FLUORIMETRIC DETECTION OF PHOSPHOLIPID VESICLES BOUND TO PLANAR PHOSPHOLIPID MEMBRANES

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ABSTRACT The first step in the fusion of two phospholipid membranes culminates in the aggregation of the two lipid bilayers. We have used a custom-built fluorimeter to detect multilamellar vesicles (liposomes) containing the fluorescent dye, 6-carboxyfluorescein (6-CF), bound to a planar lipid bilayer (BLM). Liposomes were added to one side of the BLM, and unbound vesicles were perfused out. This left a residual fluorescence from the BLM, but only when the membranes contained anionic lipids, and then only when millimolar levels of calcium were present. This residual fluorescence was consistently detected only when calcium was included in the buffer during the perfusion. This residual fluorescence originated from liposomes bound to the BLM. Breaking the BLM or lysing the adsorbed vesicles with distilled water abolished it. Free 6-CF and/or calcium in the absence of liposomes resulted in no residual fluorescence. No residual fluorescence was detected when both the liposomes and the BLM were composed entirely of zwitterionic lipids. This was found to result from the insensitivity of the fluorimeter to a small number of liposomes adsorbed to the BLM. For this system, we conclude that calcium is necessary for both the initiation and maintenance of the state in which the vesicle membrane is bound to the planar bilayer when the membranes contain negatively charged lipids. This attachment is stronger than the interaction between zwitterionic membranes.

INTRODUCTION

A new physical approximation of biological exocytosis is provided by the fusion of phospholipid vesicles with planar phospholipid membranes (Miller and Racker, 1976; Zimmerberg et al., 1980; Cohen et al., 1980). The fusion process in this model system is composed of two experimentally distinguishable steps (Akabas et al., 1984). In the first step, the vesicle approaches and adsorbs to the planar bilayer. In the second step, osmotic swelling of the vesicle enables it to fuse with the planar membrane (Cohen et al., 1982). For vesicles to approach and bind the planar bilayer when the membranes contain anionic phospholipids, millimolar amounts of a divalent cation such as calcium are required (Akabas et al., 1984). Membranes composed entirely of zwitterionic lipids, however, form attachments in the absence of divalent cations. The relative strengths of these attachments remain unknown. Our objective is to explore methods of detecting lipid vesicles bound to planar membranes to understand better the role of calcium in the adhesion process and exocytosis.

In the studies cited above, fusion was assayed by the incorporation of a vesicular membrane marker into the planar membrane (Cohen et al., 1980). Vesicle-membrane attachment was detected, albeit indirectly, as a burst of fusion after unbound vesicles were removed and the remaining vesicles were osmotically swollen.

Here, we have used liposomes containing 6-CF to detect vesicles adsorbed to the planar lipid bilayer with a custom-built membrane fluorimeter. We find that calcium is required for both the initiation and maintenance of vesicle binding when the membranes contain negatively charged lipids. Limitations in the sensitivity of our fluorimeter prevent the detection of bound vesicles made entirely of zwitterionic lipids. We also attempted to detect the fusion of vesicles with the planar lipid bilayer (BLM). Some of these results have been presented as an abstract (Niles et al., 1983).

MATERIALS AND METHODS

Dye-filled liposomes were prepared as described by Zimmerberg et al. (1980). 10 mg of a lipid mixture, containing either 8 mg egg lecithin and 2

