Fusion of synaptic vesicle membranes with planar bilayer membranes

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ABSTRACT   The interaction of synaptic vesicles with horizontal bilayer lipid membranes (BLMs) was investigated as a model system for neurotransmitter release. High concentrations (200 mM) of the fluorescent dye, calcein, were trapped within synaptic vesicles by freezing and thawing. In the presence of divalent ions (usually 15 mM $CaCl_2$), these frozen and thawed synaptic vesicles (FTSVs) adhere to squalene-based phosphatidylserine-phosphatidylethanolamine BLMs whereupon they spontaneously release their contents which is visible by fluorescence microscopy as bright flashes. The highest rate of release was obtained in KCl solutions. Release was virtually eliminated in isotonic glucose, but could be elicited by perfusion with KCl or by addition of urea. The fusion and lysis of adhering FTSVs appears to be the consequence of stress resulting from entry of permeable external solute (KCl, urea) and accompanying water. An analysis of flash diameters in experiments where Co^{+2}, which quenches calcein fluorescence, was present on one or both sides of the BLM, indicates that more than half of the flashes represent fusion events, i.e., release of vesicle contents on the *trans* side of the BLM. A population of small, barely visible FTSVs bind to BLMs at calcium ion concentrations of 100 μM. Although fusion of these small FTSVs to BLMs could not be demonstrated, fusion with giant lipid vesicles was obvious and dramatic, albeit infrequent. Addition of FTSVs or synaptic vesicles to BLMs in the presence of 100 μM–15 mM Ca^{2+} produced large increases in BLM conductance. The results presented demonstrate that synaptic vesicles are capable of fusing with model lipid membranes in the presence of Ca^{+2} ion which, at the lower limit, may begin to approach physiological concentrations.

INTRODUCTION

The fusion of membrane-bound vesicles with planar bilayers as a model system for exocytosis has been the subject of a number of investigations (1–9). The aim of these studies has been the elucidation of the mechanisms of fusion and the determination of the extent to which the conditions necessary for fusion mimic those thought to be involved in exocytosis. In the common case of lipid vesicles and planar bilayers containing phosphatidylserine, fusion requires a calcium-dependent adhesion of vesicles to the planar bilayer. Fusion is usually elicited by imposing an osmotic stress on the adhering vesicles (1–9). Although the evidence for fusion in such systems is strong, most is based upon detection of transfer of ion channels from vesicles to bilayer, and there has been a need for independent verification of such fusion, as well as for a method that, in addition to registering fusion events, allows estimation of the efficiency of the process, both with respect to extent and frequency of leakage events and to the yield, i.e., the proportion of bound vesicles that fuse.

Little attention has been given to the study of fusion of natural exocytotic vesicles with planar bilayers. We have undertaken such an investigation, using synaptic vesicles, with the objective of elucidating some aspects of the mechanism of neurotransmitter release. By freezing and thawing, synaptic vesicles may be loaded with a fluorescent dye, calcein (10, 11). A novel and important characteristic of synaptic vesicles loaded with high concentrations of calcein is that although the vesicles are easily visible by fluorescence microscopy, most of the dye is self-quenched. Thus, adhesion of such vesicles to planar bilayers can be quantified by their residual fluorescence and their fusion is revealed by bright fluorescent flashes that are the result of release and dilution of the dye. We have used this experimental system to establish the conditions under which synaptic vesicles fuse to a simple membrane lacking proteins.

MATERIALS AND METHODS

Chemicals

Decane was obtained from Eastman Kodak Co., Rochester, NY and squalene from Sigma Chemical Co., St. Louis, MO. Both were purified by passage through a silica gel column. Phospholipids were obtained from Avanti Polar Lipids Inc., Pelham, AL. Soy lipid (type IVS) was

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obtained from Sigma Chemical Co. and was acetone washed to remove neutral fats (12). Calcein, from the Hach Chemical Co., Loveland, CO was purified over lipophilic Sephadex (LHO-20, from Sigma Chemical Co.; 13). All other chemicals were reagent grade.

Synaptic vesicle preparation

Brain synaptic vesicles were isolated using the procedure of Nagy et al. (14). Electric organ synaptic vesicles were isolated by a modification of the procedure of Tashiro and Stadler (15). Electric organ from the ray *Discope ommata* was frozen and stored in liquid N_2. Before isolation, ~20–40 g of pieces frozen organ were crushed with mortar and pestle into a course powder which was then extracted with 70–100 ml cold 400 mM NaCl, 20 mM MOPS pH 7.4, 3.5 mM EGTA. After passage through four layers of cheese cloth, the extract was centrifuged at 10,000 g for 30 min (9,250 rpm in Sorvall SS34 rotor, DuPont Co., Wilmington, DE). The resulting supernatant was loaded onto a discontinuous sucrose/NaCl gradient consisting of 8 ml of 0.6 M sucrose, 0.1 M NaCl and 10 ml of 0.2 M sucrose, 0.3 M NaCl. This was spun at 67,000 g (24,000 rpm) for 3.5 h in a SW28 rotor; Beckman Instruments Inc., Palo Alto, CA. The band at the interface was collected, diluted with 0.4 M NaCl to a volume of 17 ml per tube, and loaded onto a linear isosmotic sucrose gradient consisting of a 2 ml cushion of 2 M sucrose and 16 ml of a linear gradient from 0.7 M sucrose, 50 mM NaCl to 0.2 M sucrose, 0.3 M NaCl. This gradient was spun in a SW28 rotor at 28,000 rpm for 6 h. The band at ~0.4 M sucrose, containing synaptic vesicles, was collected by syringe and kept at 0° until used.

