Nystatin-induced liposome fusion

A versatile approach to ion channel reconstitution into planar bilayers

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ABSTRACT A simple method is described for promoting and detecting fusion of liposomes with planar bilayer membranes. Liposomes containing ergosterol are doped with the pore-forming antibiotic nystatin, and the planar bilayer is kept ergosterol-free. Under these conditions, when a transbilayer salt gradient is applied, liposomes added to the high-salt side of the bilayer elicit the appearance of abrupt conductance jumps of 5–300 pS. The increase in conductance is transient, decaying back to baseline on the order of 10 s. Each of these "spikes" represents the fusion of a single liposome with the bilayer, resulting in the simultaneous insertion of many nystatin channels. Relaxation of the conductance back to baseline occurs because ergosterol, required for the integrity of the nystatin pore, diffuses away into the sterol-free planar bilayer after liposome fusion. When *Torpedo* Cl^- channels are reconstituted into liposomes containing ergosterol and nystatin, fusion spikes are observed simultaneously with the appearahce of Cl^- channels. This method allows the calculation of the density of functional ion channels in a preparation of proteoliposomes containing reconstituted channel protein.

INTRODUCTION

Reconstitution of ion channels into planar lipid bilayers provides a practical approach to studying these integral membrane proteins in a simple, chemically-defined system in which fundamental mechanistic questions may be posed more easily than in the complicated native membrane (Miller, 1986). Moreover, channels which normally reside in intracellular membranes and are inaccessible to direct cellular patch recording, may be readily observed after insertion into planar bilayers. In most applications, channels are transferred into the model membrane by fusing native membrane vesicles, or liposomes containing purified channel proteins, into the planar bilayer. Whereas the mechanism of membrane fusion is still unknown in detail, experience over the past 15 yr has led to a set of conditions under which membrane vesicles or liposomes can be induced to fuse with planar bilayers (Miller et al., 1976; Cohen et al., 1980; Cohen, 1986; Hanke, 1986). These conditions are usually chosen to satisfy the requirement for a transmembrane osmotic gradient leading to vesicle swelling (Miller et al., 1976; Cohen et al., 1980; Woodbury and Hall, 1988*a*; Cohen et al., 1989).

In spite of the wide utility of membrane fusion as the basis for ion channel reconstitution, the method has two severe drawbacks, First, fusion of vesicles into bilayers is highly selective according to vesicle type; some vesicle types fuse with great avidity, whereas others do not, and there is at present no *a priori* indicator of fusibility. For this reason, insertion of vesicles into planar bilayers is not a valid method to screen the channel types in a given vesicle population. Second, there is no rigorous way to claim that the handful of active ion channels observed in the bilayer is at all representative of the population of the 10^9–10^{10} vesicles added to the bilayer chamber. Without the ability to detect fusion of vesicles that do *not* contain active channels, we have no way of estimating the density of active channels in the preparation. This latter problem is particularly troublesome because it is known that liposomes containing ion channels are more likely to fuse than are "tight" liposomes (Woodbury and Hall, 1988*b*; Niles et al., 1989).

Here, we describe a new method that circumvents both these problems. The technique overcomes the selectivity problem by rendering all liposomes fusible, regardless of the protein channels they contain. Moreover, the method allows the detection of all liposomes fusing with the bilayer, irrespective of the presence of a reconstituted channel. It is therefore possible to determine the absolute density of functionally reconstituted channel protein in the bulk liposome population.

The method is based on the finding (Niles and Cohen, 1987) that liposomes doped with the polyene antibiotic nystatin fuse readily and nonselectively with planar bilayers in the presence of an osmotic gradient across the bilayer. Nystatin is known to form low-conductance, weakly anion-selective channels built as a high-order mixed oligomer of nystatin monomer and a membrane sterol (Andreoli and Monahan, 1968; Cass et al., 1970;

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Ermishkin et al., 1976; Bolard, 1986). The requirement for sterol is absolute for the formation of this type of channel. We show that fusion of individual liposomes can be detected as the simultaneous insertion of many nystatin channels, along with the appearance of any reconstituted integral membrane channels present in the liposome. If the planar bilayer is devoid of ergosterol, then the nystatin channels inactivate a few seconds after insertion, whereas the integral membrane channels remain active.

