PHOSPHOLIPID BILAYERS MADE FROM MONOLAYERS ON PATCH-CLAMP PIPETTES

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ABSTRACT Phospholipid bilayers were made from phospholipid monolayers at the air/water interface on patch-clamp pipettes. Lipid bilayers were characterized using the K^+ carrier nonactin and the channel formers gramicidin and alamethicin. Bilayers were also formed from monolayers spontaneously assembled in a suspension of native vesicles from cardiac sarcolemma and lobster axonal membranes and an excess of lipids. In these types of bilayers we observed several different channels including one contained in the axonal membrane that shows delayed rectifier behavior. This technique permits the study of reconstituted channels on a time scale and noise comparable to cellular patch-clamp standards.

INTRODUCTION

At present, there is considerable effort being devoted to developing techniques for the study of single channels of excitable membranes in reconstituted systems. Techniques such as insertion of purified membrane fragments into planar phospholipid bilayers (1–6) and patch-clamp pipettes (7–9) reveal interesting molecular features of channels and their modulation by the membrane phospholipid environment. We describe here the assembly of phospholipid bilayers from monolayers on standard patch-clamp pipettes and a characterization of the same with the help of two well-known channel formers, gramicidin and alamethicin. We also report the recording in these bilayers of single-channel chloride and potassium currents from calf cardiac sarcolemma and single-channel potassium currents from lobster axonal membranes. Due to the small dimensions of bilayers formed on pipettes, the technique permits the study of reconstituted channels on a time scale and noise levels comparable to cellular patch-clamp standards (10).

METHODS

Patch-clamp pipettes without fire polishing were made by the two-pull method (10) from hematocrit glass capillars (Blue-tip; Fisher Scientific Co., Allied Corp., Pittsburgh, PA). Settings in the microelectrode puller (Model 700C;) (David Kopf Instruments, Tujunga, CA) were adjusted to obtain tip diameters of ~4 μm. All pipettes were used the same day they were made. Pipettes were filled, in this case with 50 mM KCl, 5 mM Hepes-Tris, pH 7.5, and the tip was immersed in a disposable petri dish (10-cm^2 surface area) filled with the same solution. All solutions were filtered on 0.22 μm Milex-GS filters (Millipore/Continental Water Systems, Bedford, MA). After immersion of the pipette, a phospholipid monolayer was spread on the surface of the dish solution by carefully adding to the edge of the dish 0.5–1.0 μl of a 10 mg/ml solution of lipid dissolved in pentane. All lipids (Avanti Polar Lipids, Inc., Birmingham, AL) were stored in chloroform at −80°C. Chloroform was evaporated and lipids redissolved in pentane at the moment of use. Before any movement of the electrode, 2–5 min were allowed for the evaporation of the solvent from the surface of the solution. Movement of the pipette was implemented by mounting the head-stage amplifier on a coarse manipulator, and the formation process was followed without microscopic observation. In our circuit configuration, voltages are applied to the external electrode while the electrode inside the pipette is always virtually ground through the current amplifier. The rest of the recording circuit was similar to that described for cellular patch-clamp techniques (10).

Alamethicin channels were recorded by filling pipettes with 0.1 M KCl, 5 mM Hepes-Tris, pH 7.5 containing 100 ng/ml alamethicin (Upjohn Co., Kalamazoo, MI). The same buffer without alamethicin was used to spread the phospholipid monolayer of phosphatidylethanolamine (PE) and phosphatidylserine (PS) at a molar ratio of PE/PS = 1:1. Gramicidin channels were recorded after filling pipettes with 20 mM CsCl, 5 mM Hepes-Tris, pH 7.5 containing 0.1 ng/ml gramicidin (ICN Nutritional Biochemicals Div., Cleveland, OH). Bilayers were formed from monolayers spread using a mixture of phosphatidic acid (PA) and cholesterol (Chol) at a molar ratio of 1:1.

Bilayers in which the two apposing monolayers are of different composition were formed using monolayers spread from diphytanoyl phosphatidylcholine (PC)-Chol and PA-Chol at a molar ratio of 1:1. Membranes were formed by moving the pipette out of a solution that contained an interface of PC-Chol and immersing the same pipette afterwards in a solution containing an interface of PA-Chol.

