J. Membrane Biol. 109, 145–150 (1989)

The Journal of
Membrane Biology

Pure Lipid Vesicles Can Induce Channel-Like Conductances in Planar Bilayers

Dixon J. Woodbury
Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

Summary. Typical channel-like current fluctuations were observed in planar lipid bilayers following brief exposure to large concentrations of lipid vesicles *devoid of protein*. Vesicles, formed by sonication of pure lipids suspended in 150 mM salt solutions, were ejected ~0.5 mm from a planar bilayer with a pipette. Over the next several minutes the bilayer conductance changed in ways usually considered to be indicative of reconstituted protein channels including step conductance changes (both up and down), flickering, ion selectivity, and inactivation. This observation demonstrates the need for caution in interpreting conductance changes which occur following ejection of channel-containing vesicles near a membrane.

Key Words lipid channel · vesicle · bilayer · EM · conductance steps · liposome

Introduction

Planar lipid bilayers offer a simple, defined system that makes it possible to measure conductance changes resulting from the incorporation of a single, channel-forming protein into the bilayer. These proteins are usually delivered to the vicinity of the bilayer in native or reconstituted vesicles. An increase in bilayer conductance, following addition of vesicles, is interpreted as indicating that vesicles containing protein channels have fused with the planar bilayer.

One problem with this method is that vesicle addition does not always lead to a conductance increase, because vesicles may not reach the planar bilayer and those that do may not fuse. Several methods for enhancing delivery and fusion of vesicles with a bilayer have been reported. Delivery is increased many-fold by injecting vesicle-containing solution near the bilayer (Woodbury, 1986; Niles and Cohen, 1987). Vesicle fusion is enhanced by creating osmotic gradients (Miller et al., 1976; Finkelstein, Zimmerberg & Cohen, 1986) and by doping the vesicle membrane with channels of known properties (Niles & Cohen, 1987; Woodbury & Hall, 1988).

This paper reports a new problem of the opposite type. Addition of large numbers of protein-free liposomes (vesicles made by sonicating lipid) near the bilayer can induce conductance changes despite the absence of osmotic gradients and channel-forming proteins. These liposome-induced conductance steps (LICS) have an uncanny resemblance to the conductance of protein channels and are observed even when the liposomes are formed from synthetic saturated lipids. Thus, the conductance changes appear to be due to lipid structures, not protein channels.

Materials and Methods

Aqueous Solutions

Three different aqueous solutions were used (in mM): (i) 150 NaCl, 10 HEPES, pH 7.2 (NaCl solution); (ii) 150 KCl, 10 HEPES, pH 7.2 (KCl solution); (iii) KCl solution plus 10 glutamic acid, 0.5 EGTA, pH 7.6 (EB solution).

Liposome Preparation

Liposome were prepared by four different methods. (i) A mixture of lipids (Table 1) in chloroform (10 mg/ml) was dried under nitrogen. (Sometimes the lipids were redissolved in pentane and again dried.) Aqueous solution was added to give a final concentration of 1 mg/ml, and the suspension was bath sonicated for ~1 min at room temperature (ii) Small unilamellar vesicles (SUV) were made the same way but were sonicated at a lipid concentration of 10 mg/ml for 45 min (the vesicle solution became clear after about 20 min). The final vesicle solution was diluted 10× with the same aqueous solution. (iii) Lipids were prepared as in (i) but to a final concentration of 20 mg/ml. The detergent CHAPS (3-[(3-cholamidopropl) dimethylammonio] 1-propanesulfonate) was added to give a final concentration of 31 mM (2%), and the solution was again sonicated. After dilution 1 : 1 with the same aqueous solution, the detergent was removed by passing the solubilized lipid solution over a small G-50 column. The solution from the column (5–10 mg/ml lipid) was diluted 10× and briefly sonicated. (iv) Freeze-thaw-sonicated (FTS) vesicles

Table 1. Mixtures of lipids used to prepare vesicles and liposomes[a]