MATERIALS AND METHODS

Chemicals and solutions

The standard aqueous solution used in planar bilayer experiments and liposome preparation was (in millimolar): 150, NaCl; 0.5, EGTA; 8, Hepes; pH 7.5. Other solutions were made by mixing the standard solution with water or 3 M NaCl.

Phospholipids were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Natural phospholipids were phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) from bovine brain. Synthetic lipids were 1-palmitoyl-2-oleoyl PE (POPE) and the corresponding PC (POPC). Cholesterol and ergosterol, obtained from Sigma Chemical Co. (St. Louis, MO), were recrystallized from ethanol. All lipids were stored in stock chloroform solutions at −70°C. Nystatin, also obtained from Sigma Chemical Co., was stored at −20°C at 5 mg/ml in methanol and was kept in the dark.

Preparation of liposomes

Protein-free liposomes containing nystatin were formed from 1 mg lipid in 0.1 ml chloroform to which 2 μl of nystatin stock solution (10 μg) had been added. Lipid composition was (mole percent): 50, PE; 10, PC; 20, PS; 20, ergosterol. After evaporating the solvent under a stream of nitrogen, NaCl solution (0.2 ml) was added, and the mixture was bath-sonicated for 10–100 s. The liposomes were then frozen in a dry ice/ethanol bath; just before use, the mixture was thawed at room temperature and sonicated 5–15 s, to yield largely unilamellar liposomes (Pick, 1981). The final liposome solution contained 50 μg/ml nystatin. Liposome size, as measured by nystatin conductance, varied inversely with final sonication time. Under these conditions, ergosterol was much more effective than cholesterol in supporting nystatin channels. In some experiments, nystatin was added to a preformed liposome suspension just before the freeze-thaw step. In all these experiments, it was important to pay attention to the concentration of nystatin used. Because channel formation is a high-order function of nystatin concentration, too low a concentration eliminates channel activity. Nystatin at too high a concentration produces cation-selective channels which are not dependent on the presence of ergosterol. The optimal range is 50–60 μg/ml nystatin.

For liposomes containing Cl^- channels from *Torpedo* electroplax, the freeze-thaw-sonication reconstitution procedure was used (Kasahara and Hinkle, 1977). Lipid (2 mg) without nystatin was evaporated as above and sonicated for 1 min in 0.2 ml of 300 mM NaCl solution. A given amount (50–100 μg) of purified membrane vesicles derived mainly from the noninnervated face of plasma membranes of *Torpedo* electroplax (White and Miller, 1979) was added to the liposomes after the vesicle storage buffer (400 mM sucrose) was exchanged for 300 mM NaCl by spinning the vesicles through a small G-50 column equilibrated in the new buffer. Nystatin was added to the liposome-vesicle mixture to a final concentration of 60 μg/ml and the mixture was frozen, thawed, and sonicated for 15–20 s. The freeze-thaw and sonication steps were repeated two more times in order to disperse the integral membrane proteins into the excess of exogenous lipid. In some experiments, these reconstituted proteoliposomes were mixed 1:1 with 30% (wt/wt) sucrose in NaCl solution and centrifuged in an Airfuge (100,000 *g*, 5 min) under a 300-mM NaCl solution, in order to confirm that the preparation was devoid of native membrane fragments which had not been incorporated into exogenous lipid. Liposomes from the upper layer gave similar results.

Planar bilayers and liposome fusion

Planar bilayers were formed from decane solutions of PE or POPE (14 mg/ml) and PC or POPC (6 mg/ml), on a 0.15–0.25-mm hole in a polystyrene cup, according to standard methods (Miller and White, 1980). A home-built fast current-to-voltage transducer circuit was used to monitor bilayer electrical properties, and data were collected on video tape and analyzed off-line by computer. Bilayers typically had capacitances of 100–200 pF and conductances <5 pS.