ATP was determined with luciferase (16). Protein was assayed by the Lowry-SDS procedure (17) or by the Bradford procedure (18). Isolated synaptic vesicles contained 2 μmol ATP/mg protein, a concentration similar to that reported for *Torpedo* vesicles isolated using the procedure described above (1 μmol) ATP/mg protein; 15) and for *Narcine* electric organ synaptic vesicles (19) μmoles ATP/mg protein; isolated using a different procedure.

Light microscopy of vesicle bilayer interactions

Fusion of synaptic vesicle membranes to pure lipid bilayers was assessed using a procedure that relies on the formation of nonfluorescent calcein dimers at concentrations >~1 mM. Calcein was loaded into synaptic vesicles at 200 mM; its subsequent release results in a high dilution and a concomitant large increase in fluorescence. Abrupt discharge of contents is visible under the fluorescence microscope as a bright flash having a duration of ~½ s. Before loading with calcein, synaptic vesicles, prepared as described above, were collected by centrifugation at 45,000 rpm (200,000 g) for 60 min in a SW 50.1 rotor (Beckman Instruments). After resuspension in distilled water by passage twenty times through a 100 μl syringe, the vesicles were diluted with an equal volume of 400 mM calcein. The resulting suspension was subjected to three cycles of freezing in a dry ice-ethanol bath and thawing by hand warming. The vesicles that result from this procedure are denoted frozen and thawed synaptic vesicles (FTSVs).

Planar bilayers were formed as follows. Brain PS and egg PE (1/1) or brain PS and bacterial PE (1/1) were mixed in chloroform, solvent was removed under N_2 and the lipid residue put under oil pump vacuum for 1 h. Lipid was dissolved in decane or squalene to a concentration of 4–5 mg/ml, allowing 1 h for dissolution in decane and 2 to 3 h for squalene (both under N_2). The procedures used for both decane and squalene-based membranes were similar to those described by others (20, 21) except that the BLM was formed horizontally. Fig. 1 is a diagram of the
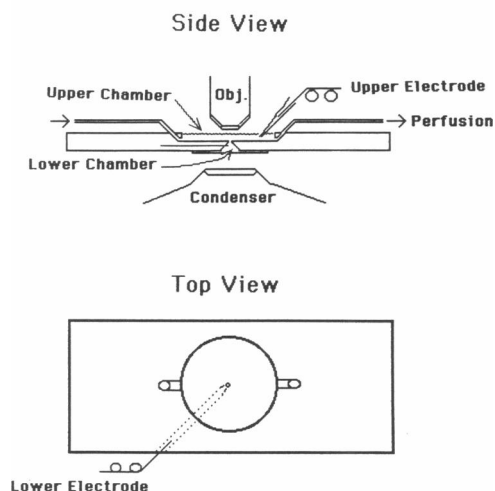
FIGURE 1 Diagram of bilayer chamber. The side view shows upper and lower compartments, electrodes, microscope objective, and condenser, and tubes for perfusion. The top view shows the placement of the lower electrode. The hole between the two compartments is the aperture on which bilayers are formed. The bottom was sealed with a coverglass.

bilayer chamber. The size of a microscope slide, the chamber was mounted on the stage of a Optiphot microscope (Nikon, Inc., Garden City, NY) equipped for phase contrast, differential interference contrast and fluorescence microscopy. Apertures on which membranes were formed were 300–500 μm in diam. The thickness of solution in lower and upper compartments was made as small as practical to maximize optical resolution. Membranes were formed from the phospholipid-hydrocarbon solutions using a Teflon paddle. Thinning was observed by phase contrast (Fig. 1), and except as noted, only completely thinned membranes were used. After thinning, the solution in the upper chamber could be changed as appropriate by perfusion using a peristaltic pump.

Membranes were normally formed in 400 mM KCl, 10 mM MOPS, pH7.4 (MOPS-buffered KCl, hereafter MbK; by freezing point depression, this solution is isosmotic to 200 mM calcein). Vesicles were added in a solution sufficiently dense to settle onto the surface of the BLM. Typically this involved FTSVs in 200 mM calcein; 1–2 μl of the suspension was added just above the BLM and, after the vesicles had adhered, the excess FTSVs and external calcein were removed by perfusion. For FTSVs, the procedure had the advantage that none of the contents of the FTSVs was lost before their adhesion to the BLM. In the case of SVs, the suspension was used directly from the density gradient because it was already in a solution (0.4 M sucrose) denser than MbK.

For fluorescence microscopy, 40X and 100X water immersion objectives were used. Reproducible epi-fluorescence illumination was obtained by positioning the field diaphragm of the illuminator condenser just in the field of view, and setting the condenser diaphragm at a fixed value selected to be low enough to minimize bleaching. Fluorescent images were recorded with a DAGE-MTI 66 SIT camera to a GYYR time-lapse video recorder. The camera black and intensity controls were set on automatic. With the 100× lens total magnification at the video monitor was 3,500.

Formation of large, unilamellar lipid bilayer vesicles

Sucrose-filled LUVs were prepared as described by Reeves and Dowben (22). Brain PS and bacterial PE (1/1) were mixed in chloroform in a large round bottomed flask, solvent was removed under nitrogen and the lipid residue put under oil pump vacuum for 1 h. 400 mM glucose was added carefully to the lipid film and large LUVs were allowed to spontaneously form for at least 4 h. The resulting suspension was mixed with 200 mM KCl, 10 mM MOPS, pH 7.4, and LUVs were collected by short (few seconds) centrifugation in a clinical centrifuge. They were then resuspended in 200 mM KCl, 10 mM MOPS pH 7.4.

