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Interaction of Charged Lipid Vesicles With Planar Bilayer Lipid Membranes: Detection by Antibiotic Membrane Probes

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A technique has been developed for monitoring the interaction of charged phospholipid vesicles with planar bilayer lipid membranes (BLM) by use of the antibiotics Valinomycin, Nonactin, and Monazomycin as surface-charge probes. Anionic phosphatidylserine vesicles, when added to one aqueous compartment of a BLM, are shown to impart negative surface charge to zwitterionic phosphatidylcholine and phosphatidylethanolamine bilayers. The surface charge is distributed asymmetrically, mainly on the vesicular side of the BLM, and is not removed by exchange of the vesicular aqueous solution. Possible mechanisms for the vesicle-BLM interactions are discussed.

Key words: antibiotics, bilayer lipid membranes, surface charge, phospholipid vesicles, fusion

INTRODUCTION

Vesicle-membrane fusion is a common event in cell biology, providing, for example, the pathway for exocytotic discharge of transmitter in the neuromuscular junction during excitation. Although the mechanisms for biological control of the fusion process, including the roles of membrane proteins and filamentous structures, are not yet understood, it has recently become apparent that phospholipid "model" membranes, under appropriate conditions, can fuse spontaneously. Thus, at least in such "artificial" situations, the fusion process is known to be mediated by the phospholipids themselves. It is of interest to ascertain whether such phospholipid interactions can provide the underlying mechanism for biological fusion, with ancillary protein-mediated interactions perhaps exerting control via modulation of factors which affect the lipids (1). Such a hypothesis requires, as a first step, thorough elucidation of the nature of "bare" phospholipid membrane fusion. In this regard, the phenomena of lipid vesicle-vesicle fusion (2–7) and spherical bilayer fusion (8–10) have been investigated by several authors.

We have chosen to examine the possibility of phospholipid vesicle fusion with planar bilayer lipid membranes (BLM). The reasons are threefold: a) The planar BLM provides a geometry more akin to that of the cell membrane (large radius of curvature), as seen by a vesicle, than does another vesicle. The structural instability of small (~ 300 Å diameter) vesicles should render the fusion of vesicles with BLM less favorable

energetically than fusion of vesicles with one another. b) Planar BLMs provide a unique geometry for investigation of membrane transport, in that convenient electrical and chemical accessibility to both sides of the BLM are possible. Thus, functional properties of the fused membrane system can be studied; for example, the voltage dependence and specificity of the conductance, or the asymmetry of the transport characteristics. c) An understanding of vesicle-BLM fusion could lead the way toward successful incorporation of biologically functional microsomes into BLM, thus permitting thorough electrochemical characterization of these membrane preparations.

The interaction of phospholipid vesicles and biological microsomes with planar lipid bilayers has now been reported several times (11–16), always with some speculation regarding the occurrence of fusion. The major purpose of this paper is to report a new technique for monitoring the interaction of charged phospholipid vesicles (or microsomes) with planar BLMs and for determining whether or not such interactions are likely to involve fusion. We shall illustrate the technique by demonstrating the interaction of phosphatidylserine (PS) vesicles with phosphatidylethanolamine (PE), phosphatidylcholine (PC), and PS BLMs.

Our technique is based on the fact that if interaction of charged vesicles with a BLM injects charged phospholipids into the BLM, then the BLM surface-charge density must be altered. For example, incorporation of anionic PS lipids into a zwitterionic PE BLM must impart negative surface charge to the BLM. A BLM surface-charge monitor could then detect such an interaction. It is well known that membrane surface-charge detectors indeed exist in the form of antibiotics such as Valinomycin, Nonactin, and Monazomycin. In the presesnce of these antibiotics the BLM conductance, which is easily monitored, is a function of the BLM surface-charge density [see McLaughlin and Eisenberg (17) for an excellent review]. Our technique, then, involves the use of standard methods for monitoring the BLM surface-charge density as charged vesicles are added to the aqueous phase.

In addition to sensing an "average" surface-charge density on the BLM, this technique can also detect asymmetries of surface-charge distributions on the two sides of the BLM. Such asymmetries are reflected in the current-voltage characteristic of the antibiotic-treated BLM. Monazomycin is particularly sensitive to these effects (18). Thus, if charged vesicles are added to one side of a BLM only, it is possible to detect the charge densities appearing on each of the two surfaces of the BLM separately. Appearance of surface charge on the side opposite to which charged vesicles are added is a likely indication of fusion (9, 19–21).