For recordings of single-channel currents from cell membrane fragments, the bilayers containing calf cardiac sarcolemma or lobster axonal membranes were formed from monolayers spontaneously assembled in a suspension of native vesicles and an excess of lipids. The vesicle-liposome suspension was prepared as follows: 6 mg of phospholipid composed of brain PE/PS at a molar ratio of 1:1 (cardiac sarcolemma experiments) or

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PE/PS/Chol = 1:1:1 (axon membrane experiments) were dried under N_2 and sonicated in 1 ml of 100 mM KCl, 0.2 mM $CaCl_2$, 5 mM Hepes-Tris, pH 7.5. To this suspension 0.6 mg of vesicular protein purified as described elsewhere (3–4) was added. The mixture was quickly frozen in an ethanol dry-ice bath and thawed at room temperature. Freeze-thaw vesicles were added to a Teflon chamber (1 cm^2 surface area, 500 μl volume) that had been previously coated with a thin film of dried lipids. This film was made by evaporating thoroughly at the bottom of the chamber 1 mg of phospholipid dissolved in chloroform. The composition of this layer of lipid was the same as that of sonicated liposomes. Pipettes were filled with 50 mM KCl, 5 mM Hepes-Tris, pH 7.5 and immersed into the suspension. Usually, 2–3 movements through the interface were required to form a bilayer in the 1–5 GΩ range. Coating of the chamber with phospholipids and dilution of native vesicles with exogenous liposomes increased the resistance and stability of bilayers.

RESULTS AND DISCUSSION

The steps of bilayer formation and a model diagram that accounts for the current waveforms observed during assembly of membranes is shown in Fig. 1. Bilayers are formed by moving a saline-filled patch-clamp pipette out and into a solution that sustains a lipid monolayer at the air-water interface. The monolayer can be spread from lipid solutions in volatile solvents, from dry lipids, or from vesicle suspensions. Formation of membranes is monitored by changes in resistance that take place during movement of the pipettes out, into the air, and back into the solution. According to the scheme of Fig. 1, by moving the pipette through a phospholipid monolayer out of the solution, a film of phospholipid is adsorbed to the pipette. Reentry of the pipette through the monolayer allows the mechanical contact of the hydrophobic tails of both monolayers and the formation of a stable membrane. Upon reentry of the pipette, a resistance in the giga-ohm range develops instantaneously. Capacitance ranged from 10 to 40 pF and is due to the glass pipette rather than to the membrane itself. For example, the same range in capacitance was obtained by filling about half a millimeter of the pipette tip with decane. Resistance of bilayers varies with both phospholipid composition and ionic strength of the solution. In 0.1 M

