FUSION OF NATIVE OR RECONSTITUTED MEMBRANES TO LIPOSOMES, OPTIMIZED FOR SINGLE CHANNEL RECORDING

ANA M. CORREA AND WILLIAM S. AGNEW
Department of Cellular and Molecular Physiology, Yale University, School of Medicine, New Haven, Connecticut 06510

ABSTRACT We here describe a protocol for fusing vesicles into large structures suitable for patch clamp recording. The method may be used with native membrane vesicles or with liposomes containing reconstituted/purified ion channels. The resulting unilamellar membranes exhibit high channel surface abundance, yielding multiple channels in the average excised patch. The procedure has been used to record voltage-sensitive Na channels from three native membrane preparations (eel electroplax, rat skeletal muscle, squid optic nerve), and from reconstituted protein purified from eel electroplax. Channels treated with batrachotoxin (BTX) displayed characteristic activation voltage dependence, conductances, selectivity, and sensitivity to saxitoxin (STX).

INTRODUCTION

A particularly important advance for the analysis of the conformational mechanisms of ion channels has been the extension of single channel recording techniques to biochemical preparations, including channels from native membrane vesicles and from liposomes containing reconstituted/purified proteins. There remain certain limitations to these techniques. Planar bilayers, in most configurations, exhibit large capacitance transients and recording bandwidths that restrict their use with transient voltage-activated ion channels (for review, Montal et al., 1981). Patch-clamp recording methods have overcome some of these problems (Hamill et al., 1981). Two approaches are frequently used: recording from a bilayer formed from an interfacial monolayer at the tip of a patch pipette (Coronado and Latorre, 1983), and the excision of patches from expanded, reconstituted liposomes. The first technique appears not to be universally applicable, perhaps because of deterioration of some proteins at the air-monolayer interface. Also, when vesicles are fused into preformed patch-pipette bilayers, the incorporation efficiency can be low, especially when pure lipid-containing liposomes are employed. With the second approach, reconstituted liposomes are expanded by rapid freezing and thawing (FT) to form giant structures, and has been widely used (Tank et al., 1982; Rosenberg et al., 1984*b*). However, it has not proven efficient to incorporate native membrane vesicles into these structures without a solubilization step. More recently, Keller et al. (1988) have described the formation of giant reconstituted liposomes by dehydration and rehydration of samples in the presence of ethylene glycol. Successful recording from isolated patches containing K channels from skeletal muscle sarcoplasmic reticulum, as well as from other sources, has been reported. It will be important to learn whether this method can be applied to very labile proteins like the voltage-activated Na channel.

The FT protocol has previously been employed in our laboratory to study the function of purified, reconstituted, voltage-sensitive Na channels from the eel *Electrophorus electricus* (Rosenberg et al., 1984*b*). These studies have been hampered by a consistently low encounter frequency of the channels in excised patches, despite the presence of extremely high levels of active sodium channel protein in the reconstituted liposomes, evidenced both by the binding of [^{3}H]-tetrodotoxin and by neurotoxin-activated ion flux (Rosenberg et al., 1984*a*). Several lines of evidence suggested the problem reflected a nonuniform surface abundance of the protein. We describe here an optimized protocol whereby suspensions of small pure-lipid vesicles and protein-containing vesicles (reconstituted liposomes or native membrane vesicles) can be fused to form very large, primarily unilamellar structures with high channel surface abundance, permitting convenient patch recording. A preliminary report has been presented elsewhere (Correa et al., 1988).

MATERIALS AND METHODS

Preparation of Liposomes

Pure lipids (egg phosphatidylethanolamine (PE), bovine brain phosphatidylserine (PS) (disodium salt), and L-α-lecithin (egg phosphatidylcholine

[PC]; Avanti Polar Lipids, Inc., Birmingham, AL), in chloroform were dried in a stream of N_2 or Ar. The dry lipids were hydrated by resuspension in ⅕ the final volume of 10 mM Tris-HCl, pH 7.2, 1 mM $CaCl_2$ (buffer A). After thorough vortexing, the sample was sonicated to clarity in a bath sonicator (model G112SP1T, Laboratory Supplies, Hicksville, NY). Ionic strength was then raised by adding 0.5 M NaCl in buffer A to a final concentration of 0.4 M. This was done in two additions of first ⅕ and then ⅗ of the final volume. After each addition, samples were vortexed and sonicated to clarity. The procedure was performed at room temperature, under N_2 or Ar.

