reassembly. *Proc. Natl. Acad. Sci. USA.* 78:2302–2306.

Smit, J., Y. Kamio, and H. Nikaido. 1975. Outer membrane of *Salmonella typhimurium:* chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. *J. Bacteriol.* 124:942–958.

Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry.* 150:76–85.

Tank, D. W., C. Miller, and W. W. Webb. 1982. Isolated-patch recording from liposomes containing functionally reconstituted chloride channels from *Torpedo* electroplax. *Proc. Natl. Acad. Sci. USA.* 79:7749–7753.