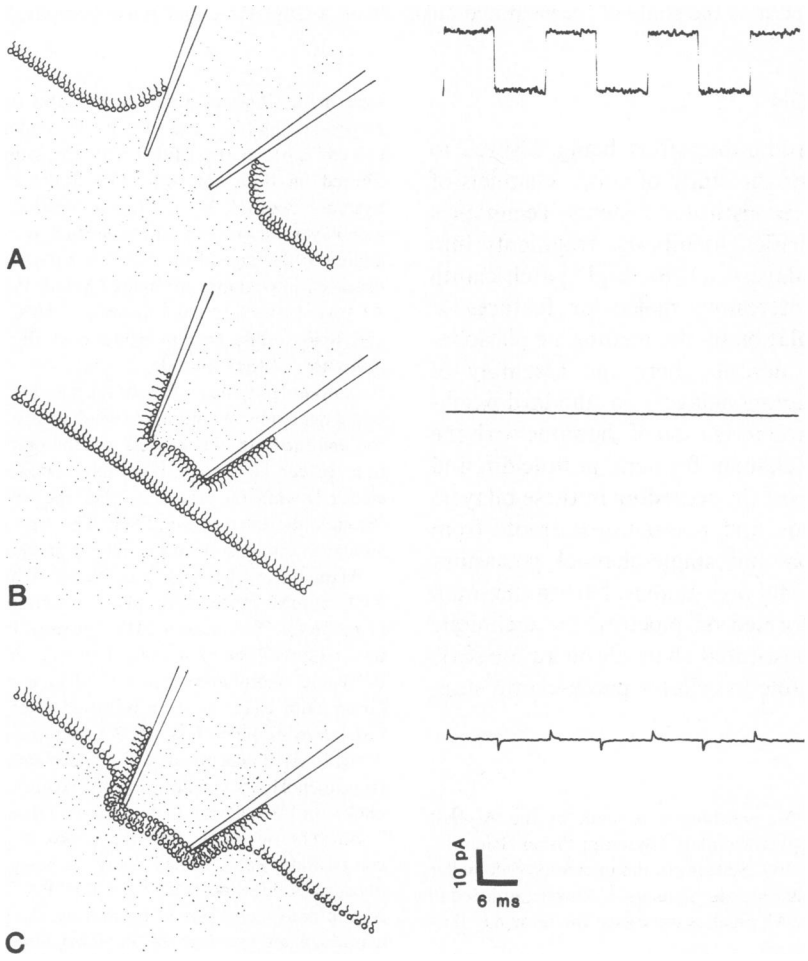

FIGURE 1 Current waveforms associated with formation of membranes and model of assembly. The current waveforms, *A*, *B*, and *C* are in response to an 0.5mV square voltage pulse applied to the command electrode. *A* shows leakage current when the pipette is immersed in the solution, which corresponds to a tip resistance of 15 MΩ. In *B* the two electrodes are out of contact when the pipette is moved into the air; and in *C* upon reentry of the pipette into the solution, a 5-GΩ membrane is formed. The bilayer was formed from a PC monolayer. For PC and PC-Chol mixtures, the process, as described here, is about 80% successful at first trial and more than 90% at the second trial in case of failure of the first, with the same pipette. Leakage currents are larger, however, after formation of several membranes on the same pipette.

KCl and for monolayers of PC, or bovine brain PE, or for mixtures of PC, PE, and Chol, resistances are typically 10–20 GΩ. When charged phospholipids such as bovine brain PS are included in the monolayer, resistance values are 2–5 fold lower. Chol was included in most lipid mixtures because it decreases leakage currents.

The model of bilayer assembly in pipettes assumes a mechanism similar to that described for bilayers made from monolayers across Teflon partitions (11). We have tested this model in several ways. First, the surface density of phospholipids in the monolayer required to form pipette bilayers can be lower than 1 molecule per 50 $Å^2$, the limiting area of phospholipids in monolayers (12). Thus, all molecules in the surface are in the monolayer configuration. Using PE we determined that the lowest surface density that forms bilayers is 1 PE/85 $Å^2$. A second test is the recording of gramicidin and alamethicin single-channel currents as shown in Fig. 2. These pore formers are only active in bilayers; thus, the recordings verify that the film assembled at the tip of the pipette is actually bilayer and not multilayer. As described in Fig. 2, gramicidin and alamethicin have similar conductances to those reported in other bilayer systems under similar conditions (13–15). Third, we tested the model by bringing into contact monolayers of different lipid composition and thus forming an asymmetric membrane (16–18). We implemented the formation of asymmetric membranes by moving the pipette out of a monolayer and reentering the same pipette into a different monolayer. Two lipid mixtures that generate stable asymmetric membranes are described in the Methods section and shown in Figs. 3 *A* and *B*. As indicated, when the pipette is moved out of PC-Chol and into PA-Chol, the asymmetry of surface charge due to negatively-charged PA generates asymmetric current-voltage relations for both gramicidin and for the K^+ carrier nonactin (16, 18). In agreement with the model of Fig. 1, the asymmetry is such that most of the PA is located in the final membrane of the monolayer, which is external to the pipette. From the asymmetry of nonactin current-voltage curves, we calculated a surface-potential difference of 70 mV, negative on the outside of the pipette (see legend, Fig. 3). This surface potential is lower than that calculated on the basis of surface density of charges in a PA-Chol monolayer. Thus, it is possible that some mixing of the two monolayers occurred during membrane assembly or in the assembled membrane. Nevertheless, the measured asym-