Native Membrane Vesicles

Three sources of native membranes were used: collagenase-treated, sucrose gradient purified electroplax membranes from *Electrophorus electricus* (M. Emerick, manuscript in preparation); squid optic nerve membranes (Condrescu et al., 1984; courtesy of Dr. F. Bezanilla); and rat skeletal muscle membranes (Guo et al., 1987; courtesy of Dr. E. Moczydlowski). Native membrane vesicles (~5 mg protein/ml) were washed by 10–20-fold dilution in buffer A followed by centrifugation in an Airfuge at 150,000 *g* for 5 min (Beckman Instruments, Inc., Fullerton, CA). This served to remove sucrose or undesired ions present in the original suspensions. Pellets were resuspended at the desired protein concentration in 0.4 M NaCl solution containing liposomes (PE:PS, 7:3; 10 mg lipid/ml) prepared as described before.

Purified Na Channel Protein

Studies with purified electroplax sodium channel protein were facilitated by a rapid affinity isolation procedure according to James and Agnew (1988, and manuscript in preparation). Electroplax membranes, 10–30 ml, were solubilized in 1% Lubrol-PX and purified by DEAE-Sephadex ion exchange chromatography (cf. Rosenberg et al., 1984*a*). This was followed by lectin affinity purification on Sepharose 6B conjugated with *Limax flavus* agglutinin (5 mg/ml resin), specific for sialic acid residues. Material eluted from the DEAE-Sephadex was adsorbed to the resin for 90 min, in 375 mM NaCl, 50 mN Na phosphate, pH 7.2, 0.02% NaN_3, containing 0.1% Lubrol-PX, 0.183 mg/ml phosphatidylcholine (Sigma Chemical Co., St. Louis, MO), and protease inhibitors as described. The protein, assayed for [^{3}H]-TTX binding, was eluted with 10 mM *N*-acetyl-neuraminic acid (NANA) in the same buffer. Peak binding fractions were pooled. Specific activities of 1,000 to >2,000 pmol [^{3}H]-TTX binding sites/mg protein were regularly obtained with this procedure. Before reconstitution, residual NANA was removed by dialysis against 0.4 M NaCl, 10 mM Tris-HCl, pH 7.2, 1 mM $CaCl_2$, 0.02% NaN_3, three times for 1 h each. All steps were at 4°C.

Reconstitution

The reconstitution was modified from that of Rosenberg et al. (1984*b*). Before removing detergent, the lipid concentration in the solubilized protein sample was raised to 4 mg/ml by stepwise addition of liposomes (PE:PS:PC, 5:4:1; 40 mg/ml, prepared as described before), followed by careful mixing and 10 min of incubation on ice after each addition. This likely exceeds slightly the level of lipid that can be solubilized or disrupted by 0.1% Lubrol-PX. Detergent was then removed as before with BioBeads SM-2. Controls with radiolabeled detergent demonstrated that detergent removal is slowed slightly, but is complete under these conditions. After this reconstitution, lipid was then raised to a final level of 10 mg/ml by further liposome addition.

Fusion Procedure

Both native membrane and reconstituted preparations were fused as follows. Samples were mixed and sonicated briefly (<1 s), under N_2 or Ar, at room temperature. They were then frozen slowly in a −80°C freezer (30 min or more), and then thawed at room temperature without agitation. To create expanded structures, aliquots (2 μl) were placed at the bottom of the recording chamber and diluted 20-fold by careful addition of a hypoosmotic solution, normally 250 mM NaCl, 10 mM Hepes, pH 7.2. Swelling of the mixed protein-lipid vesicles begins immediately upon dilution and continues for 10–15 min. Additional bath solution can then be added to the chamber. Vesicles were allowed to settle to the bottom of the chamber before beginning electrical recordings. Vesicles were visualized with an inverted microscope (Olympus Corporation of America, New Hyde Park, NY) with a 40× objective and Nomarski optics.