Fusion of liposomes was induced by establishing a transbilayer osmotic gradient (Cohen et al., 1980; Woodbury and Hall, 1988*a*). After a bilayer was formed in symmetrical 150 mM NaCl, an osmotic gradient across the bilayer was formed by adding 3 M NaCl to give a final concentrationof 450–750 mM on the *cis* side. Liposomes (20–100 μg/ml) were then added, with stirring, to the *cis* side. Fusion was detected as sudden jumps in bilayer conductance, as packages of nystatin channels were incorporated via fusion of single liposomes. To obtain reproducible fusion rates, it is important to stir the bilayer chamber continually; conversely, fusion could be suspended simply by stopping the stirrer.

RESULTS

The object of these experiments is to develop conditions under which unilamellar liposomes can be induced to fuse reliably and nonselectively with planar bilayers. Previous work has shown that liposomes fuse with planar bilayers if two conditions are satisfied: a transbilayer osmotic gradient and increased permeability of the liposomes to aqueous solutes (Woodbury and Hall, 1988*b*; Niles et al., 1989). In these studies, the solute permeability was increased by incorporating either bacterial porin or nystatin channels into the liposome membrane. As a fusion-inducing channel, nystatin has several advantages for practical use. First, it is a small, hydrophobic molecule which spontaneously inserts into lipid membranes and second, nystatin channels are stable only if the membrane in which they reside contains a sterol such as ergosterol. The sterol is apparently required as a glue to hold together ~10 nystatin monomers in a barrel-stave arrangement (Cass et al., 1970; Moreno-Bello et al., 1988).

Fig. 1 shows the effect on membrane conductance of adding nystatin to the aqueous solution bathing an ergosterol-containing planar bilayer. Upon addition of nystatin to the aqueous phase, the conductance steadily increases, as nystatin channels spontaneously insert; we did not detect individual channels at the sensitivity used

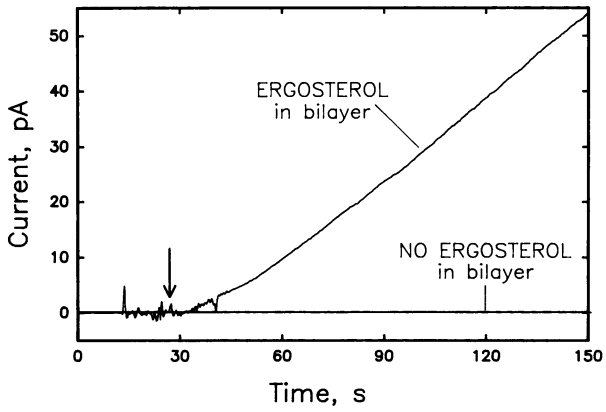

FIGURE 1 Ergosterol-dependence of nystatin-induced conductance. Transmembrane current was monitored at zero voltage across a planar bilayer separating 600 and 150 mM NaCl solutions. Bilayer was formed from a POPE/POPC mixture, with or without 20 mol% ergosterol, as indicated. At the arrow, nystatin was added to both sides of the bilayer, with stirring, to a final concentration of 2 μg/ml. Current was filtered at 40 Hz.

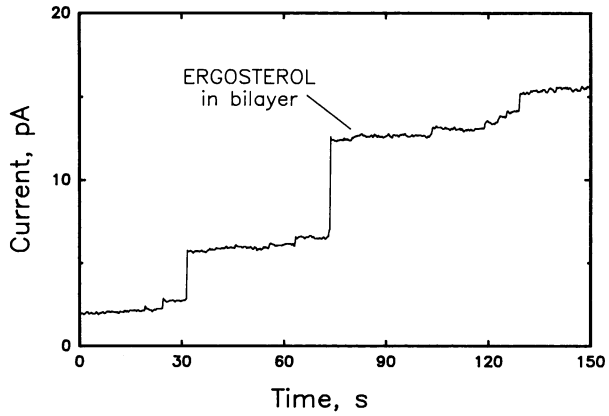

FIGURE 2 Fusion into bilayers of nystatin-containing liposomes. Liposomes containing nystatin and ergosterol were formed as in Methods. A planar bilayer containing 20 mol% ergosterol separated solutions with a NaCl gradient, 450 mM on the *cis* side, and 150 mM on the *trans* side. Just before the beginning of the trace, liposomes were added to the *cis* side, to a final lipid concentration of 20 μg/ml.

here. Ergosterol in the planar bilayer is required for the nystatin-induced conductance rise; the control trace of Fig. 1 shows that the conductance of an ergosterol-free phospholipid bilayer remains unaffected by nystatin, even at fivefold higher concentration of pore-former. As shown below, the nystatin-induced conductance is weakly anion-selective, with a reversal potential of 14 mV in a 450-mM/150-mM NaCl gradient (E_{Cl} = 27 mV).