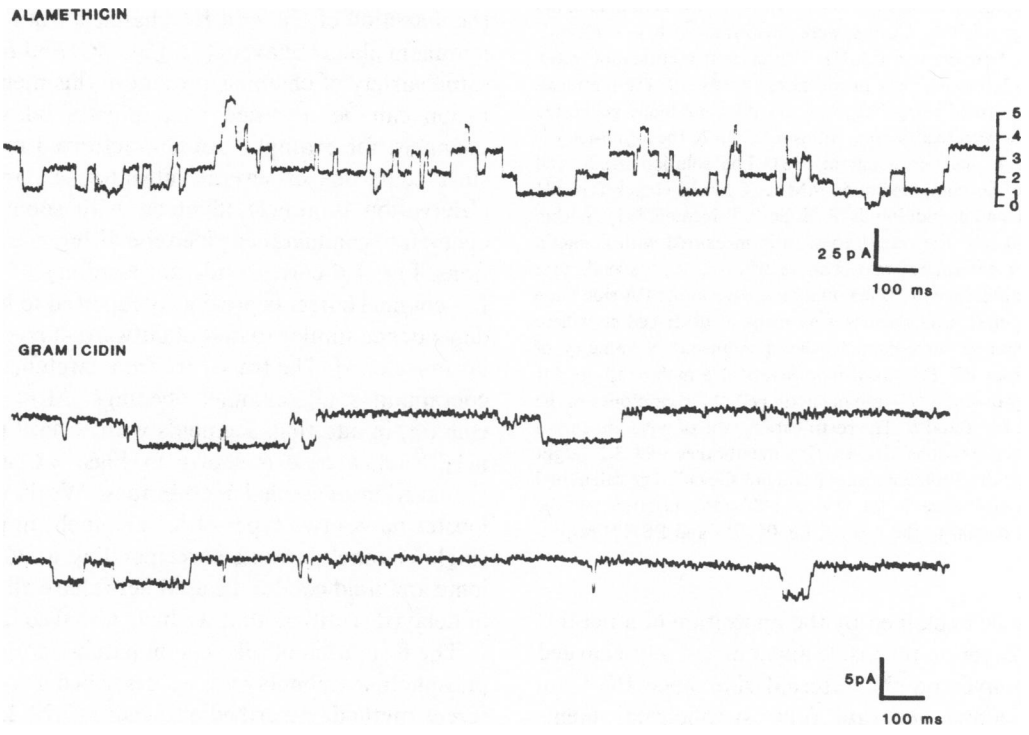

FIGURE 2 Records of currents through a single alamethicin and gramicidin channels. The holding voltage for the alamethicin recording was +60 mV and recordings were low-pass filtered at 1 KHz. Indicated on the recording are fluctuations from the lowest observed level (labeled 0) to the highest observed level (labeled 5). The indicated levels have conductance values of 30 pS, 130 pS, 290 pS, 610 pS, 910 pS, and 1,230 pS, respectively. When these values are normalized with respect to the 0 level, the ratio of conductance increase from one level to the next higher gives the sequence 1:4.3:9.6:20.3:30.3:41.0. This sequence agrees well with the same sequence for the first five levels of alamethicin in bilayers made from monolayers (14), 1:4.3:9.3:15.7:23, and for alamethicin in decane films (15), 1:5.6:13.7:24.4:35.8. Gramicidin recordings were made at +158 mV and were low-pass filtered at 500 Hz. The mean single-channel conductance under these conditions is 25 pS. This value is within the limits of conductance of gramicidin channels at the same Cs^+ concentration in fully charged PS bilayers (50 pS) and neutral PC bilayers (6 pS) (13). For more details, see text.