Electrophysiological Recordings

Single channel events from excised patches were recorded following conventional patch-clamp recording techniques (Hamill et al., 1981). Excised patches were formed as described by Tank et al. (1982). A detailed description will be published elsewhere. Patch pipettes were pulled from Corning 7052 (Garner Glass Co., Claremont, CA) glass capillaries, coated with Sylgard, and fire-polished to tip resistances of 10–50 Mohms (<1 μm) in 250 mM NaCl solutions. Pipettes under slight positive pressure, were rapidly brought close to vesicles at the bottom of the chamber. The pressure was slowly released as the pipette was moved to the vesicle surface. Seals of 5–20 Gohms form either immediately or after a few seconds. Patches were excised by withdrawing and passing the tip through the air-water interface, returning to the same or to an adjacent chamber. Data obtained through continuous acquisition with a Yale patch clamp was stored in video cassette tapes. Holding potentials were applied through an A/D converter driven by a PDP 11/23 computer (Digital Equipment Corp., Maynard, MA). All recordings were at room temperature.

RESULTS AND DISCUSSION

Rationale

The protocols described here are distinct from simple freeze thaw (FT) of pure lipid liposomes with reconstituted vesicles. In that case, fusion to form multilamellar structures, with occasional unilamellar blebs, occurs during the FT cycle. Our experience has been that the frequency of encountering a channel was far less than expected based on the levels of active ([^{3}H]-TTX binding) protein in the sample. Raising the protein-to-lipid ratio increased a population of small, aggregated vesicles, with little change in the surface abundance in the blebs amenable to patch formation. In addition, when native membrane vesicles were added to pure lipid vesicles, FT cycles did not produce efficient native membrane fusion. The latter remained as a separate population of small vesicles while the exogenous liposomes did fuse to form large multilamellar structures, essentially devoid of protein. The current protocols result in fusion during swelling after slow FT, and take into account several factors, including effects of Ca^{2+}, lipid composition, uniform distribution of protein, ionic strength, and osmotic gradients.

Calcium Concentration

In our experience, elevated calcium at the time of FT prevents formation of giant multilamellar structures, but does promote formation of conglomerates of small heterogeneous vesicles. Ca^{2+}, furthermore, retards swelling and fusion after dilution into hypoosmotic buffer. Last, Ca^{2+} promotes rapid formation of high-resistance seals. Thus,

FT of liposomes with either reconstituted vesicles or native membrane vesicles was performed in 1 mM $CaCl_2$, but the subsequent dilution was 20-fold into buffer containing no Ca^{2+}. If Ca^{2+} is to be used to facilitate seal formation, it is added after fusion and swelling are complete.

Lipid Composition

Pure liposomes underwent slight fusion and swelling when a composition of PE:PS:PC of 5:4:1 was used (Fig. 1 *A*). Pure PE:PS (7:3) liposomes aggregated more and swelled less (Fig. 1 *C*). However, when native membranes were included with PE:PS liposomes, swelling improved dramatically, indicating true incorporation (Fig. 1 *D*). Likewise, fusion of reconstituted vesicles with PE:PS:PC liposomes also resulted in intense swelling upon dilution (Fig. 1 *B*). Results from pilot experiments suggested that PS favors fusion, whereas PC inhibits it. The inclusion of protein with associated lipid clearly favors fusion and swelling. It appears that protein itself might have an active role in the process.

Sonication

By sonicating pure liposomes to small size before adding protein-containing vesicles, and sonicating again just before freezing, a more homogeneous distribution of protein in a well-mixed population of small vesicles was ensured. This appeared to markedly improve encounter frequency with the reconstituted protein and fusion efficiency with native membranes. In addition, the modified reconstitution procedure described before was also aimed at improving the uniform surface dilution of the protein.