Mix #	Natural lipids				Synthetic lipids			Vesicle type
	PE	PC	PS	CHOL	POPE	POPC	PC-sat	
I	46	9	27	18	—	—	—	1 (liposome)
II	70	30	—	—	—	—	—	"
III	—	—	—	—	70	30	—	"
IV	—	—	—	—	—	—	100	"
V	—	—	—	—	—	—	100	2 (SUV's)
VI	46	9	27	18	—	—	—	3 (detergent extract)
VII	46	9	27	18	—	—	—	4 (FTS)

[a] Each number in the table is the mole percent of lipid used to make vesicles. "Vesicle type" refers to one of the four methods used to prepare vesicles (*see* Materials and Methods). Phospholipids, both natural and synthetic, were obtained from Avanti Polar Lipids (Birmingham, AL) and stored at −70°C. Natural phospholipids were bovine brain phosphatidylcholine (PC), brain plasmalogen phosphatidylethanolamine (PE), and bovine brain phosphatidylserine (PS). Synthetic phospholipids were 1-palmitoyl-2-oleoyl PE (POPE), 1-palmitoyl-2-oleoyl PC (POPC), and diphytanoyl PC (PC-sat). Cholesterol (CHOL), obtained from Sigma Chemical (St. Louis, MO) was recrystallized from ethanol and stored at −70°C in chloroform (10 mg/ml).

were made as in the previous method, but the solution from the column was frozen in a dry ice/ethanol bath, thawed at room temperature and then briefly sonicated before diluting. These last two methods resemble those used for preparing protein-containing liposomes for reconstitution studies.

Planar Bilayers

Rudin-Mueller type planar bilayers (Mueller et al., 1962) were formed similar to the method of Miller and White (1980). Briefly, both sides of the bilayer chamber were filled with aqueous solution. The bilayer was formed by brushing a lipid solution (20 mg/ml decane) over a small hole (0.35 mm) between the two chambers with a small, rounded glass rod. Bilayers were usually formed of POPE/POPC 7:3 (mix III in Table 1) but were also formed of mix I, II and IV (to match vesicles composition). Bilayers typically had a capacitance of 250–500 pF and a conductance <10 pS (<20 nS/cm^2).

Addition of Liposomes to the Bilayer

Liposomes were added to the bilayer chamber by ejecting 1 μl of liposome solution (0.5–1.0 μg of lipid) about 0.5 mm from the hole. The liposomes were ejected from an "ultra micro" pipette tip (Eppendorf Cat. No. 22 35 153-2) inclined 20–30° away from the normal to the bilayer. The pipette tip was slid along the hard polystyrene partition by hand until the tip was in position by the hole. Liposome solution was ejected from the pipette quickly (<0.5 sec). Care was taken to avoid disturbing the membrane with the pipette tip.

Electron Microscopy

Vesicle solution (5 μl) was placed for 30 sec onto a 400-mesh copper grid coated with a thin carbon film. The preparation was stained with 3 drops of 1% uranyl acetate aqueous solution. Excess stain was drained with filter paper. Grids were viewed with a Philips EM420T transmission electron microscope.

Results

The standard experimental protocol was to make liposomes (as listed in Table 1) using either NaCl, KCl or EB solution. The bilayer chamber was filled with the same solution, and a bilayer was formed from one of the lipid mixtures listed in Table 1 (usually the same mixture as the liposomes). Liposome solution (1 μl) was delivered near the bilayer with a small pipette and bilayer conductance (current at constant voltage) recorded. The recorded conductance changed in ways characteristic of protein channels, including step-wise conductance changes, inactivation, flickering, and cation selectivity.

Figure 1 shows the bilayer conductance following four consecutive additions of small unilamellar vesicles (SUV's). Note that the conductance starts to flicker and rise in a step-wise fashion within 1 min following the first, second and fourth vesicle additions. (The second and fourth additions lead to a conductance that saturates the amplifier.) The rise is followed by a step-wise decrease in conductance which drops to base-line within 4 min (inactivation). Although an increase in bilayer conductance did not occur with every vesicle addition[1] (e.g., third addition of Fig. 1), the remaining additions demonstrate that changes can occur following addition of sonicated lipid. Similar results were obtained with every type of vesicle or liposome listed in Table 1.