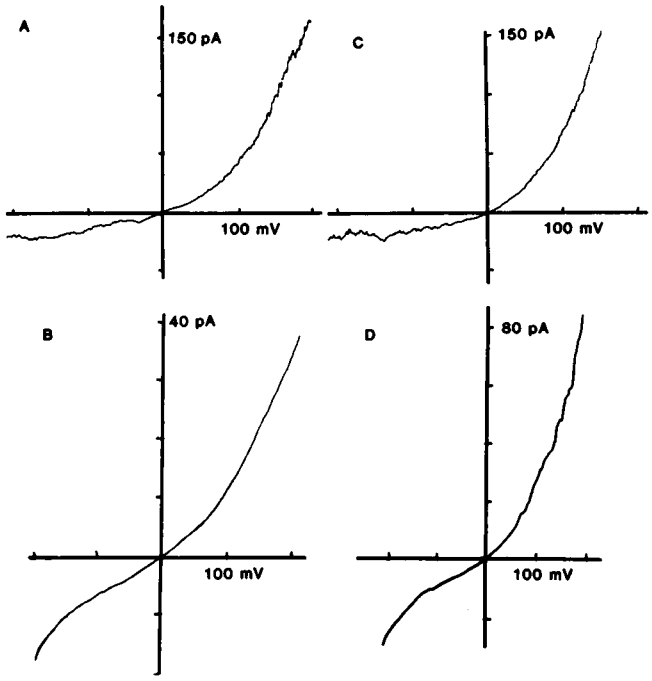

FIGURE 3 Formation of asymmetric membranes. In *A* the asymmetry of PC/PA membranes is probed by measuring current-voltage curves of asymmetric membranes containing gramicidin (18). The solutions inside and outside of the pipette contained 10 mM KCl, 5 mM Hepes-Tris, pH 7.5, 10^{-9} g/ml gramicidin. Curves were measured with a ±200-mV voltage ramp at a frequency of 0.1 Hz. The current rectification ratio, $i(+200\text{ mV})/i(-200\text{ mV})$, was in all cases≥8 (five determinations). Membranes were formed using PC-Chol and PA-Chol alone generated symmetrical curves with rectification ratios <1.5. In *B*, the asymmetry is probed using the K^+-carrier nonactin (16). The solution inside and outside the pipette was composed of 50 mM KCl, 5 mM Hepes-Tris, pH 7.5, 2×10^{-7} M nonactin (Squibb, E. R. & Sons, Princeton, NJ). Within the range of ±200 mV, the rectification ratio measured with nonactin averaged 2.7 (four determinations). This rectification corresponds to a surface potential difference of 70 mV more negative in the PA side (side external to the pipette). Calculations were made as described elsewhere (16). Curves *C* and *D* correspond to the spontaneous asymmetry of bilayers formed from PC/PS mixed monolayers at a molar ratio of 1:1 and probed with gramicidin (*C*) and nonactin (*D*). All conditions are the same as described for *A* and *B*. The rectification ratio of nonactin curves was 3.8 (four determinations) for PC/PS membranes and 3.2 (eight determinations) for PE/PS membranes (data not shown). The calculated surface potential difference is 93 mV and 83 mV, negative on the monolayer more external to the pipette, for PC/PS and PE/PS respectively.

metry can only be explained by the apposition of a neutral PC-Chol monolayer on the inside and a negatively-charged PA-Chol monolayer on the external side since PC-Chol and PA-Chol alone generate fully symmetrical membranes, as probed with gramicidin and nonactin (not shown).

Unlike the formation of membranes on inert Teflon partitions, we have found that some phospholipids appear to interact strongly with glass pipettes. For example, a mixed monolayer of PC/PS = 1:1 generates bilayers that are spontaneously asymmetric (Figs. 3 *C* and *D*). The surface-potential difference is 93 mV more negative on the external monolayer. At the ionic strength of the experiments, this surface potential indicates that an excess of 0.005 $e/Å^2$ exists in the external monolayer (16). A possible interpretation is that the structure of glass and/or surface charge of glass (19) act to exclude PS molecules from the monolayer adsorbed to the pipette during the movement through the interface. The effect, however, is rather selective because phosphatidic acid does not distribute asymmetrically. The implications of this exclusion of lipids in bilayers formed in glass electrodes are numerous and warrant a more detailed study in both bilayers and cell-membrane patches.

Perhaps the most exciting result that we have obtained in pipette bilayers is the insertion into these small membranes of channels from purified membrane fragments of muscle and nerve cells. Fig. 4 describes a procedure to record single-channel currents from isolated membranes. Our experience with several types of purified membranes indicates that the same rules used in conventional planar bilayers can be used to incorporate channels in pipette bilayers. Thus, the inclusion of PS (1–4), the formation of monolayers from vesicle-liposome suspensions (5, 6), and the establishment of an osmotic gradient (20) are all conditions used here to enhance the transfer of channels from vesicles to pipette bilayers. We previously reported the detection of Cl^- and K^+ channels from cardiac sarcolemma in planar bilayers (4). Figs. 4 *A* and *B* show that the same variety of channels present in this membrane preparation can be recorded from pipette bilayers. Chloride channels, for example, are characterized by a noisy open state composed of several substates (4). In pipettes, the observation is almost identical with some variations in open-state conductance given the difference in ionic conditions. Fig. 4 *B* corresponds to recordings of single cardiac K^+-channel currents previously reported to have a voltage-dependence similar to that of outward currents of ventricular muscle (4). The traces are from stretches of recordings containing single-channel openings. Most of the traces contain, in addition, segments with several types of channels. Such a case is shown in Figs. 4 *C* and *D* from a preparation of axonal membranes. We have detected in lobster nerves two types of K^+ channels; in particular, the single-channel currents corresponding to 26 pS under the ionic and lipid conditions used here share all the properties of delayed rectifiers that we have tested so far (21).