Electrical measurements

BLM's were formed as outlined above except that an electrical potential was applied across the bilayer and the resulting current was measured with a high-speed picoammeter (model 416; Keithley Instruments Inc., Cleveland, OH) and recorded on a chart recorder or a storage oscilloscope. A typical experiment consisted of spreading a squalene-based BLM in MbK and allowing the film to thin. The potential across the bilayer was held at −25 mV (with respect to the grounded upper [cis] compartment) via a battery-driven voltage divider. When the membrane had thinned, MbK containing various concentrations of calcium chloride or EDTA was perfused into the chamber. After the solution in the upper chamber was changed, FTSVs or synaptic vesicles were added above the BLM and allowed to settle onto the bilayer. During these procedures the current across the BLM was recorded. BLMs having high conductances and/or liposome-like membranes over the BLM were not used. Resistances of acceptable squalene-based membranes were 3–30 × 10⁶ ohm-cm². Squalene-based BLMs do not have lenses and their capacitances are in the range expected for solvent-free membranes (~0.7 μF/cm², calculated from membrane time constant and measured resistance). Onset of thinning as indicated by capacitance increase corresponded exactly to that observed under the microscope.

RESULTS

Synaptic vesicles can be loaded with self-quenched calcein by freezing and thawing

Freeze-thawing was examined as a method to load synaptic vesicles with calcein because this process was known to induce disruption and fusion of liposomes and natural membranes (23, 24). Electron microscopy of brain synaptic vesicles before and after freeze-thawing revealed that the process produced an increase in vesicle diameter of four to five times. The increased size of FTSVs is an advantage because they are more visible than SVs and, more importantly, they have much larger encapsulated volumes. Because our assay could not be applied without freezing and thawing synaptic vesicles, the possibility must be acknowledged that freezing, thawing, or both alter the fusion properties of the vesicle membranes.

Encapsulation of calcein within FTSVs was initially verified by fluorescence measurements as a function of time before and after detergent lysis of the vesicles. Lysis

of FTSVs by detergent leads to dilution of trapped calcein and relief of self quenching. Such measurements are shown in Fig. 2 for synaptic vesicles that were freeze-thawed in 200 mM calcein and eluted from a G-75 Sephadex column with MbK. The eluate was passed through a flow cell in a fluorometer, and the vesicle peak was collected. For the experiment represented by the first trace of Fig. 2, FTSVs were diluted into MbK. The initial fluorescence declines slighlty because the very low concentrations of calcein represented here are susceptible to the trace amounts of quenching ions present in reagents and leached from glassware. Addition of detergent (Triton X-100) to lyse the FTSVs causes a large increase in fluorescence. Addition of cobalt chloride to a final concentration of 10 μM results in quenching of almost all the fluorescence. In the case represented by the second trace, FTSVs were suspended in a solution containing 10 mM EDTA. With time, the fluorescence of suspended FTSVs increases and addition of Triton yields a large, stable increase in fluorescence. Although these experiments demonstrate that FTSVs encapsulate self-quenched calcein, the vesicles are sufficiently leaky that, after chromatography, the contents are diluted to the point where self-quenching is only ~95%. To avoid loss of contents and reduction in the ability to detect release, FTSVs for bilayer experiments were used directly from stock suspensions in 200 mM calcein.
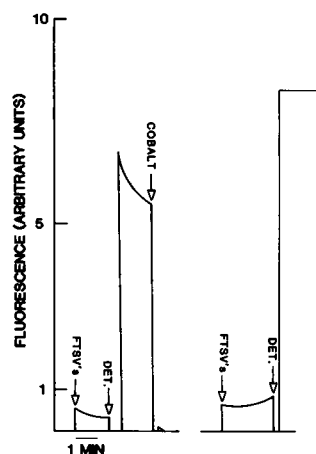

FIGURE 2 FTSVs contain self-quenched calcein which is immediately accessible to external cobalt ions only when their membrane is disrupted. Synaptic vesicles were loaded with self-quenched calcein by freezing and thawing three times in 200 mM calcein followed by gel exclusion chromatography in 400 mM KCl, 10 mM MOPS, pH 7.4. Samples in the absence (*first trace*) or presence (*second trace*) of 10 mM EDTA were added to the fluorometer cuvette. Subsequent addition of detergent releases calcein and produces a rapid increase in fluorescence that can be quenched by cobalt ions. The final levels of fluorescence correspond to a calcein concentration of ~10 nM.

FTSVs and SVs bind to decane-based bilayers and induce the formation of solvent lenses