Nystatin-induced fusion of liposomes has been previously observed by direct observation of transbilayer transfer of fluorescent dye packaged inside the liposomes (Niles and Cohen, 1987). Fig. 2 shows a similar experiment, but using electrical detection of the fusion events. Here, both liposomes and planar bilayer contain ergosterol, so that nystatin channels can form. With a transbilayer osmotic gradient, addition of nystatin-containing liposomes causes the bilayer conductance to increase in discrete steps. The step-conductance displays a wide distribution (as does the liposome size), with a mean value of ~60 pS, equivalent to the simultaneous insertion of ~100 nystatin channels. The appearance of these steps is absolutely dependent upon a transmembrane osmotic gradient, and also upon the presence of both nystatin and ergosterol in the liposomes. The conductance induced by these steps is weakly anion-selective, giving reversal potentials in a salt gradient indistinguishable from those seen for nystatin added directly to the aqueous phase (data not shown).

These results strongly support the idea that each conductance step represents the fusion of a single liposome carrying a package of preformed nystatin channels. A rough calculation of the absolute step size expected for such a fusion further supports this picture. If the liposome mean diameter is 100 nm, and if all nystatin molecules exist in the decameric channel complex (Moreno-Bello et al., 1988), then the average liposome should contain ~100 channels. Because the single-channel conductance of nystatin is ~0.5 pS at 500 nM salt (Ermishkin et al., 1976; Kasumov et al., 1979), the average step size should be ~50 pS, in agreement with the observed steps in the range 5–300 pS (steps smaller than 5 pS occur but are near the experimental noise level).

Nystatin-induced fusion as employed above would be useless for studying reconstituted ion channels in planar bilayers because the nystatin channels, which are required for the fusion process, would swamp out the currents due to any other channels that were transferred in the fusion event. However, we can eliminate this problem by exploiting the ergosterol-dependence of nystatin (Bolard, 1986). In Fig. 3, liposomes are made with nystatin and ergosterol, but now the planar bilayer is ergosterol-free. Under these conditions liposome fusion displays a profoundly different time course. The nystatin channels raise the bilayer conductance at the moment of insertion, as in Fig. 2, but then the conductance returns to baseline within tens of seconds. The appearance of these "spikes" is naturally explained by assuming that as soon as the ergosterol-rich liposomes fuse into the ergosterol-free bilayer, the nystatin-associated ergosterol dissociates from the channel complex and diffuses away into the huge excess of bilayer lipid. As a result, the channel falls apart and ceases to conduct ions.

This explanation is supported by several additional

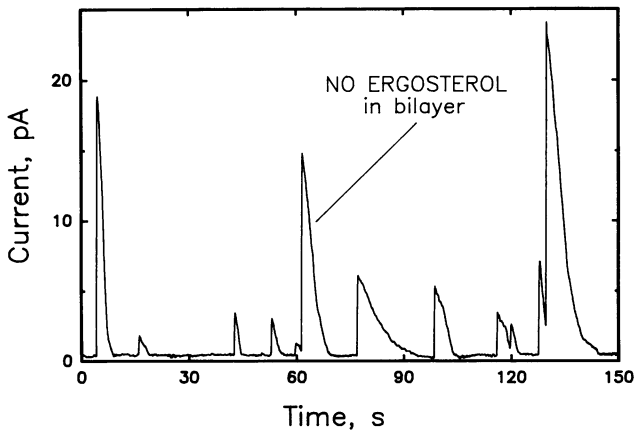

FIGURE 3 Fusion spikes in ergosterol-free bilayers. Liposomes containing both ergosterol and nystatin were made exactly as in Fig. 2. A POPE/POPC bilayer not containing ergosterol separated a 600 mM/150 mM NaCl gradient, and liposomes (25 μg/ml) were added to the *cis* side just before the start of the record. In extended experiments (>100 spikes), the bilayer conductance no longer returns all the way to baseline after a spike; this is probably due to extensive transfer of ergosterol into the bilayer. Similar results are observed with bilayers containing 20 mol% cholesterol, except incomplete return to baseline occurs more frequently.