METHODS

Lipid bilayers were formed on a 1 mm diameter hole in a Teflon partition separating 5 and 10 ml aqueous compartments. Electrical measurements were made with a pair of Ag/AgCl electrodes connected to the aqueous solutions through KCl-agar bridges. A standard op-amp circuit was used to clamp the membrane voltage and monitor the membrane current. Each membrane was formed in an aqueous solution initially consisting of 10 mM KCl buffered at pH 7.0 with 5 mM Mops/Tris buffer at 22°C. In some cases, 0.2 mM EDTA was also present.

Membranes were made from bacterial phosphatidylethanolamine (Supelco), egg phosphatidylcholine (Sigma, Type VI), and bovine phosphatidylserine (PL Biochemicals).

Each lipid was dissolved in n-decane (Sigma) to give a 1% (w/v) solution. For the Valinomycin measurements the Val was added directly to the lipid-decane solutions in a concentration range of 0.05–0.3 mg/ml. The Valinomycin-treated membranes typically achieved conductances of 2×10^{-6} to 2×10^{-5} ohms^{-1} cm^{-2}. These values were sufficiently high to provide a convenient baseline above the unmodified bilayer conductance but sufficiently low to avoid the undesirable effects of diffusion polarization or interface-limited kinetics (28). All Valinomycin results were confirmed with Nonactin. Monazomycin, when used for asymmetric surface-charge determinations, was added to the aqueous phase of one side of the BLM at a concentration of 3.1 μg/ml. The Monazomycin was a generous gift from Dr. E. L. Patterson, Lederle Laboratories, and Nonactin from Drs. H. Bickel and F. Jenny, Ciba-Geigy Ltd.

Phosphatidylcholine vesicles were prepared by sonicating an 8.3 mg/ml lipid suspension, buffered to pH 7.0, until a clear bluish solution was obtained. Sonication usually took about 25 min at 40–50°C. Phosphatidylserine vesicles were similarly prepared but required only 5–10 min of sonication at 25°C to give a clear opalescent solution. Sonication was carried out under nitrogen using a Kontes probe-type sonicator. Unless otherwise indicated, all vesicle additions to the aqueous BLM compartments gave 13 μg/ml final concentration.

Aqueous solutions were exchanged in the presence of a bilayer with a peristaltic pump (Cole-Parmer) that provided gentle enough action to avoid excessive disturbance of the bilayer. Exchange rates of ~ 5 ml/min were typically used.

RESULTS

In these measurements Valinomycin was used as the BLM surface-charge detector. The action of this antibiotic has been well characterized (22–24), and its utility in sensing BLM surface-charge density is well known (17). Briefly, Valinomycin (Val) imparts K^+ specificity to the BLM conductance, which is then proportional to the K^+ concentration (or activity) at the BLM-water interface. If the BLM has surface charge, then $[K^+]$ at the interface is altered relative to $[K^+]$ in the bulk aqueous phase by electrostatic attraction or repulsion. Thus, the observed Val-mediated BLM conductance (G^+) is enhanced by negative, and suppressed by positive, surface charge. Quantitatively, $G^+ \propto [K^+]_{\text{interface}} = [K^+]_{\text{bulk}} \exp(-F\Psi_0/RT)$, where Ψ_0 is the BLM surface potential. Ψ_0 is in turn determined by the surface-charge density and the bulk electrolyte concentrations by the Gouy equation (for simple electrolytes) or Grahame equation (for more complex electrolytes) (25). Changes in surface-charge density can thus be calculated from observed changes in G^+ *if* it can be established that the G^+ changes are indeed electrostatic in origin.

Figure 1 shows the conductance of a Val-treated PE BLM as a function of time upon addition of 13 μg/ml PS vesicles (A) or PC vesicles (B) to both aqueous compartments. The conductance increases markedly upon addition of the PS vesicles, indicative of anionic surface charge appearing on the BLM. The possibility of this conductance increase arising from PS-induced BLM leakage was investigated by adding PS vesicles to a *non*-Val-treated PE BLM under otherwise identical conditions. The dashed line segment in the lower right corner of Fig. 1A indicates the maximum conductance increase that can be attributed to leakage. The electrostatic origin of the G^+ increase in Fig. 1A was further confirmed by the addition of 0.1 M LiCl (final concentration) to both 10 mM KCl