[1] The number of conductance steps following addition of vesicles varied considerably (0 to >100). This variability most likely reflects changes in the exact placement of the dispensing pipette tip, which can greatly alter the percent of vesicle solution delivered to the bilayer.

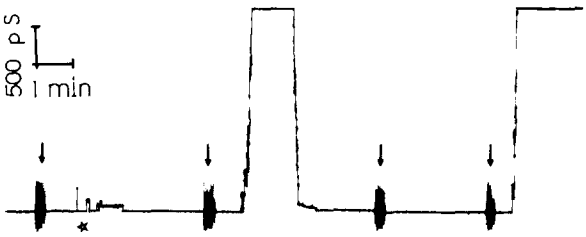

Fig. 1. Bilayer conductance record during four consecutive liposome additions. The arrows indicate the time of addition. This trace is typical in that it shows the four frequently observed outcomes that follow addition of vesicles (SUV's made from saturated PC for this experiment) to a bilayer (also made of saturated PC). The first addition led to just a few conductance steps. These steps (star) are shown in expanded scale in the first three traces of Fig. 2. The second and fourth additions led to a conductance that saturated the amplifier (in the fourth trace the bilayer broke after saturation). The third addition caused no conductance changes. The holding voltage in this and all experiments was 20 mV (liposome side positive); thus the conductance scale bar of 500 pS corresponds to a current of 10 pA. The trace was filtered at 100 Hz

Table 2. Selectivity[a]

Solution (mM)		Reversal potential (mV)			Ratio K^+/Obs.
Cis	*Trans*	K^+	Cl^-	Observed	
50	200	+35	−35	+20	+0.58
"	"	+35	−35	+18	+0.52
150	50	−27	+27	−16	+0.56
"	"	−27	+27	−21	+0.77

[a] The selectivity of the observed conductance was measured by determining the reversal voltage of the conductance with asymmetric KCl. The reversal potential was determined only when the first conductance step after liposome addition lasted at least several seconds (the time required to ramp the voltage). The table indicates that the conductance is cation selective. All four determinations were done with liposomes made of lipid mix I (Table 1). The bilayer also had the same mix of lipids.

Figure 2 shows, in expanded scale, the stepwise nature of many of the conductance changes that occur following liposome addition. Both upward and downward steps of many different sizes and life times are clear in this figure. Sometimes downward steps of a certain magnitude follow upward steps of the same magnitude, but usually the traces are too complicated to attempt careful comparison of step heights. One exception is shown in Fig. 3. This trace shows repeated transitions between the same conductance state (flickering).

The conductances were also characterized as to their ion selectivity and found to be mildly cation selective. Liposomes were added with asymmetric solutions across the bilayer and the reversal potential was determined by measuring the current through a single "channel" during a voltage ramp. Table 2 lists the results from four separate determinations.

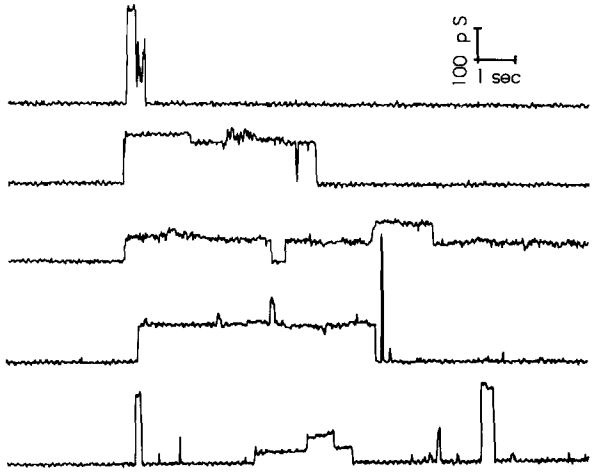

Fig. 2. Similar to Fig. 1 but with expanded scales. These traces clearly show step-wise conductance changes but of many different sizes and life times. The traces were selected from experiments with only a few conductance steps. The bottom two traces are with liposomes (and bilayer) made of POPE/POPC 7 : 3 (mix III in Table 1). The top three traces are expanded from the first addition shown in Fig. 1 (mix V). Similar step sizes and life times are also observed in experiments with many steps (as in the second and fourth addition of Fig. 1)