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mg bovine phosphatidylserine (4:1 PC/PS) or 8 mg egg lecithin and 2 mg bacterial phosphatidylethanolamine (4:1 PC/PE) in chloroform (all lipids were from Avanti Polar Lipids, Birmingham, AL), was dried down in a round-bottom glass flask by rotary evaporation. 1 ml of dye buffer containing 16 mM 6-CF, 68 mM KCl, 10 mM Mes, and 1 mM EDTA (pH 8.0) was added along with three glass beads, and the flask was swirled gently for 2 min. After the liposomes were allowed to swell for 30 min at ice-bath temperature, the liposomes were separated from the untrapped dye by filtration through a 1 cm × 30 cm column of Sepharose 4B. The column was eluted with our standard buffer consisting of 100 mM KCl, 10 mM Mes, and 1 mM EDTA (pH 6.5). 0.6 ml fractions were collected, and the three most turbid fractions were pooled. The density of liposomes in this preparation is $\sim 10^{10}$ vesicles/ml (Zimmerberg et al., 1980). In those experiments in which the ion-channel porin was incorporated in the vesicle membranes, the lipid mixture, containing 50 μg of porin, was dissolved in hexane and dried down by rotary evaporation. Dye was entrapped in these liposomes as described above.

Liposomes were detected with a membrane fluorimeter consisting of a light source for excitation, a membrane chamber, and detectors for transmitted and emitted light, as shown in Fig. 1. This membrane fluorimeter is an improvement over one referenced earlier (Veatch et al., 1975).

The light source consisted of a 150 W Xe arc lamp focused onto the entrance slit of a monochromator (Jobin-Yvon H2). A circular exit opening of the monochromator was focused onto the center of the planar BLM in the membrane chamber. This resulted in an illuminated spot ~320 μm in diameter. A wavelength of 493 nm (1.5-nm bandwidth) was used to maximize excitation of 6-CF.

The cubical membrane chamber, completely made from 1% carbon black PTFE to minimize light scattering, was divided into two compartments by a diagonal septum with an oval orifice (1.1 vertical × 1.4 horizontal mm) in which the BLM was formed. The plane of the membrane was at a 45° angle with respect to both the directions of excitation and emission. We used 5% asolectin or bacterial PE in decane as the membrane-forming solutions. Each compartment initially contained 8.6 ml of chamber buffer. Ag/AgCl electrodes were submerged in each compartment and connected to a voltage-clamped–current-meaning amplifier. A planar membrane was formed by brushing the orifice under a 50× stereo-microscope's view, and formation of a stable membrane was determined by monitoring its thinning to a black film and its low, steady conductance. All windows for the light path within the chamber and all lenses in the optical paths were ultraviolet (UV) grade quartz.

The emission of fluorescence was measured at a 90° angle with respect to the direction of excitation. The emitted light was filtered through a sharp-cut long-pass filter (C3-68; Corning Glass Works, Corning Science Products, Corning, NY) to minimize scattered light. This filtered fluorescence emission was measured with a high-gain, photon-counting photomultiplier tube (#31034; RCA, RCA New Products Div., Lancaster, PA) cooled to −20°C. The resulting photoelectron pulses were fed into a discriminator capable of resolving pulses separated by at least 22 ns (Photon Amplifier/Discriminator #1120, Princeton Applied Research, Princeton, NJ) and counted by a computer (Digital Synchronous Computer #1110, Princeton Applied Research). Pulses were added over 1 s intervals. This detected fluorescence emission rate (photon counts per second) was displayed on an X-Y plotter (#17005A; Hewlett-Packard Co., Palo Alto, CA) operating with a time-base on the x axis.

Transmitted light was measured with a photodiode (HUV 4000B; EG &G, Electro-Optics Div., Salem, MA) placed in the direction of the excitation beam behind the membrane chamber. This enabled aligning the excitation beam and monitoring its intensity stability. It also provided a measure of the radiant intensity of the exciting light beam, which amounted to $\sim 10^{14}$ photons $cm^{-2}\ s^{-1}$.