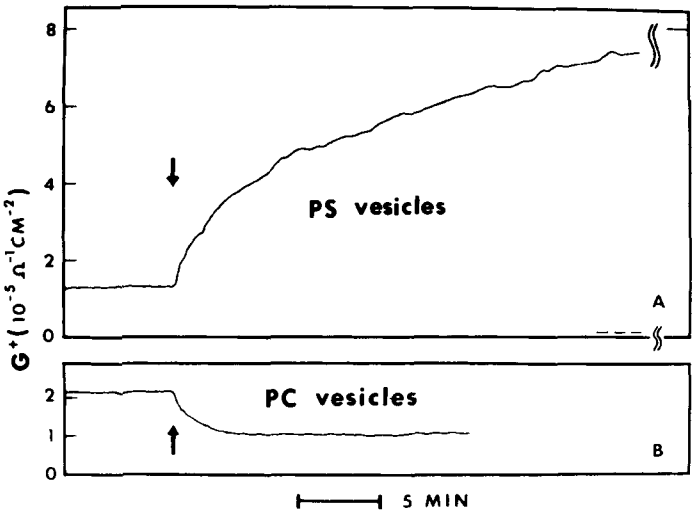

Fig. 1. Conductance of Val-treated PE BLM vs time. Arrows indicate the addition of 13 μg/ml PS vesicles (A) or PC vesicles (B) to both aqueous compartments of the BLM. Voltage clamp = +50 mV. Solutions are 10 mM KCl and 0.2 mM EDTA buffered at pH 7.0 with 5 mM Mops/Tris at 22°C. Dashed line segment in (A) indicates the conductance observed after addition of PS vesicles to a non-Val-treated PE BLM as discussed in the text.

aqueous compartments of the Val-PE BLM. The Li^+ cannot contribute significantly to G^+ due to Val's pronounced K^+ selectivity (24); hence its presence serves merely to increase the ionic strength of the aqueous medium. From the Gouy equation, this should screen the negative surface charge, decrease the magnitude of the surface potential, and hence cause G^+ to drop. In fact, G^+ in Fig. 1A decreases markedly (by ~ 67%) when the LiCl is added, in approximate concordance with the Gouy theory.

In Fig. 1B the PC vesicles are seen to cause a G^+ decrease of ~ 50%. Ideally, zwitterionic PC vesicles should not change G^+ at all, since they cannot add surface charge to the BLM. We attribute this G^+ decrease to loss of Val from the BLM. (This idea is confirmed later in Fig. 3.) Conceivably aqueous vesicles deplete the aqueous Val concentration, which then causes Val to diffuse out of the BLM faster than it can be replenished from the torus. This nonelectrostatic effect is undoubtedly superimposed upon the surface-charge-related G^+ increase for PS vesicles in Fig. 1A and must be accounted for in quantitative determinations. Estimates from the steady-state conductances in Fig.1 indicate a negative PS surface-charge density of ~ 1 charge/700 $Å^2$ appearing on both sides of the PE BLM. The current-voltage relation is slightly superlinear and symmetric.

When PS vesicles are added to one side only of a Val-PE BLM, a conductance increase similar to that of Fig. 1A, but of smaller magnitude, is observed. Now the current-voltage relation is asymmetric, as seen in Fig. 2. (This asymmetry is further evidence against leakage.) The properties of Valinomycin-like carrier transport under asymmetric membrane conditions have been discussed by Stark (23) and by Hall and Latorre (26). The asymmetry seen in Fig. 2 is consistent with the majority of negative surface charge appearing on the side of the BLM to which the vesicles are added. This conclusion is

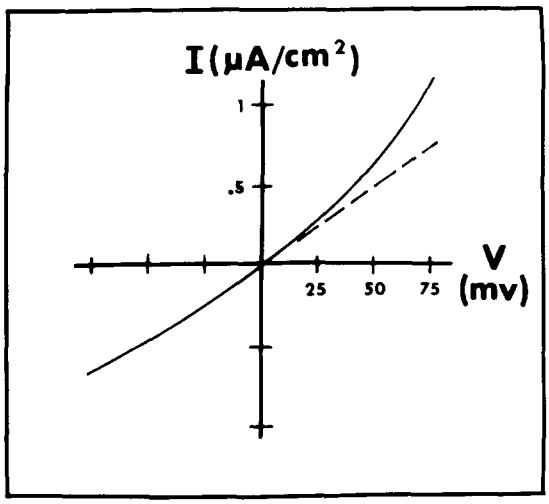

Fig. 2. Current-voltage relation for Val-treated PE BLM after addition of 13 μg/ml PS vesicles to one aqueous compartment. Conditions are the same as for Fig. 1. Dashed line is the tangent at zero voltage. Sign convention: V as indicated = V (vesicle side) −V (non-vesicle side).