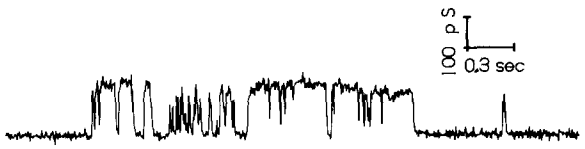

Fig. 3. Similar to Fig. 2 but showing a rare example of flickering that appears similar to protein channel gating. The liposomes were made of PE/PC/PS/CHOL 5 : 1 : 3 : 2 (mix I in Table 1) and the bilayer POPE/POPC 7 : 3 (mix III)

Discussion

In the original experiments that led to this work, protein-containing liposomes were added to the bilayer chamber near the bilayer. The subsequent conductance jumps that followed liposome addition were thought to represent a rich diversity of channel proteins just waiting to be studied. However, this thought was abandoned, when control experiments using liposomes without protein gave similar results.

The liposome-induced conductance steps (LICS) were then attributed to a mechanical distur-

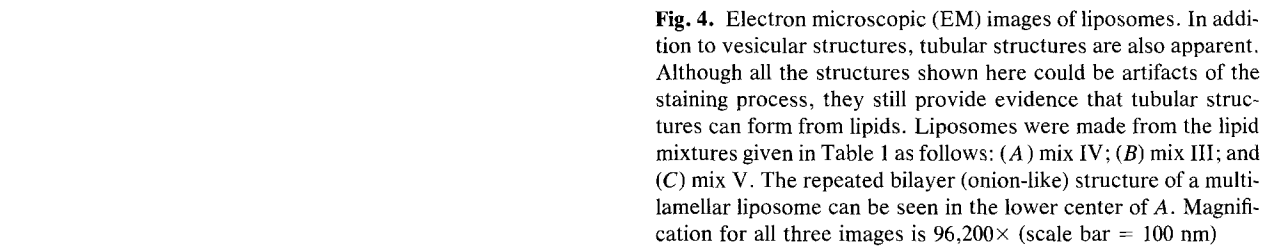

Fig. 4. Electron microscopic (EM) images of liposomes. In addition to vesicular structures, tubular structures are also apparent. Although all the structures shown here could be artifacts of the staining process, they still provide evidence that tubular structures can form from lipids. Liposomes were made from the lipid mixtures given in Table 1 as follows: (*A*) mix IV; (*B*) mix III; and (*C*) mix V. The repeated bilayer (onion-like) structure of a multilamellar liposome can be seen in the lower center of *A*. Magnification for all three images is 96,200× (scale bar = 100 nm)

bance of the bilayer caused by the pipette during liposome addition. This possibility is unlikely since LICS first appear after (not simultaneous with) liposome addition. Also, addition of solution without liposomes ($n = 12$) or addition of liposomes diluted 10× ($n = 5$) did not cause any changes in bilayer conductance.

A second possibility was that LICS were due to contaminants in the liposome preparation. The only possible sources of contaminants are the ionic solution, the extracted detergent or the lipid mix. The first two possibilities are ruled out since addition of solution alone did not cause LICS, but addition of liposomes made in the absence of detergent (*see* Materials and Methods) did cause LICS. The final possibility, contaminants in the lipid mix, requires careful consideration.