RESULTS AND DISCUSSION

Liposomes attached to the planar bilayer were detected as a residual fluorescence of the BLM after unbound vesicles were removed. This residual fluorescence was abolished by osmotic lysis of the adsorbed liposomes. The measured values of a typical experiment are shown in Fig. 2. Fluorescence emission rates (in photon counts per second) are plotted against time in each indicated condition. First, the initial background fluorescence of the asolectin BLM bathed in chamber buffer was measured. This was found to equal the dark count rate of the photomultiplier tube (shown at the end of the experiment), which indicates that the BLM and the buffer made no intrinsic contribution to the measured fluorescence. Liposomes made of 4:1 PC/PS

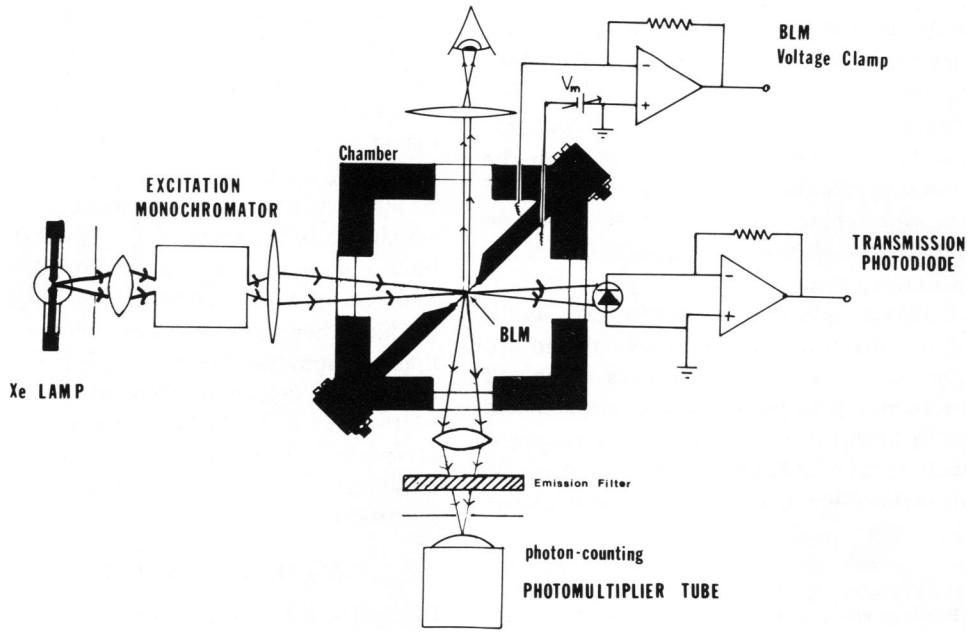

FIGURE 1 Schematic diagram of the membrane fluorimeter.