Fig. 3 is a series of photomicrographs of FTSVs adhering to a decane-based PS/PE 1:1 BLM. Because of ease of formation, our initial investigations were of the interaction of SVs and FTSVs with bilayers spread from decane solutions. We concluded that these membranes are unsuitable for our purposes, but they do exhibit some interesting characteristics and it is useful to contrast these with those of the squalene-based membranes that were found more suitable. Upon adhesion, which occurred in the presence of 15 mM $CaCl_2$, FTSVs induce formation of lenses, which, as shown in the progression of photographs (Fig. 3) coalesce. It is of interest that, as the lenses grow, the punctate fluorescence of the individual vesicles gives way to a near-uniform fluorescence of the entire
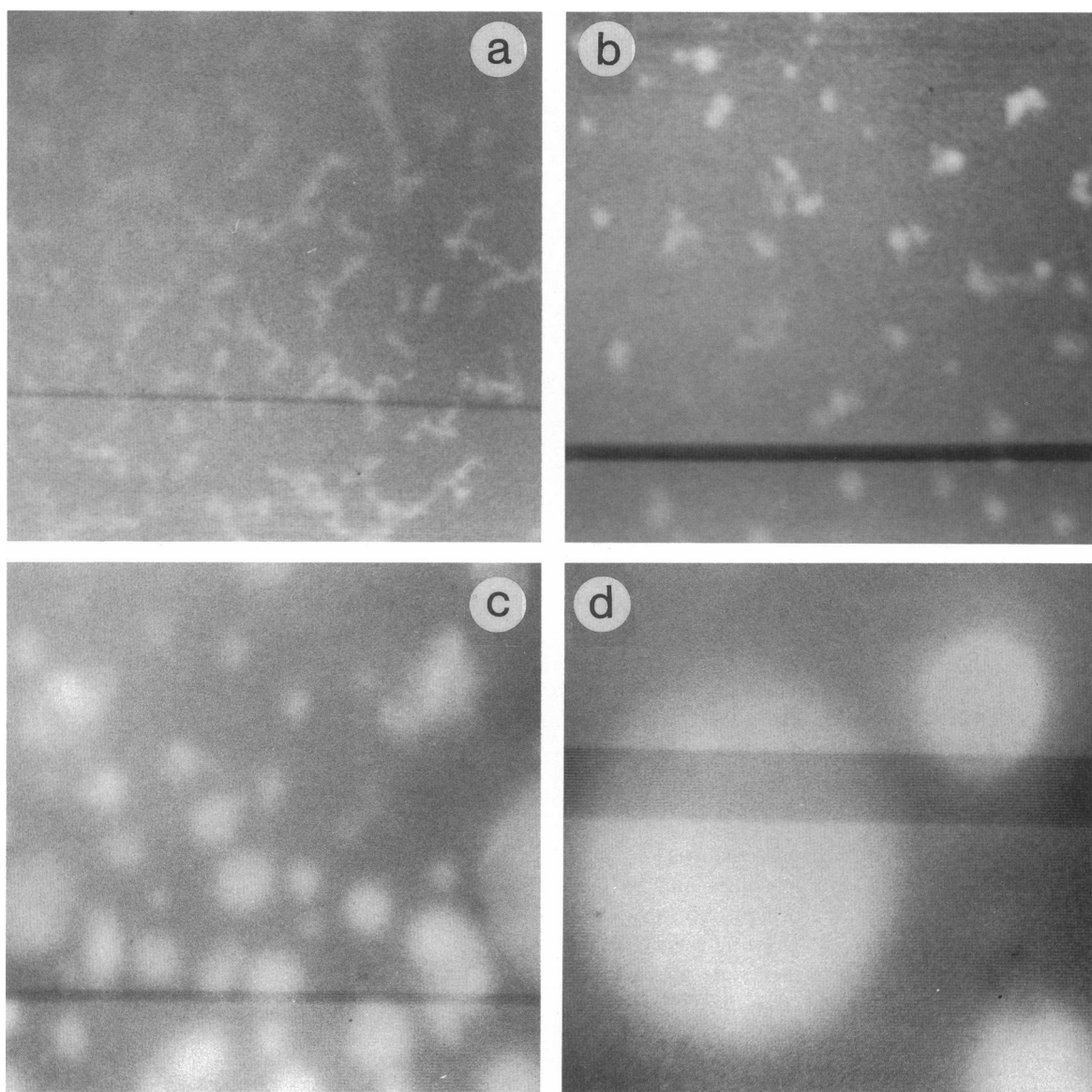
FIGURE 3 Fluorescence photomicrographs of FTSVs adhering to decane-based BLMs. FTSVs adhere to BLMs, forming aggregates. Lenses form under the aggregate (a) and, over the course of several minutes, the lenses coalesce (b–d). Decane-based PS-PE BLMs were painted and allowed to thin in MbK. MbK containing 15 mM $CaCl_2$ and 10 μM $CoCl_2$ was perfused into the upper chamber. FTSVs were then added and allowed to settle onto the BLM, after which unbound FTSVs and external calcein were removed by perfusion with MbK containing 15 mM $CaCl_2$ and 10 μM $CoCl_2$. The width of each photograph corresponds to 47 μm. The pictures were recorded with a silicon-intensified target video camera. Single frames from videotape were photographed.

lens. Although the nature of the FTSV-BLM interaction is unclear, it is effectively irreversible; removal of calcium does not release adherent FTSVs. Lenses were not induced by Ca^{+2} in the absence of vesicles.

We also examined the adhesion of intact synaptic vesicles to decane-based BLMs (Fig. 4). In this case, the lenses induced are smaller than those produced by FTSVs. They form in large numbers at $CaCl_2$ concentrations above 5 mM. Because the SVs are themselves invisible, either before or after adhesion, we could not establish whether a single SV is sufficient to induce the formation of a lens. Given the relative uniformity of the lenses and the paucity of visible aggregates in SV suspensions, however, it appears probable that one SV is sufficient.