Contamination of the lipid mix might be the result of channel-forming compounds that co-purify with the lipids (Gögelein & Koepsell, 1984) or from oxidation of the lipids themselves. Initially, liposomes were formed from natural lipids (lipids isolated form bovine brain). It is conceivable that LICS are due to some polypeptides that were co-purified with the lipids. This possibility is eliminated by the finding that liposomes made from synthetic lipids (Table 1, mix III) also produce LICS. LICS cannot be explained by oxidation (or reaction) of unsaturated lipids since liposomes made from fresh PC with fully saturated chains (synthetic diphytanoyl PC) also caused LICS (Table 1, mixes IV and V).

Another possible explanation for LICS is that they are related to spontaneous bilayer fluctuations previously reported (Boheim, Hanke & Eibl, 1980; Yoshikawa et al., 1988). These fluctuations are attributed to boundary defects in the bilayer due to phase domains. It is reasonable to suspect that LICS are also due to boundary defects that form when liposomes, formed from one mixture of lipids, fuse with bilayers, formed from a different mixture of lipid. However, LICS are observed even when the liposomes and bilayer are formed from the same lipids. It is also noteworthy that the reported bilayer fluctuations require conditions that LICS do not. Yoshikawa et al. (1988) reported that fluctuations were observed only when the bilayer was made from lipids with unsaturated chains. Boheim et al. (1980) reported fluctuations with saturated lipids, but only near the phase transition. In both cases,

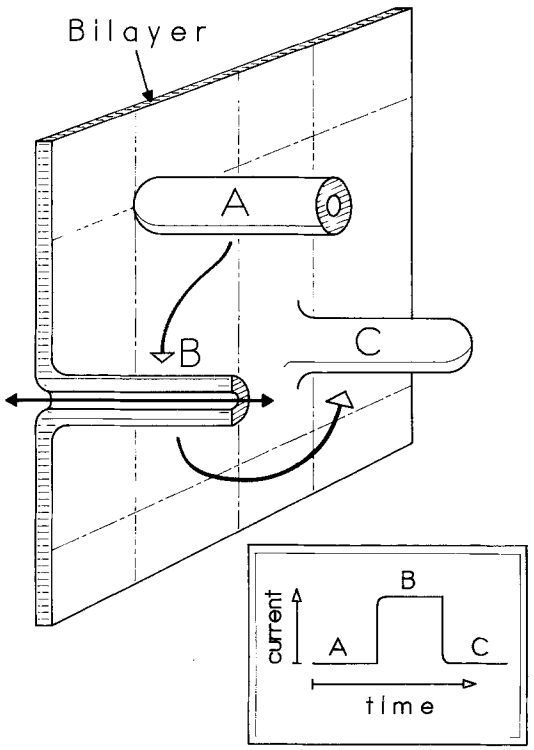

Fig. 5 Cartoon of a bilayer and the postulated tubular structure responsible for LICS (liposome-induced conductance steps). The lipid tube is based on structures visible in EM images of liposomes formed several different ways (Fig. 4). If a lipid tube with one closed end and one open end (*A*) were to fuse at its closed end with a planar bilayer, an aqueous channel would be formed (*B*). The channel would close when the unstable open end of the tube sealed (*C*). The insert shows the bilayer conductance expected for each of the structures

fluctuations were observed only at high concentrations of salt (0.5–1 M KCl or NaCl). LICS are observed at low concentrations of salt (0.15 M) and with unsaturated or saturated lipids far away from their phase transition.

Thus, LICS are not explained by a mechanical disturbance, nonlipid contaminants, or lipid boundary defects. It follows that there must be channel-forming structures made of lipid that form when liposomes are made (or during ejection of liposomes through a pipette). If such structures exist, what do they look like and how do they change bilayer conductance? Figure 4 shows electron microscopic (EM) images of several different liposome preparations used here. In every case, tubular structures are observed in addition to spherical liposomes. If these tubular structures are real (and not artifacts of staining), then they could be the structures responsible for LICS.