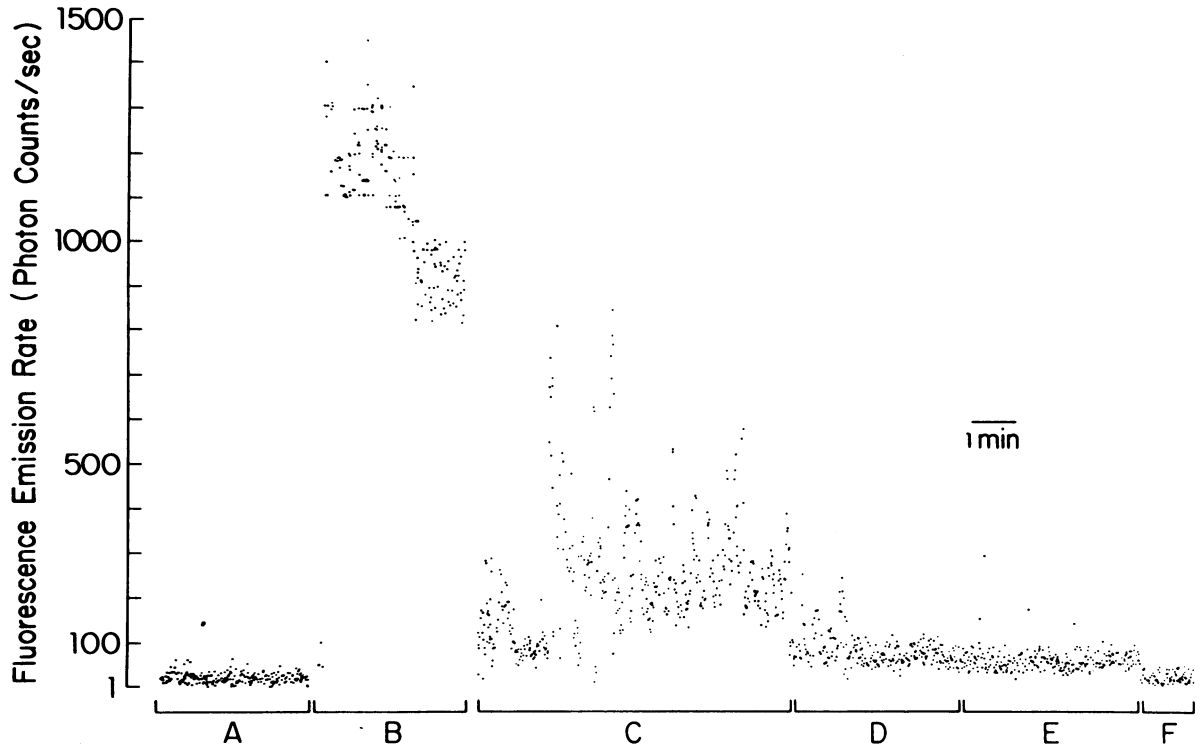

FIGURE 2 Detection of 6-CF–filled liposomes made of 4:1 PC/PS bound to an asolectin BLM as a residual fluorescence. Each point is the number of photons detected by the photomultiplier tube during a 1-s period of time in the indicated conditions: (*A*) The BLM bathed in buffer. (*B*) After the addition of fluorescent liposomes and 20 mM $CaCl_2$ to the *cis* compartment. (*C*) After perfusion of the *cis* compartment with vesicle-free buffer containing 20 mM $CaCl_2$ to remove unbound liposomes. (*D*) After perfusion of the *cis* compartment with distilled water containing 20 mM $CaCl_2$ to lyse osmotically the bound liposomes. (*E*) After breaking the BLM. (*F*) The dark count rate of the photomultiplier tube. The residual fluorescence of the BLM, remaining after unbound liposomes were removed, was abolished by osmotic lysis of the liposomes. This confirms that the residual fluorescence originated from 6-CF encapsulated in liposomes bound to the BLM.

were then added to one compartment, termed the *cis* compartment, to a density of $\sim 10^8$ vesicles/ml. This produced an increased fluorescence of 1,000 photon counts/s. Enough $CaCl_2$ was also added to the *cis* compartment to achieve a concentration of 20 mM, and the chamber was stirred for 5 min. While this produced no change in the fluorescence, we found in our earliest experiments that detection of bound vesicles depended on the presence of calcium (see below). Unbound liposomes were then removed by perfusing the *cis* compartment with eight volumes of vesicle-free buffer containing 20 mM $CaCl_2$. This left a residual fluorescence that ranged from 150 to 400 photon counts/s. Liposomes remaining attached to the BLM were osmotically lysed by perfusion of the *cis* compartment with eight volumes of distilled water containing 20 mM $CaCl_2$. This resulted in a decreased fluorescence ranging from 50 to 100 photon counts/s. Subsequent breakage of the BLM produced no change in fluorescence. In control experiments, no residual fluorescence was detected after the free dye and calcium were added to the chamber and then perfused out. This ruled out the possibility that the residual fluorescence originated by transfer of the dye or by partition of a lipophilic contaminant into the BLM. These results indicate that the residual fluorescence originated from liposomes attached to the BLM.