We have studied the induction, by purely lipid vesicles, of lenses in decane-based bilayers M. S. Perin and R. C. MacDonald, manuscript submitted for publication) and

FIGURE 4 Differential interference contrast photomicrograph of lenses formed by the adhesion of intact synaptic vesicles to a decane-based BLM. The procedures were as in Fig. 3 except that synaptic vesicles, as isolated from the linear sucrose gradient, were allowed to settle onto the BLM. The height of the photograph corresponds to 117 μm.
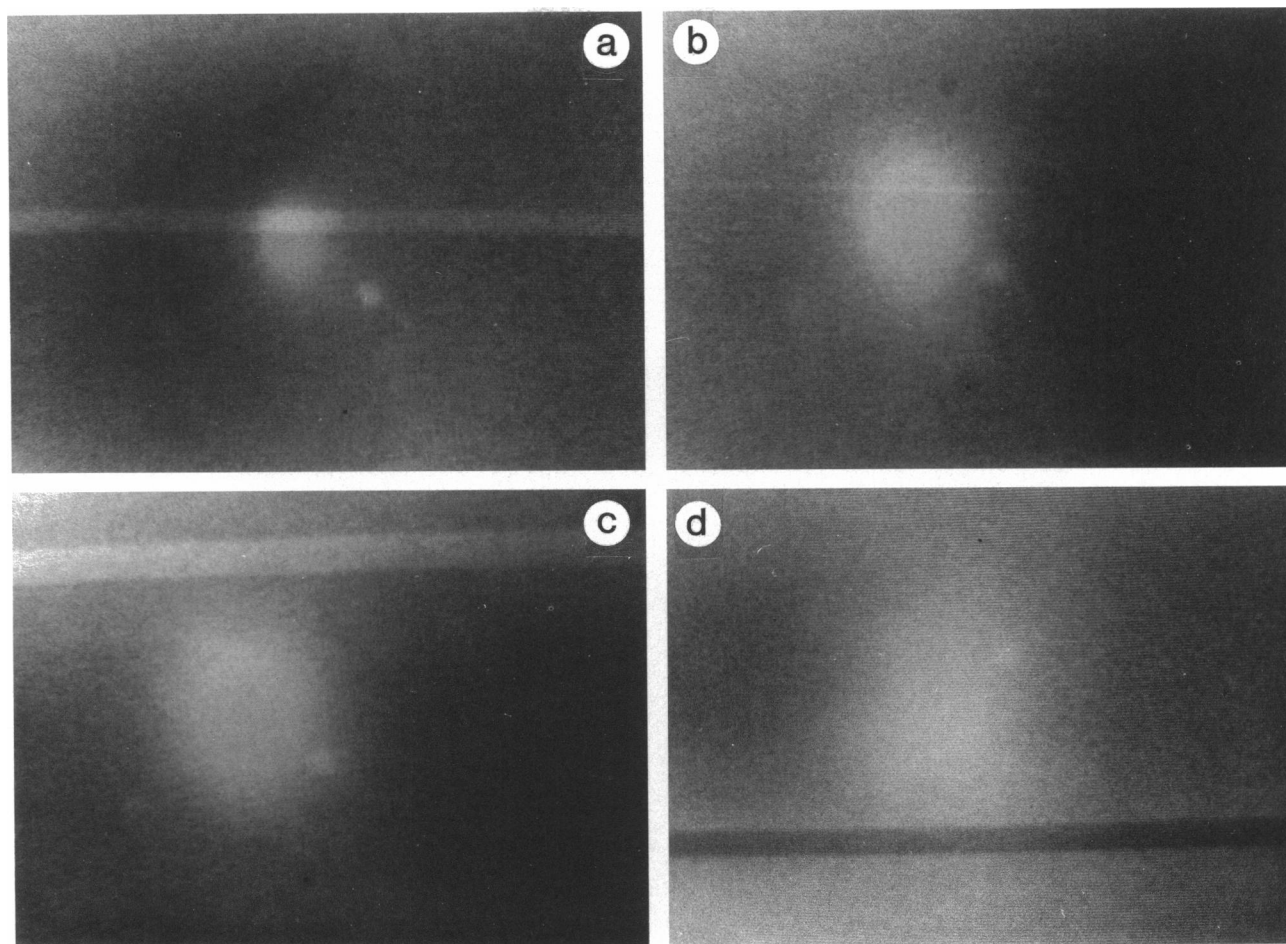
FIGURE 5 Photomicrographs of FTSVs adhering to a squalene-based BLM and showing a flash of fluorescence due to the abrupt release of calcein from one adherent vesicle. The photographs *a–d* represent successive video frames, corresponding to slightly over 0.1 s. The procedures were similar to those used for the experiments of Figs. 3 and 4 except that the membrane was formed from a squalene solution of PS and PE.

concluded that the phenomenon is a consequence of the fact that the upper monolayer of the planar bilayer must partially conform to the curvature of the adhering vesicle. Disproportionation of decane into a lens at the attachment site obviates the necessity that both monolayers become distorted.

Because of the probable interference of decane lenses with fusion, we substituted for decane, squalene, a lipid solvent that is almost completely excluded from the bilayer region (21). Success was most consistently achieved with the lipid combination, phosphatidyserine:phosphatidylethanolamine, 1:1. Although such squalene-based membranes take an inconveniently long time to thin, their interaction with FTSVs was not complicated by the formation of lenses and these membranes were used for all experiments described below. Fig. 5 *a* shows FTSVs allowed to adhere to a squalene-based BLM in the

presence of 15 mM $CaCl_2$. In contrast to the situation with decane-based BLMs, FTSVs do not undergo 2-dimensional aggregation on squalene-based BLMs and when they attach, lenses do not appear. As with decane-based BLMs, adhesion is essentially irreversible; no adherent FTSVs are released upon perfusion of a calcium-free solution.

Adhesion of synaptic vesicles to squalene-based BLMs can also be detected under epi-fluorescence at high magnification (2,400) using fluorescamine (25) labeled synaptic vesicles. The vesicles that adhered to BLMs in MbK-15 mM $CaCl_2$ were visible to the eye, but could not be recorded on video tape because our equipment did not allow the extraction of their image from the noise before they bleached.