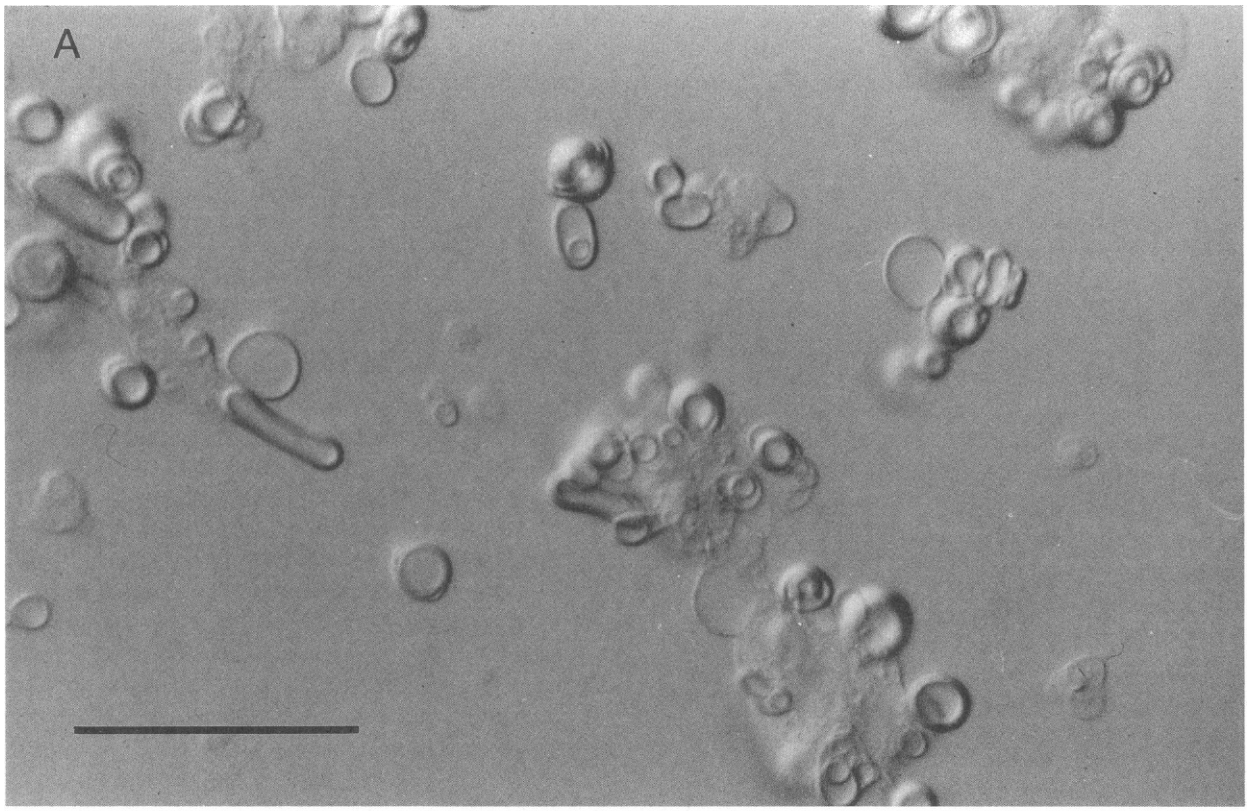
Hypoosmotic Swelling

Before dilution and after FT, the sonicated lipid-protein samples (PE:PS:PC, 5:4:1 with reconstituted vesicles, or PE:PS 7:3 with native membranes) appeared as aggregated conglomerates of small vesicles, less closely packed than was seen with pure lipid vesicles alone. Upon 20-fold dilution the vesicles were observed to swell like balloons during a period of 10–15 min. This dilution reduced liposomes and Ca^{2+} by 20-fold and ionic strength by about two-fold. The structures were usually 10–50 μm in diameter. Whereas some seemed to be multilamellar, apparently unilamellar structures predominated. The best swelling was observed with hypoosmotic chloride salts. Replacement of chloride with other anions (e.g., glutamate or aspartate) resulted in similar structures, but these consistently did not settle well to the bottom of the chamber.

In our experiments seals of 5–20 Gohms were normally obtained; resistance usually increased slightly after excision. Resistances higher than 20 Gohms often indicated the presence of a small vesicle at the pipette tip, and attempts to break one side of the vesicle were usually unsuccessful. Though not required, seals were more readily formed in the presence of Ca^{2+}. When Ca^{2+} was raised after fusion and swelling the vesicle morphology changed dramatically, but large structures were still present for convenient patching. In addition, pipette solutions slightly hypoosmotic (~10%) to the bath facilitate seal formation. Examples of data obtained with these methods are as follows.