Figure 5 is a cartoon of how a lipid tube (as seen in EM) could explain LICS. Although this is speculative, it is not hard to envision the rounded end of a tube (Fig. 5*A*) fusing, like a vesicle, with the planar bilayer (Fig. 5*B*). If the nonfusing end were open or broken, then an aqueous channel through the bilayer would be formed. The channel would close when the broken end closes (Fig. 5*C*). Although the fused tube (channel) structure shown in Fig. 5*B* might be unstable, the long life of many LICS observed here (seconds) could be due to aggregated vesicles that stabilize the base and mouth of the fused tube. Alternately, tube stability could be due to insertion of tubes into decane lenses or the torus.

A simple calculation provides additional support for the hypothesis that LICS are due to fused lipid tubes. Based on the EM structures observed (Fig. 4), lipid tubes are about 15 nm in diameter and 50–300 nm long. Assuming a bilayer thickness of 5 nm, this would mean lipid tubes form an aqueous pore 5 nm in diameter. The specific conductance of the KCl solution used in these experiments was measured to be about 20 mS/cm. From these numbers the conductance of a fused lipid tube can be calculated to be 100–800 pS (depending on tube length). This compares well with the observed sizes of LICS, 30–600 pS.

Regardless of the lipid structure involved, it is concluded that the interaction of lipid vesicles (liposomes or SUV) with planar membranes can induce conductance changes that closely mimic ion channel behavior during the first 5 min after vesicle addition. The main condition that made LICS repeatedly observable here is that liposomes were added at high concentrations near the bilayer. This should serve as warning to anyone interested in reconstituting protein channels: if enough liposomes are added conductance steps will be observed, but the conductances may be due to lipid, not protein channels.

The author is grateful to Drs. Chris Miller and J. Walter Woodbury for invaluable comments on the manuscript and to Diana Cherbavaz for helpful discussion and independently repeating these results. The author also wishes to thank Linda Melanson for help with the EM. Funds to purchase and maintain the Philips EM420T transmission electron microscope were obtained from a Shared Instrumentation grant 1-S10-RR02464-01 awarded to Carolyn Cohen by the National Institutes of Health. This research was carried out during the tenure of a Postdoctoral Fellowship from Muscular Dystrophy Association, and was additionally supported by NIH grant #AR-19826.

References

Boheim, G., Hanke, W., Eibl, H. 1980. Lipid phase transition in planar bilayer membrane and its effect on carrier- and pore-mediated ion transport. *Proc. Natl. Acad. Sci USA* **77**:3403–3407

Finkelstein, A., Zimmerberg, J., Cohen, F.S. 1986. Osmotic swelling of vesicles: Its role in the fusion of vesicles with planar phospholipid bilayer membranes and its possible role in exocytosis. *Annu. Rev. Physiol.* **48:**163–174

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

Miller, C., Arvan, P., Telford, J.N., Racker, E. 1976. Ca^{++}-induced fusion of proteoliposomes: Dependence on transmembrane osmotic gradient. *J. Membrane Biol.* **30:**271–282

Miller, C., White, M.M. 1980. A voltage-dependent chloride conductance channel from *Torpedo* electroplax membrane. *Annu. N. Y. Acad. Sci.* **341:**534–551

Mueller, P., Rudin, D.O., Tien, H.T., Wescott, W.C. 1962. Reconstitution of excitable cell membrane structure *in vitro*. *Circulation* **26:**1167–1171

Niles, W.D., Cohen, F.S. 1987. Video fluorescence microscopy studies of phospholipid vesicle fusion with a planar phospholipid membrane. *J. Gen. Physiol.* **90:**703–735

Woodbury, D.J. 1986. Fusion of Vesicles with Planar Bilayers, Membrane Fusion and Content Release. Ph.D. Thesis. University of California, Irvine

Woodbury, D.J., Hall, J.E. 1988. Role of channels in the fusion of vesicles with a planar bilayer. *Biophys. J.* **54:**1053–1063

Yoshikawa, K., Fujimoto, T., Shimooka, T., Terada, H., Kumazawa, N., Ishii, T. 1988. Electrical oscillation and fluctuation in phospholipid membranes: Phospholipids can form a channel without protein. *Biophys. Chem.* **29:**293–299

Received 11 November 1988; revised 14 February 1989