Because FTSVs settle onto horizontal BLMs before vesicle aggregation occurs, it was possible to investigate

the divalent cation concentration requirements for vesicle binding, without interference from vesicle aggregation. FTSVs in 200 mM calcein were allowed to settle through a divalent cation-containing buffer onto the BLM. Some vesicles, depending upon the divalent cation and its concentration in such a suspension, adhere strongly enough to resist removal during the perfusion time required to eliminate external calcein.

As may be seen from Fig. 6, Ca^{+2} supports adhesion at concentrations two orders of magnitude below those where Mg^{+2} is effective. It is surprising and perhaps significant that vesicles adhere at 100 μM Ca^{+2}, for both PS/PE vesicles and synaptic vesicles do not themselves aggregate below 1–2 and 4–5 mM Ca^{+2}, respectively (26–28). The vesicles that remain bound at 100 μM Ca^{+2} are also unusual with respect to size. In contrast to those that bind at higher calcium ion concentrations, these are too small to be resolved as vesicles. They reveal themselves as such, however, by the fact that they regularly disappear into a burst of light, indicative of release of their contents. The extremely rapid rate (few seconds) at which these small vesicles bleached and became invisible made their study extremely difficult and consequently most of our investigation of fusion subsequent to adhesion was done with the larger FTSVs that bind at millimolar concentrations.

FTSVs fuse with BLMs but they also lyse without fusing

As soon as FTSVs adhere to the BLM, bursts of light due to calcein released from adherent FTSVs is observed. To
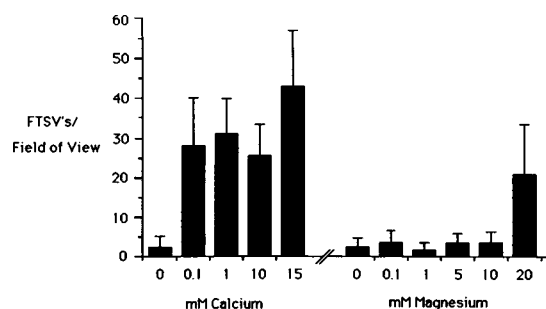
FIGURE 6 Binding of FTSVs to BLM depends upon the concentration of $CaCl_2$ and $MgCl_2$. BLMs were spread and allowed to thin in MbK and solutions containing calcium or magnesium chloride were perfused into the upper chamber. FTSVs were then added and allowed to settle down onto the BLM. The number of FTSVs bound per field of view was counted under epi-fluorescence illumination using a 100× water immersion lens. Bars represent the mean number of FTSVs per field of view and the lines represent one standard deviation. The field of view was 85 μm in diam.

determine the relative contributions of these flashes to release of calcein into the *trans* compartment and simple rupture of the adherent vesicle, i.e., fusion and lysis, we determined maximum flash diameters for three situations: 100 μM cobalt *cis*, 100 μM cobalt *trans*, 100 μM cobalt on both sides of the bilayer (*cis* and *trans*). Calcein chelates Co^{+2} very tightly, whereupon calcein fluorescence is virtually eliminated. Under the conditions of our experiments, cobalt is expected to reduce flash diameters by ~90% (see Discussion), so that the effect on flash size of including cobalt ion in one or both compartments can be used to differentiate fusion from lysis. Flash diameters for cobalt *cis* and for cobalt *cis* and *trans* are presented in Fig. 7. When cobalt is present on both sides of the BLM, all release events are quenched and no flashes with diameters larger than 10 μm are observed. In contrast, when cobalt is present in the *cis*, but not in the *trans* compartment, flashes of up to 30 μm in diam are observed. The flashes >10 μm in diam are evidently due to fusion and release of calcein to the *trans* compartment. In the case of *trans* cobalt ion, we found that 12% of the flashes were in the large (>10 μm diam) categories. These data are somewhat less reliable than the other two sets because, in the absence of *cis* cobalt, the background fluorescence is high and the measurement of flash diameters is difficult.

Water bubbles trapped in the torus of BLMs also can
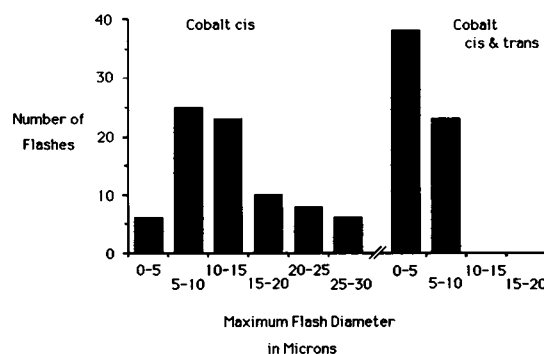
FIGURE 7 Fusion and lysis are differentiated by the effects of quenching agent on one or both sides of the BLM. Shown in the figure are histograms of maximum diameters of the bursts of light seen under the fluorescence microscope that are produced by release of calcein from FTSVs bound to planar bilayers with 100 μM $CoCl_2$ present on either the *cis* side or on both the *cis* and *trans* sides of the BLM. Cobalt ion present in the *cis* compartment reduces flashes due to rupture of adherent FTSVs, cobalt ion on the *trans* side should reduce the size of flashes due to fusion of FTSVs to the BLM, whereas cobalt in both compartments reduces all flashes. Experiments were performed as described for Fig. 5, except for the compartment in which Co^{+2} was present. Maximum flash diameters were determined from videotape using single frame advance.

be targets for the fusion of adherent FTSVs. The top and bottom surface of these bubbles often thin to bilayers and if they are sufficiently small, their fusion with FTSVs is revealed by the presence of a fluorescent bubble after a flash originates from an adherent FTSV. Similarly, lysis of FTSVs adhering to the torus is represented by a flash and the absence of residual fluorescence.