Batrachotoxin (BTX) is a potent alkaloid neurotoxin which, among many effects, modifies Na channel gating properties to produce chronic opening at resting and depolarized potentials (Catterall, 1980). This toxin was used for rapid screening for Na channels in different preparations. In the presence or absence of BTX, excised patches from each of the three tested native membrane vesicles (electroplax, squid nerve, and rat muscle membranes) revealed several types of channels. Under the appropriate conditions, however, a single type of channel can be studied. As an example, Fig. 2 shows selected records at three potentials from a patch containing one BTX-modified Na channel excised from a rat muscle membrane preparation. Though more extensive analysis is not presented in this communication, the channel exhibits normal BTX-modified gating kinetics, voltage-dependence, and conductance (20 pS in buffer containing 250 mM NaCl). In the absence of BTX, with excised patches from vesicles prepared with squid optic nerve membranes we have recorded several types of channels; employing this technique in addition, colleagues have readily observed one to several well-behaved, BTX-modified squid optic nerve Na channels per patch (Bezanilla and Latorre, personal communication).

Single channel activity in patches excised from liposomes containing purified electroplax Na channels was observed both in the presence and absence of BTX. Fig. 3 displays some results obtained with BTX-modified Na channels. Fig. 3 *A* shows an array of records at voltages ranging from -100 to $+100$ mV obtained in a patch with three channels. As expected, the channels tended to remain opened at depolarized potentials and to close at hyperpolarized potentials. This is also illustrated in the probability of opening vs. voltage curve shown in Fig. 3 *B*. Patches with more than one channel were normal. At the protein concentration used (100–300 μg/ml), from one to four or five channels were routinely seen. The channel orientation was essentially random, as expected (cf. Rosenberg et al., 1984*a* and *b*), but channels in patches with more than one channel tended to be all in the same orientation. The BTX-modified channels displayed normal kinetics (Fig. 3, *A* and *B*) and conductances estimated from current vs. voltage curves like that in Fig. 3 *C*. Channels were selective for Na over K (P_{Na}/P_K = 5–10), and were blocked in a voltage-dependent manner by nanomolar STX (manuscript in preparation). In addition, channel activity induced by voltage steps alone have been recorded (not presented here). Purely voltage-activated events were observed less frequently. When detected, however, one to several channels could be recorded. A more detailed account of the electrophysiological properties of the BTX-treated and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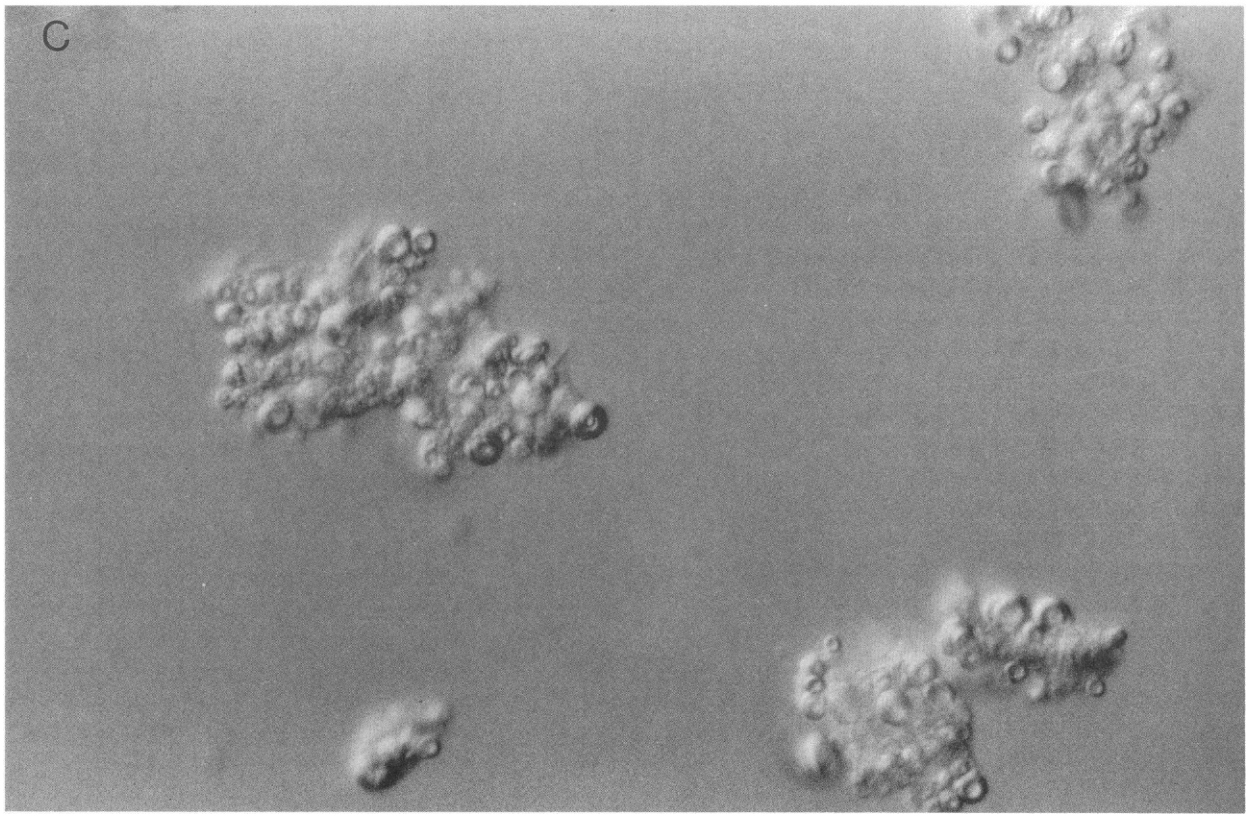
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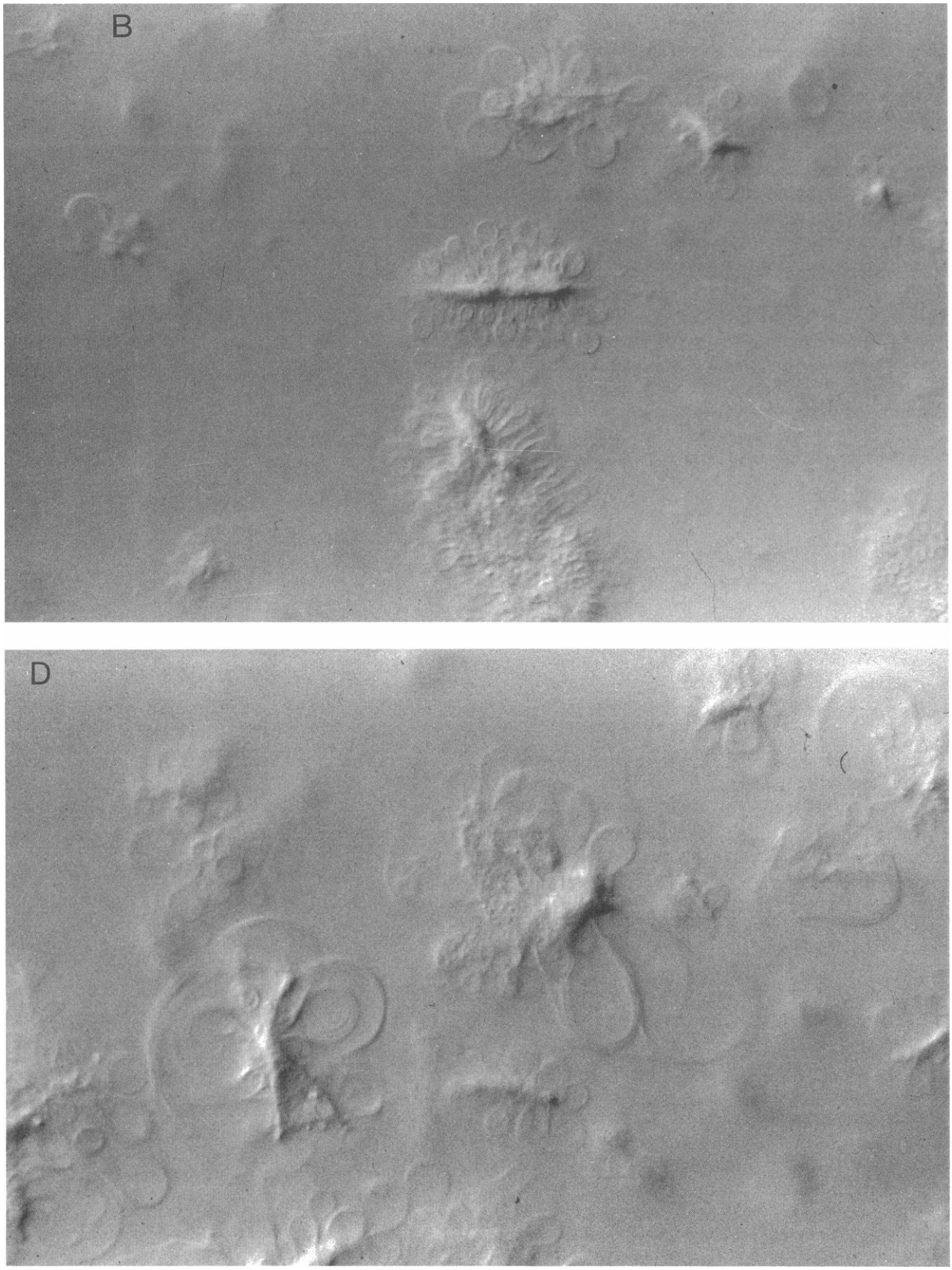