Binding of liposomes made of 4:1 PC/PS to asolectin planar bilayers was detected only when calcium was also present in the buffer. The procedure for these experiments was similar to the one depicted in Fig. 2, except that the presence of calcium was varied. Instead of osmotic lysis of the bound liposomes, however, the BLM was broken after the measurement of the residual fluorescence. The final background fluorescence was then recorded to determine how much of the residual fluorescence actually originated from the BLM. To compare the results of experiments with different initial background fluorescence rates, the residual fluorescence was expressed as a multiple of the initial background rate. This was calculated for each experiment as the ratio of the difference between the average fluorescence rates recorded after perfusing out unbound liposomes and after breaking the BLM to the average initial background fluorescence rate. In three experiments in which calcium was omitted from the buffers during the entire experiment, these residual fluorescence ratios were 0.6, 0.2, and 0.0. When 20 mM $CaCl_2$ was included in the *cis* compartment along with the liposomes, yet was omitted from the perfusion, the ratios obtained in three experiments were 2.3, 0.5, and 0.0. In six experiments in which 20 mM $CaCl_2$ was present both when the vesicles were added and during the perfusion, the average of the ratios

was 2.9 ± 1.3 (± the standard deviation of the observations). These data indicate that the formation of attachments between negatively charged membranes depended on the presence of calcium. This calcium-mediated interaction between the membranes must be fairly strong, since a residual fluorescence signal was detected after perfusion with a large volume of calcium-free buffer. The continued presence of calcium, however, was necessary for a sufficient number of liposomes to remain bound to the BLM to allow the consistent detection of a residual fluorescence. This reveals that the interaction between a vesicle and the planar membrane is stabilized in the presence of calcium.

We attempted to detect fusion in this system by a decrease in the residual fluorescence. Vesicles fusing with the BLM should expel their fluorescent contents into the *trans* compartment and would no longer be concentrated at the surface of the BLM. Liposomes attached to the BLM were osmotically swollen by making the *cis* compartment hyperosmotic with urea, glycerol, or KCl. In all experiments, this produced an increased residual fluorescence of 20 to 50% as compared with the residual fluorescence prior to the addition of the osmoticant. Breaking the BLM produced little diminution of the residual fluorescence. This increased residual fluorescence probably resulted from the release of dye to the *cis* compartment by the liposomes as they initially shrank in the hypertonic buffer (F. S. Cohen, personal communication; Homan, R., and M. Eisenberg, unpublished results).

To determine whether the vesicles were fusing with the BLM in these conditions, we used liposomes prepared with the ion channel porin as a vesicular membrane marker in addition to 6-CF. When these bound liposomes were osmotically swollen by the addition of osmoticant to the *cis* compartment, fusion was evident as a burst of current required to clamp the voltage of the BLM at +20 mV. This was accompanied by an increased residual fluorescence. We also created the osmotic gradient by initially including 500 mM urea in both compartments during the addition of liposomes and during the perfusion to remove unbound liposomes. The *trans* compartment was then perfused with urea-free buffer. Although fusion was evident by the burst in the voltage-clamp current, the residual fluorescence remained unchanged. These results indicate that the residual fluorescence of the BLM originated from a large number of adsorbed liposomes. The insensitivity of the fluorimeter to the disappearance of a small fraction of these liposomes prevented the detection of fusion.

We also attempted to detect the binding of fluorescent liposomes to planar lipid bilayers when both membranes were composed entirely of zwitterionic phospholipids. This was motivated by the finding that calcium or other divalent cations are unnecessary for the initiation and maintenance of attachments between these neutral membranes (Akabas et al., 1984). We detected no residual fluorescence above the initial background rate after liposomes made of 4:1 PC/PE were perfused away from the *cis* side of PE planar bilayers. This difference between our results and those of Akabas et al. (1984) may have originated at least from the following four sources: (*a*) The decane in our BLMs may have hindered the stability of the vesicle-membrane attachment. Akabas et al. (1984) formed planar bilayers by the union of two, relatively solvent-free phospholipid monolayers. (*b*) Our multilamellar vesicles may have bound less tightly to the BLM than their unilamellar vesicles. (*c*) Leakage of the anionic dye from the liposomes and its subsequent adsorption to the membranes may have prevented vesicle binding. (*d*) Our fluorimetric method was less sensitive to bound liposomes than their method, which was capable of detecting the fusion of a single vesicle remaining attached to the planar bilayer.