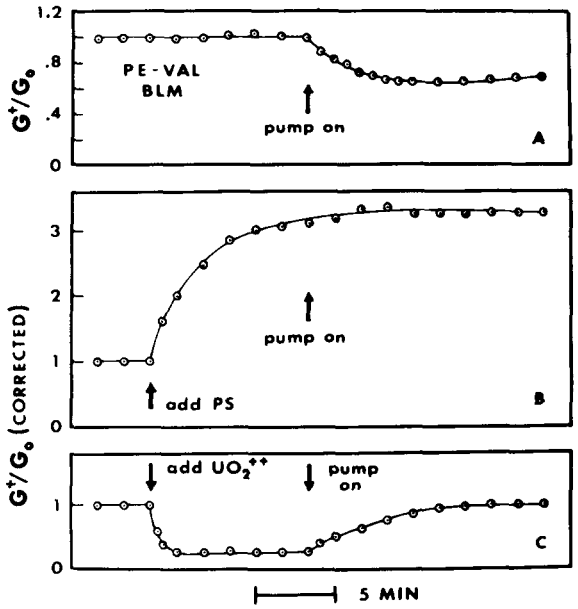

Fig. 3. Perfusion experiment. Normalized conductance vs time for Val-treated PE BLMs. Voltage clamp = +50 mV (sign convention as in Fig. 2). "Pump on" indicates start of exchange of solution in one aqueous compartment with buffered 10 mM KCl. Curve (A) shows decrease in steady-state conductance resulting from loss of Val during exchange. Curves (B) and (C) are corrected for this loss of Val during exchange. (B) shows increase in BLM conductance following addition of PS vesicles (13 μg/ml) to one side of the BLM. Conductance remains unaffected by exchange of vesicle solution. (C) shows membrane conductance drop following addition of 3 μM UO_2^{++} to one compartment. BLM conductance returns to initial value when UO_2^{++} solution is exchanged.

substantiated by screening the highly-charged side of the BLM with 0.1 M impermeant LiCl, which causes the current-voltage curve to become symmetric and the conductance to return to near its original value.

A more quantitative measurement of the asymmetric charge distribution was made with the voltage-sensitive pore-former Monazomycin. The advantage of Monazomycin arises from its extreme sensitivity to asymmetric changes in membrane surface potentials (18). In particular, if Monazomycin is added to one side of a bilayer (which we call the *cis* side), it can readily detect changes of several millivolts in surface potential on the opposite (*trans*) side of the membrane, while remaining relatively insensitive to changes on the cis side. Using this approach, we added PS vesicles to the cis side of a Monazomycin-treated PE bilayer. If surface charge were to appear on the trans side, a significant increase in the Monazomycin conductance would be expected. We found the conductance to increase by a factor of approximately 3 after addition of the vesicles. If all of this conductance increase were attributed to charge appearing on the trans side, the trans charge density would be $\sim$ 1 charge/10,000 Å^2. However, as mentioned above, the Valinomycin data indicate that most of the charge appears on the vesicle side of the BLM. Thus, the charge density on the trans side must in fact be much smaller than the above maximal value. A reasonable estimate is $\leqslant$ 1 charge/50,000 Å^2 on the trans side of the membrane or $\leqslant$ 1 charge/1,000 phospholipids. This value is $\leqslant$ 2% of the charge estimated to be on the vesicle side of the BLM.

We find that Val-treated egg-PC BLMs act similarly to Val-PE BLMs with respect to acquisition of surface charge from aqueous PS vesicles. However, Val-PS BLMs do not acquire additional surface-charge density in the presence of PS vesicles. This result is not unexpected and reflects either a) a lack of PS vesicle – PS BLM interaction, conceivably due to charge repulsion, or b) a "saturated" BLM charge density ($\sim$ 1 charge/phospholipid) that cannot be exceeded by further PS incorporation.