FIGURE 1 Photomicrographs of liposomes prepared as described in the presence (*right*) and absence (*left*) of protein. (*A* and *C*) Pure lipid vesicles made with 5:4:1 PE:PS:PC and 7:3 PE:PS, respectively, after dilution in 250 NaCl, 10 mM Hepes, pH 7.2. (*B*) Mixed protein-lipid vesicles prepared by fusion of reconstituted vesicles containing solubilized, purified eel Na channels (≈100 μg/ml protein) to 5:4:1 PE:PS:PC liposomes. (*D*) Mixed liposomes after fusion of native eel electroplax collagenased membranes (0.7 mg/ml protein) to 7:3 PE:PS liposomes. Magnification, 150. Scale bar, 50 μm.

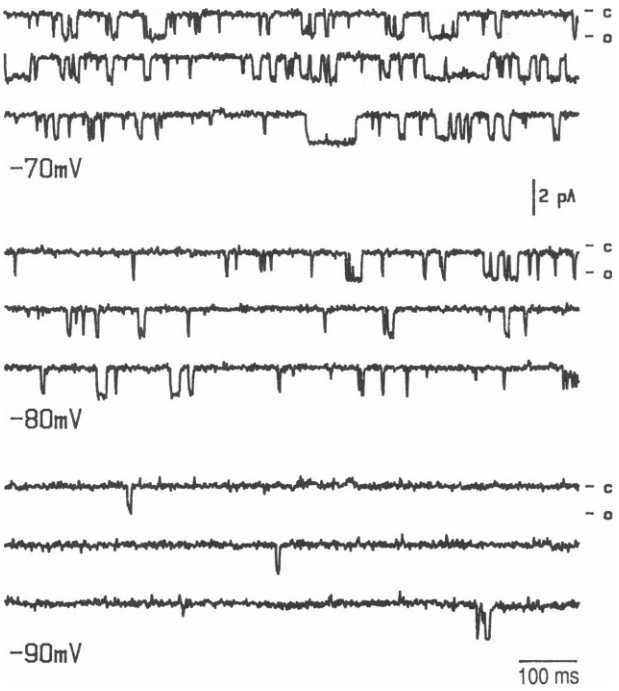

FIGURE 2 Selected records at different holding potentials showing a BTX-modified channel in a patch excised from vesicles formed with rat skeletal muscle membranes and PE:PS 7:3 liposomes. Recording conditions: symmetrical 250 NaCl, 10 Hepes, pH 7.2; filter set at 500 Hz; voltage polarity determined from the characteristic pattern of BTX activation kinetics.

unmodified electroplax channels is the subject of a future report.

In summary, we provide here a method for creating large liposomes with high surface abundance of channels derived either from purified, reconstituted channel preparations, or native membrane vesicles. The procedure can be performed in a short time (60–90 min) in the absence of fusogenic substances, detergents, or organic solvents. The procedure reproducibly yields a homogeneous population of large, primarily unilamellar structures, and the average excised patch has from one to several channels. This is potentially a useful preparation with which to study large populations of channels in whole-cell (liposome) configuration. Not less appealing is the possibility of incorporating components of transport systems or channels from different sources at high densities in the same vesicle. These large structures may facilitate biochemical flux studies of components from native membranes, which may be too small or too leaky for more conventional methods, such as those derived from intracellular organelles.