To test these possibilities, we prepared dye-filled liposomes containing the ion channel porin as a vesicular membrane marker. These liposomes, made of 4:1 PC/PE, were added to the *cis* side of a PE BLM to the same density that was used in the fluorimetry experiments. After the compartment was stirred for 5 min unbound liposomes were removed by perfusing the compartment with 10 volumes of buffer. The *cis* compartment was then made hyperosmotic by the addition of KCl. The subsequent burst of fusion of vesicles remaining attached to the BLM was manifested as an explosive increase in the current required

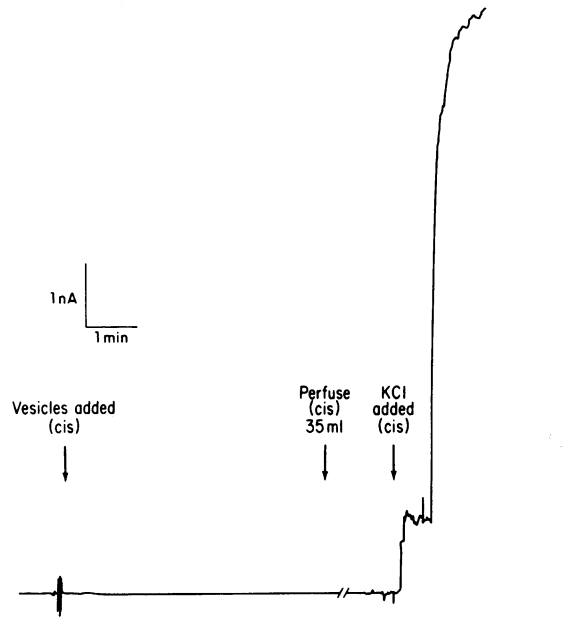

FIGURE 3 Voltage-clamped current record of the fusion of liposomes made of 4:1 PC/PE bound to a PE BLM. The liposomes, containing the ion-channel porin incorporated as a vesicular membrane marker, were added to one side of the BLM. This compartment was then perfused with vesicle-free buffer to remove unbound vesicles. The voltage across the BLM was clamped at 20 mV (vesicle-containing side positive). The *cis* compartment was made hyperosmotic by the addition of KCl. The fusion of liposomes bound to the BLM is manifested by the increase in the voltage-clamp current as the porin is reconstituted in the planar bilayer. Under these same conditions, no bound liposomes were detected by the fluorimeter.

to clamp the voltage of the BLM at 20 mV (Fig. 3). Thus, liposomes were bound to the BLM under conditions in which no residual fluorescence was detected by the membrane fluorimeter.

The above experiment shows that fluorimetry of dye-filled liposomes is a much less sensitive technique for detecting vesicles bound to planar bilayers than electrophysiological methods. This is not surprising, since the magnitude of the detected residual fluorescence is dependent on both the number and the size of the liposomes bound to the region of the BLM illuminated by the exciting light beam. This size dependence is related to the quantum cross-section of the vesicle. A given liposome offers ten times as many dye molecules per incident photon as a liposome one-tenth its diameter. The number of bound liposomes, however, is determined by the free energy of the binding interaction.

Our results indicate that the calcium-mediated attachment between negatively charged membranes is stronger than the interaction between zwitterionic membranes. It has been suggested that calcium forms a complex with two negatively charged lipids, one from each bilayer, that dehydrates the head groups and holds the lipids in close apposition (Portis et al., 1978). The interaction between zwitterionic membranes is unknown, but it may involve electrostatic attraction between complementary charges on the head groups of lipids in the two bilayers. This attraction must be weak because the interbilayer separation amounts to several nanometers (LeNeveu et al., 1976). Thus, the liposomes made of zwitterionic lipids are probably loosely bound to the BLM. A significant number detach from the planar membrane during the extensive perfusion necessary to remove unbound liposomes. The fluorimeter is insufficiently sensitive to detect the few vesicles remaining bound.

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