It is interesting to consider whether the BLM surface charge acquired from PS vesicles is actually incorporated irreversibly into the bilayer structure or, alternatively, is loosely bound on the surface. We attempted to answer this question by perfusing the vesicle-containing aqueous compartment with fresh vesicle-free electrolyte while monitoring the BLM conductance; that is, we attempted to "flush off" the surface charge. The results are given in Fig. 3. Fig. 3A shows the effect of perfusion on a plain Val-PE BLM having no surface charge. The G^+ decrease indicates that about one-third of the Val is lost from the BLM. (This result is similar to that observed in Fig. 1B and supports the mechanism described there; i.e., depletion of aqueous Val indeed causes a G^+ decrease.) Figs. 3B and 3C compare the effects due to addition of PS liposomes and UO_2^{++} to one side of Val-PE BLM, with subsequent perfusion. These curves are corrected for the loss of Val shown in Fig. 3A. Uranyl ion is known to bind strongly to lipid membranes ($K_D \sim 10^{-5}$ M for PE) and therefore to impart positive surface charge to BLMs (25). This effect is evident in Fig. 3C from the sharp G^+ decrease upon addition of 3 μM uranyl acetate to one aqueous compartment. However, perfusion removes the positive surface charge completely, implying the existence of a reversible equilibrium between aqueous and membrane-bound UO_2^{++}. Thus the uranyl, although bound strongly, is not bound irreversibly. On the other hand, the negative surface charge from the PS liposomes is *not* removed by perfusion (Fig. 3B). The PS surface charge seems to be irreversibly incorporated into the PE BLM structure.

DISCUSSION

Our results have shown that addition of PS vesicles to the aqueous phase of Valinomycin-treated PE or PC BLMs causes negative surface charge to appear on the BLMs. This charge is irreversibly bound to the BLM and appears mainly on the side of the BLM to which the vesicles are added.

There are several a priori mechanisms that could explain these effects: a) lipid monomer transfer from vesicles to BLM via the aqueous phase; b) lipid transfer from vesicles to BLM via direct contact, either transitory or long-lived; c) irreversible adhesion of vesicles to BLM; d) semi-fusion, in which apposing vesicle and BLM monolayers fuse, but the back monolayers do not; e) full fusion; f) assorted combinations of the above.

In order to test for possibility a), we added one-tenth the normal concentration of PS dispersion to the aqueous phase of Val-PE BLM, the rationale being that since our PS solution ($\sim$ 16 μM) is far above the PS critical micelle concentration, a tenfold reduction in lipid concentration should reduce the vesicle population but not affect the aqueous monomer population. Thus, a decreased effect would indicate the interaction to be vesicle-mediated (or micelle-mediated), and an unchanged effect would indicate the interaction to be monomer-mediated. We found that the effect virtually disappeared for the reduced PS situation, implying vesicle or micelle mediation. We do not, however, feel that this simple test entirely rules out the possibility of aqueous monomer transfer.

Mechanism b) is fully consistent with our observations. The possibility of exchange of hydrophobic molecules from one membrane to another upon close contact was considered by Pohl et al. (11). Exchange of amphiphilic lipids should be equally possible.

It is difficult to envision how mechanism c) could affect the BLM conductance significantly, unless it also involved lipid transfer, that is, mechanism b). A vesicle adhered to the BLM would simply remove the vesicle-BLM adhesion area from contact with the electrolyte, thus reducing the conductive area of the BLM. Possibly that part of the vesicular surface charge lying outside the contact area, but within a Debye length of the BLM surface, could affect local ionic concentrations at the BLM-water interface over small regions, giving rise to small conductance effects.

In mechanism d) PS lipids could spread over the "front" side of the BLM by lateral diffusion, producing the types of effects we have seen. Under the assumption that adhered or semi-fused vesicles might fuse fully with BLM if suitably perturbed, we tried adding decane "chasers" (16), lysolecithin, and heating to 50°C. These manipulations produced no discernible change in the Val-BLM charge distributions.

Full fusion, mechanism e), should deposit some surface charge on the "back" side of the BLM, that is, the side opposite to which the vesicles are added. Monitoring charge density on the back BLM surface is thus the most reliable indicator of fusion provided by our technique. Other mechanisms listed above cannot deposit surface charge on the back of the BLM unless they also manage to enhance lipid flip-flop rates significantly. Our semiquantitative determination of back-side surface-charge density for PS vesicles and PE BLM ($\leqslant$ 1 charge/50,000 Å^2 and $\leqslant$ 2% of the front charge density) indicates that most of the observed interaction is not fusion.

In conclusion, we have shown that surface-charge-sensitive antibiotic probes can be used to detect and monitor the interaction of charged vesicles with planar BLMs. For the case of PS liposomes with PE or PC BLMs, a definite interaction occurs and implants

anionic surface charge irreversibly on the BLM. The charge appears mainly, if not completely, on the side of the BLM to which the vesicles are added. Thus this interaction is *not* likely to be fusion.

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