We are very grateful to Jiuying Zhou for collaborating in the electrophysiological studies. We greatly appreciate Dr. J. Daly's generous gift of BTX. We also want to thank Dr. D. Landowne, M. Emerick, Dr. F. Bezanilla, Dr. E. Moczydlowski, Dr. F. Sigworth, Dr. R. Rosenberg, E. Cooper, and Dr. H. Affolter for their different contributions during the development of this work.

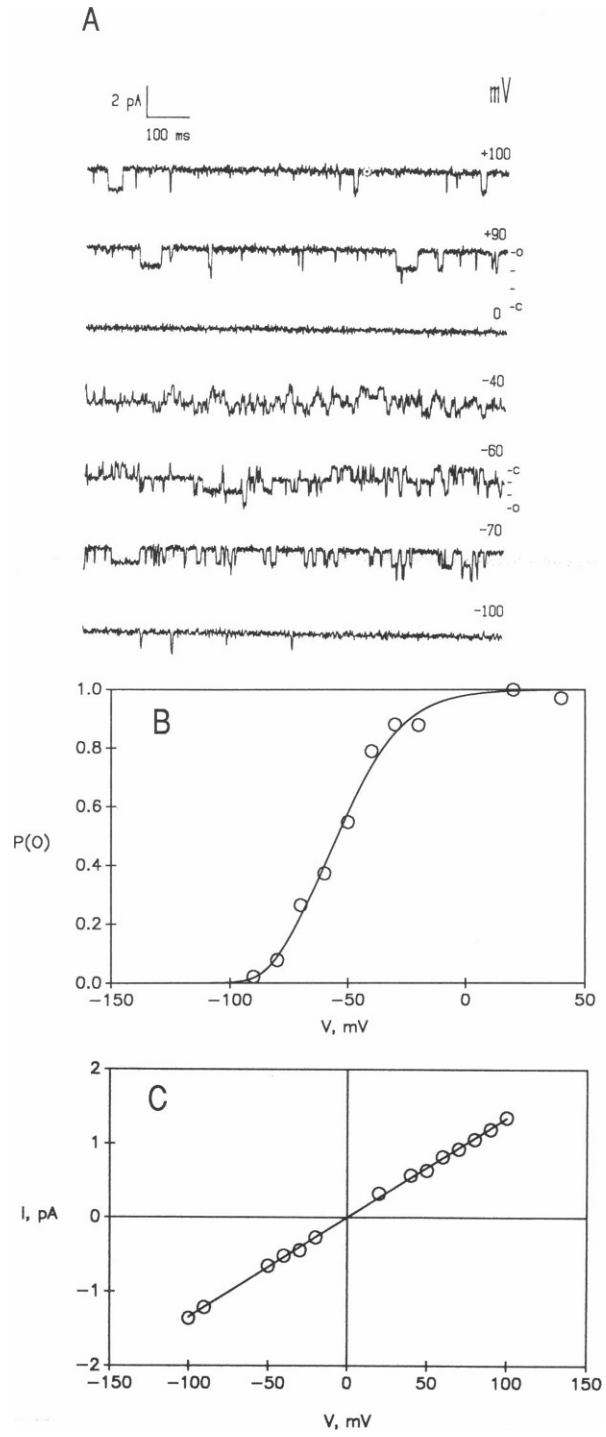

FIGURE 3 BTX-modified reconstituted, purified Na channels from eel electroplax. (*A*) Array of selected records obtained at different voltages in a patch containing three BTX-activated Na channels. Filter, 1 kHz. (*B*) Probability of opening as a function of the imposed holding potential obtained from a patch with only one channel. The solid line is the fit to the data after a sequential model of two closed states and one open state. (*C*) Current-voltage relationship determined from a patch with only one channel. The channel conductance calculated from the slope of the I-V curve was 13.8 pS. All data shown were recorded in symmetrical solutions: 250 mM NaCl, 10 Hepes, pH 7.2. Voltage polarity assessed from channel gating kinetics.

This project was funded by National Institutes of Health grants NS-17928 and HL-38156, and a grant from the National Multiple Sclerosis Society to W.S.A.; A.M.C. was supported by a James Hudson Brown-Alexander B. Coxe Fellowship.

Received for publication 28 March 1988.

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