Fusion of FTSVs to planar bilayers is facilitated by osmotic stress

In suspension, FTSVs slowly lyse spontaneously. A possible explanation of such behavior is that the vesicle membrane is permeable to KCl and as it enters the FTSV, water follows and the vesicle bursts. Because it had been reported that osmotic stress is necessary for fusion of lipid vesicles to BLMs (5), it was of interest to examine the possibility of a similar driving force for FTSV fusion.

As one test of whether FTSVs are indeed permeable to KCl, we examined FTSVs adhering to BLMs in the presence of solutes potentially less permeant than KCl, because if release results from the osmotic stress of influx of solute and accompanying water, release should be slowed in the presence of less permeant solutes. For these tests it was unnecessary to determine the proportion of flashes that were due to fusion, so we simply included enough (10 μM) cobalt chloride in the upper chamber to reduce background fluorescence and counted total flashes. One solute that greatly reduces the number of release events is glucose. When a membrane in MbK, 10 μM CoCl$_2$, and 15 mM CaCl$_2$ to which FTSVs are bound and are releasing normally is perfused with isotonic glucose, the flash rate falls abruptly (Fig. 8, compare bar d with bar a). When the glucose solution is replaced by KCl, the vesicles again release their contents (Fig. 8, bar f). The rate of release is lower after such a replacement, but this is probably due to the normal reduction in rate that would occur in the time required for a second perfusion.

Inhibition by glucose solutions of calcein release from FTSVs greatly diminished when the CaCl$_2$ concentration was reduced from 15 to 0 mM (Fig. 8, bar e). One interpretation of this observation, suggested by the common finding that divalent cations are often required to reseal disrupted biological membranes such as red cell ghosts (29), is that in the absence of Ca^{+2}, the FTSV membrane remains permeable even to glucose.

Because the effect of glucose solutions could have been due to their low ionic strength, we examined release in 400 mM solutions of LiCl, NaCl, KCl, and cholinecl. Because the density of these solutions is, in contrast to that of glucose, low enough that FTSVs in 200 mM calcein sink down to the membrane, they allowed us to follow both the adhesion and release processes. As is
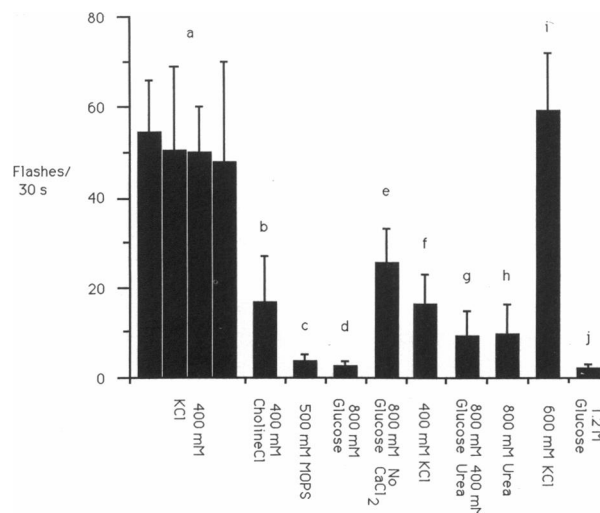
FIGURE 8 The rate of release of FTSV contents depends upon the composition of the solution in which they reside. The figure shows the rate of flashes observed FTSVs adhere to BLMs in various aqueous solutions. For a–c and i, BLMs were spread and allowed to thin in either KCl, choline Cl or MOPS solution without calcium chloride. The corresponding solutions containing 15 mM calcium chloride were perfused into the upper chamber and then FTSVs were added and allowed to settle onto the BLM. Independent experiments showed that the same number of FTSVs bind to BLMs in KCl, choline Cl and MOPS. In the cases of the glucose solution (d, e, j), these were perfused into the chamber after vesicles had adhered in 400 mM KCl, 15 mM calcium chloride. For the experiments of f, g and h, vesicles were allowed to adhere to the BLM in 400 mM KCl, 15 mM calcium chloride; release was then inhibited by perfusion of 800 mM glucose, 15 mM calcium chloride; finally 400 mM KCl or 800 mM glucose 400 mM urea or 800 mM urea was perfused into the chamber. All solutions, except the 800 mM glucose solution of e, contained 15 mM calcium chloride. All solutions from which flashes were counted contained 10 μM cobalt chloride in the cis compartment to eliminate background fluorescence and contained at least 10 mM MOPS as a buffer. The number of flashes per field of view in 30 s was counted under epi-fluorescence illumination using a 40× water immersion lens. Bars represent the mean number of flashes per field of view per 30 s. Means were derived from values of five experiments. The four bars for the KCl experiments represent four different preparations of FTSVs. Error bars represent the standard deviation. The field of view was 245 μm in diam.

shown in Fig. 8 (*bar b*), only the largest cation, choline, caused any reduction in the number of flashes.

We also sought to test whether anion size, and hence permeability, affected the rate of calcein release from FTSVs. Most anions examined were unsuitable because they precipitated with Ca^{+2} or caused membrane instability. We were able to form and investigate membranes in 500 mM solutions of MOPS. Although the torus of such membranes tended to extrude interfering myelin figures, it was evident that MOPS also reduced the rate of FTSV contents release (Fig. 8, *bar c*). Counts of FTSVs bound to membranes formed in choline Cl and MOPS revealed

that the reduction in flash rate seen in these solutions was not due to a reduction in the density of bound FTSVs.