characteristics of the conductance spikes. The spike amplitudes are similar to the step-sizes seen in ergosterol-containing bilayers (Fig. 2). In addition, the ionic conduction properties of the spikes are identical to those induced by nystatin added to the aqueous phase of a sterol-containing bilayer, as is demonstrated in Fig. 4. Here, we plot the current-voltage relation (*I-V curve*) for the spike amplitudes, under conditions of a threefold NaCl gradient across the bilayer. These data can be fit well by the

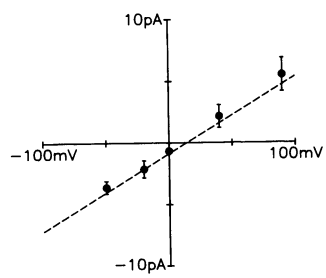

FIGURE 4 Current-voltage relation for fusion spikes. Fusion spikes were collected at different holding voltages, with a salt gradient 450 mM/150 mM present. At each voltage, the mean of the spike peak current (±SE) was plotted (n = 3–21). Dashed curve is the I-V relation of nystatin channels in ergosterol-containing bilayers, under identical ionic conditions; this I-V curve was measured after addition of nystatin to the aqueous phase, as in Fig. 1, and was scaled to the spike data at −20 mV. Reversal potential was 14 mV, and E_{Cl} was 27 mV in these experiments.

I-V curve of the conductance induced in a sterol-containing bilayer into which nystatin has been incorporated from the aqueous phase (*solid curve*). In particular, the reversal potential of the nystatin I-V curve agrees with that of the spikes, and indicates a weak anion selectivity favoring Cl^- over Na^+.

The time courses of the relaxation phase of the spikes shown in Fig. 3 are clearly not all identical; some spikes inactivate more quickly than others. We have not found an adequate explanation for this heterogeneity in inactivation kinetics, nor have we identified any systematic dependence of these kinetics on spike amplitude. However, it may be related to the channel assembly-disassembly rate (Kasumov and Malafriev, 1984) and sterol concentration (Andreoli and Monahan, 1968; Kasumov et al., 1979) studied by others. It is certain, however, that the time course of inactivateion is *not* rate-limited by diffusion of ergosterol away from the vicinity of the channels. Assuming a lateral diffusion coefficient of 10^{-8} cm^2/s, ergosterol originally present in a patch of membrane the size of a typical liposome (e.g., 100–150 nm diameter) would exchange into the planar bilayer with a characteristic time of at most 5 ms, much less than the observed relaxation times in the order of 10 s. We therefore imagine that the spike relaxations are limited by dissociation of ergosterol off the channel complex.

The above results immediately suggest a practical way to transfer reconstituted integral membrane channel proteins into planar bilayers. We demonstrate this method using the Cl^- channel of *Torpedo* electroplax as a test case. This channel, found in the noninnervated face of the *Torpedo* electric organ, has been extensively studied by transferring native membrane vesicles into planar bilayers (Miller and White, 1980; White and Miller, 1979; Miller and Richard, 1990). Despite diligent efforts over the past ten years, we have never been able to transfer these channels reliably into planar bilayers after reconstitution into liposomes. Possibly, this failure might be a consequence of the channel's very high selectivity towards Cl^- (Miller and White, 1980); liposomes into which it is incorporated may not be leaky to both anions and cations, as is required for liposome-bilayer fusion.

Ergosterol-containing proteoliposomes carrying Cl^- channels were prepared by the freeze-thaw-sonication reconstitution procedure (Kasahara and Hinkle, 1977), using purified native *Torpedo* membranes to "dope" the liposomes at a low protein/lipid ratio. (Because the Cl^- channel has not been purified, other membrane proteins are present in these preparations; however, because this is the major ion channel in these membranes, it is expected to predominate in the proteoliposomes.) When added to a sterol-free planar bilayer in the presence of an osmotic gradient, these proteoliposomes induce nystatin spikes similar to those seen with protein-free liposomes (Fig. 5).