The formation of bilayers on patch-clamp pipettes from phospholipid monolayers, as described here, and by different methods described elsewhere (7,22,23), are powerful techniques for the reconstitution of channels of excitable membranes. There are differences with the previous methods that are difficult not to mention. The process of bilayer assembly from monolayers does not require at any step either the pretreatment of pipettes with *n*-alkane solvents or the presence of solvents in the monolayer. For example, the recordings of Fig. 4 were made using monolayers formed from dry lipids and a suspension of vesicles.

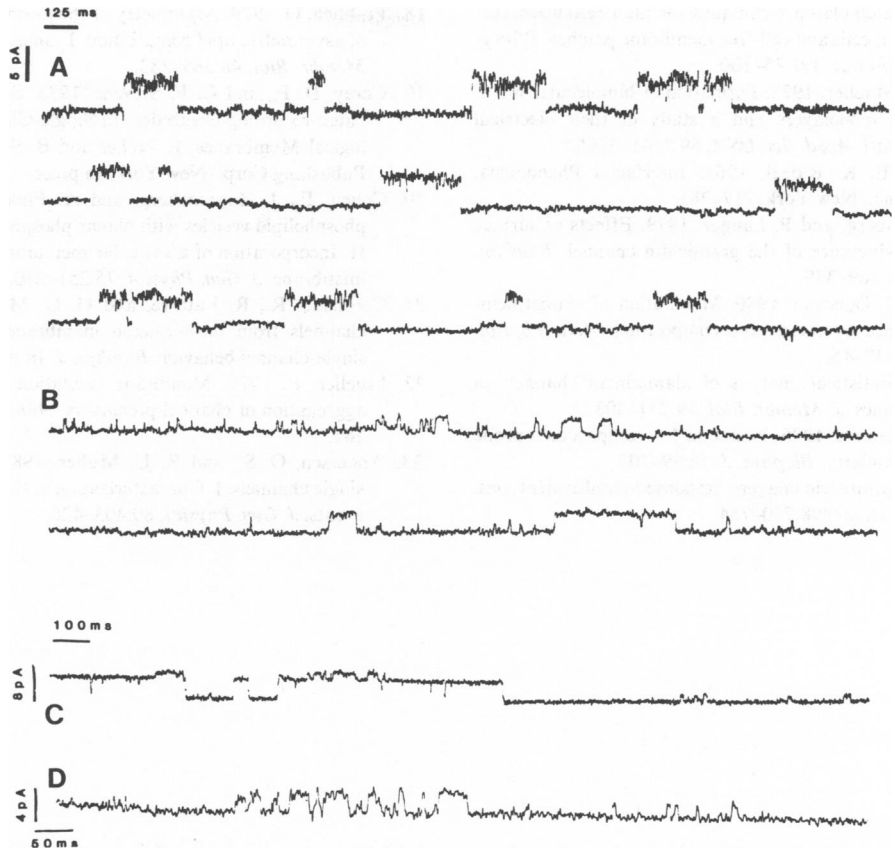

FIGURE 4 Recordings of single-channel currents from cell membrane fragments. Recordings *A* and *B* are from membranes that contained stretches in which cardiac single-channel currents were clearly observed. *A* corresponds to 70 pS chloride channels at a holding voltage of −50 mV; *B* corresponds to 35 pS potassium channels at a holding voltage of −80 mV. *A* and *B* were selected from recordings of 120 s (*A*) and 900 s (*B*) and were low-pass filtered at 1 KHz and 500 Hz, respectively. Recordings *C* and *D* correspond to channels from lobster axonal membranes at a holding voltage of +50 mV. *C* shows unitary currents of amplitudes corresponding to 85 pS and 26 pS. A stretch of 26-pS channels is presented in *D* at a higher resolution. The total recording time in *D* was 350 s and recording was low-pass filtered at 700 Hz.

Thus, this technique represents a substantial improvement in the use of planar phospholipid bilayers for the reconstitution of channels.

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