In this figure, the first four spikes represent the fusion of "pure" nystatin-containing liposomes, but the appearance of the fifth spike is clearly different. This spike is much noisier than the first four because three Cl^- channels were inserted along with the package of nystatin channels. After ~20 s, the nystatin channels had all inactivated, and the Cl^- channels were left in the low-baseline membrane. As with protein-free liposomes, the observed spikes absolutely required both nystatin and ergosterol in the liposomes.

Our hypothesis that Cl^- channels are inserted via fusion of proteoliposomes demands that the appearance of Cl^- channels should occur simultaneously with nystatin spikes. Fig. 6 confirms this expectation. Here we display at higher time resolution a nystatin spike which brought a single Cl^- channel into the bilayer. It is clear that the insertion of the Cl^- channel and the rise of the nystatin-spike are simultaneous within the time resolution of the measurement (5 ms).

Because the nystatin-mediated conductance disappears after 2–20 s, this method can be used to examine in detail the properties of single reconstituted Cl^- channels. Fig. 7 illustrates this capability. Here we monitored a membrane into which a single Cl^- channel was inserted (at the second fusion spike). After two subsequent nystatin-spikes had relaxed to baseline, the Cl^- channel could be observed in isolation. The characteristic three-state gating process (Miller, 1982; Hanke and Miller, 1983) is apparent.

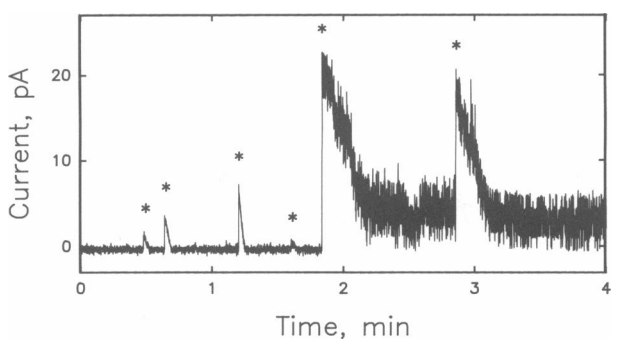

FIGURE 5 Fusion of proteoliposomes containing reconstituted *Torpedo* Cl^- channels. Proteoliposomes reconstituted with *Torpedo* Cl^- channels were prepared as in Methods, with a lipid/protein ratio of 32. Final concentrations in the preparation were: lipid, 10 mg/ml; protein, 0.31 mg/ml; nystatin, 60 μg/ml. Experiment was started by addition of 10 μl of the proteoliposome preparation to the *cis* chamber. Individual fusion spikes are marked by an asterisk. The fifth fusion event incorporated three Cl^- channels into the bilayer (current traces at higher time resolution are shown in Fig 6 and 7). There are four fusions before and one after that contained only nystatin channels. Current fluctuations, as seen after the fifth fusion event, were never seen with liposomes made without protein. Holding potential was −90 mV. Current was filtered at 1 kHz.

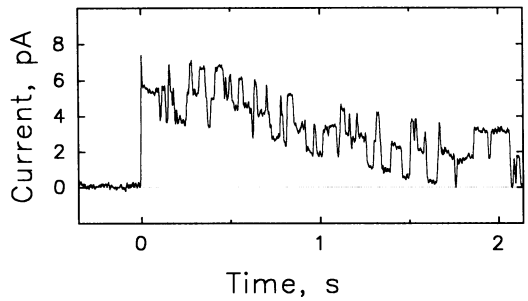

FIGURE 6 High time resolution of the fusion of a Cl^- channel-containing liposome. Conductions are as in Fig. 5, but the fusing vesicle contained only one Cl^- channel. Discrete current fluctuations between three conductance states, typical of the *Torpedo* Cl^- channel, start at the same time as the nystatin-induced conductance spike. Holding potential was −90 mV. Current was filtered at 400 Hz.

By using this technique, we have been able to observe in planar bilayers Cl^- channels prereconstituted into liposomes. Because the Cl^- channels are not purified, we have also observed the appearance of unrecognized channels in these experiments, but the Cl^- channel is most frequently observed. We have also been able to adjust the average number of channels incorporated per spike by varying the protein/lipid ratio in the reconstitution mix. The cleanest results are obtained with low protein, as in Figs. 5 and 7, where the majority of spikes have no channels at all.

DISCUSSION

This report describes a simple, practical, and generally applicable technique for transferring liposome-reconsti-

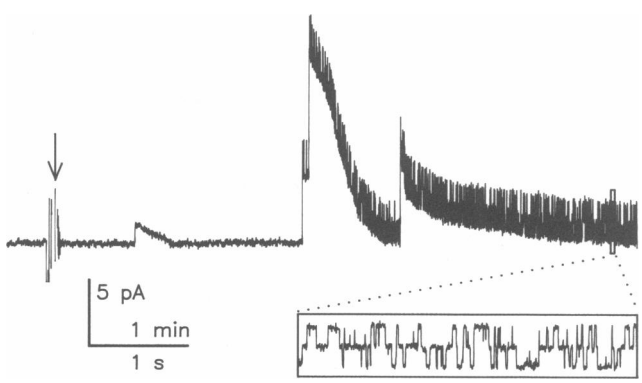

FIGURE 7 Reconstitution of a single Cl^- channel. Conditions are as in Fig. 5, but only one Cl^- channel was incorporated into the bilayer (with the second fusion event). At the arrow, proteoliposomes were added to the *cis* chamber. Stirring was stopped after Cl^- channel insertion. (*Insert*) Current record shown at expanded (60×) time scale.

tuted ion channel proteins into an electrically accessible planar lipid bilayer. Although the idea of using ion channels to promote fusion is not new, the present method has a novel feature: the fusion of individual liposomes is revealed by a transient spikelike conductance change mediated by nystatin, whether or not the liposome carries a functionally reconstituted integral membrane channel. Because the nystatin channels inactivate after fusing into the sterol-free bilayer, the reconstituted integral membrane channels fusing along with them may be studied cleanly, unsullied by a nystatin background. A practical bonus of this method is that it makes it easier to interpret negative experiments in which active channels are not observed. In the past, it was impossible to know if a failure to observe channels was due to poor fusion conditions or to a poor channel preparation. Now, however, the fusion process may be observed via nystatin-dependent spikes, independently of the functional health of the integral membrane channels.

This method makes possible a new type of measurement previously impossible with planar bilayer approaches: the absolute density of functional channels in the reconstituted proteoliposome preparation. Previously, one could never know if the channel most frequently observed in the planar bilayer was also the most abundant channel in the liposome population, or if it was derived from a minor population of highly fusogenic liposomes. Now, because all liposomes are rendered fusogenic by nystatin, an estimate of channel density in the bulk liposome population can be made. We illustrate this capability of "quantitative reconstitution" by estimating the density of *Torpedo* Cl^- channels in our preparations. Under conditions of Fig. 5, in which native protein has been diluted by a factor of 32 into excess lipid, we observe ~0.15 channels per spike, a value leading to a calculated density of 5 channels/μm^2 of proteoliposome area (assuming an average liposome diameter of 100 nm). If the channels are homogeneously dispersed into the excess lipid by the reconstitution procedure, then the original native vesicles would carry Cl^- channels at a density of ~150 channels/μm^2, in good agreement with 200–1000 channels/μm^2 independently estimated from fusion of native membrane vesicles (Miller and White, 1980). This agreement argues strongly that the Cl^- channels are well dispersed in the liposome preparation, and that they do indeed represent a major population of channels in the native membrane vesicles. In claiming to calculate the absolute density of Cl^- channels in native membrane, we acknowledge that all liposomes may not be equally fusible. For example, fusion is almost certainly affected by liposome size, which may be coupled to liposomal protein composition. However, no estimate could have been made if we did not have the ability to detect all the liposomes fusing with the bilayer, including those carrying no Cl^- channels.

The generality of this technique remains to be established, but it is likely that the method will be suitable for the transfer to planar bilayers of many types of ion channels. Our reason for optimisim on this point is the identical behavior of protein-free liposomes and proteoliposomes with reconstituted *Torpedo* Cl^- channels. The fusion process itself does not appear to be altered by the presence, in the liposome membrane, of reconstituted channels along with the fusion-inducing nystatin pores. By taming the process of liposome fusion into planar bilayers, this method opens the way to rational, quantitative ion channel reconstitution work.

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