Additional evidence that glucose exerts an osmotic effect was obtained from experiments in which either 800 mM glucose, 400 mM urea, 10 mM MOPS, 15 mM calcium chloride, 10 μM cobalt chloride, or 800 mM urea, 10 mM MOPS, 15 mM calcium chloride, 10 μM cobalt chloride, both at pH 7.4, was perfused onto membranes in place of the release-inhibiting glucose solution. The result, as shown in Figure 8, *d*, *g*, and *h*, was a fourfold increase in frequency of flashes. Although the absolute rate remains low as a consequence of the depletion of intact vesicles during the three preceding perfusions, the significant increase in the presence of urea is consistent with the effect of glucose being due to its low permeability and not to an absence of electrolyte.

An increase in KCl concentration to 600 mM slightly increases the flash rate (Fig. 8, *bar i*) while the rate remains low when the glucose concentration is increased, even to 1.2 M (Fig. 7, *bar j*).

FTSVs fuse with giant unilamellar vesicles

Our initial attempts to examine fusion between FTSVs and lipid vesicles under conditions compared with those most favorable for FTSV-planar bilayer interactions were not successful because of the formation of aggregates (SVs and PS/PE vesicles aggregate at 4–5 and 1–2 mM Ca^{+2}, respectively (26–28). With the recognition of FTSV-bilayer binding at 0.1 mM Ca^{+2}, it became apparent that at such concentrations, the study of vesicle-vesicle fusion is feasible. Using preparations of GUVs in which 10–50 μm diam vesicles predominate, we had no difficulty observing fusion with FTSVs at 0.1 mM $CaCl_2$. Although such events are not common, perhaps 1% of the GUVs become fluorescent, the process is dramatic, being marked by the sudden appearance of a brightly fluorescent vesicle such as that shown in Fig. 9. The fused GUVs are leaky, presumably due to the high permeability of the incorporated FTSV membrane at the low Ca^{+2} concentrations that obtain in these experiments, and their fluorescence fades with time. In addition, they lose the sucrose trapped inside when they were formed and so the fused vesicles are also characterized by a change in contrast under the phase microscope and a change in bouyant density.

## Fusion with FTSVs and SVs produces an increase in planar bilayer electrical conductance

Because it was evident from the experiments described above that FTSVs fuse to planar bilayers and that the
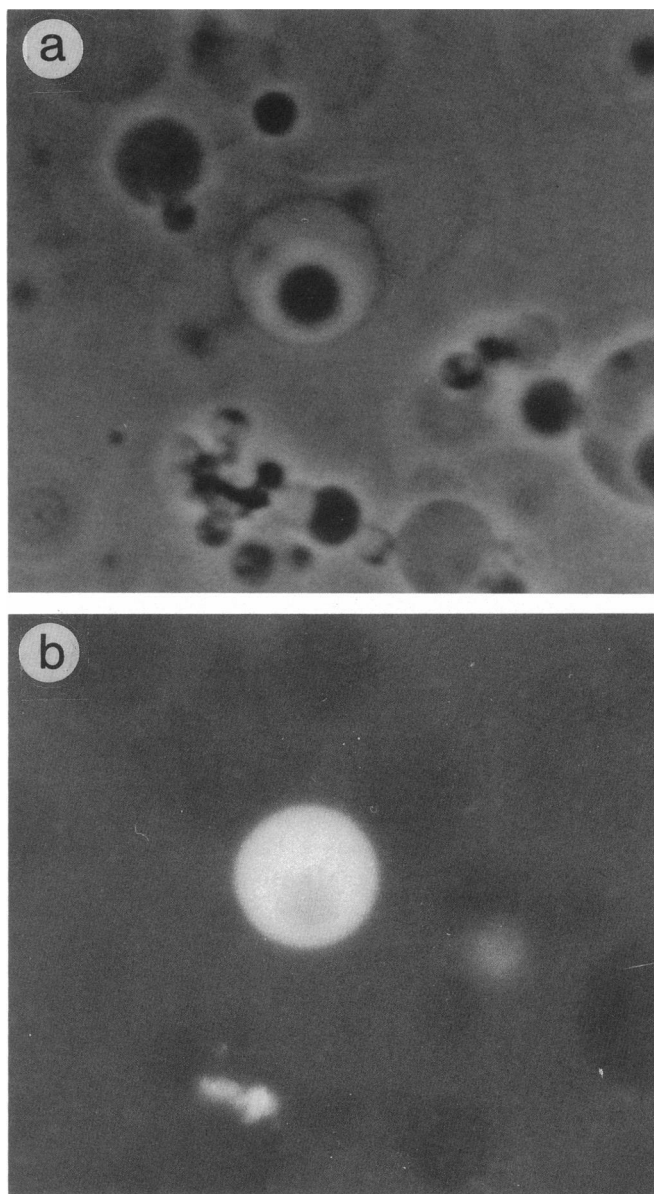
FIGURE 9   Fusion of FTSV to giant unilamellar, PS/BPE vesicle. The *lower* photograph (fluorescence) shows that a FTSV has fused with one of the GUVs shown in the *upper* photograph (phase). PS/BPE GUVs were prepared by slow hydration in 400 mM sucrose. FTSVs were prepared in 100 mM calcein and chromatographed over G-75 Sephadex. Chromatographed FTSVs were added to sucrose GUVs in 200 mM KCl, 10 mM MOPS pH 7.4, 0.5 mM calcium chloride, 20 μM cobalt chloride. The width of the photographs corresponds to 177 μm.

vesicle membrane has a significant permeability to KCl, the consequence of fusion should then be an increased bilayer conductance. To test this hypothesis, the bilayer chamber was modified to allow measurement of electrical current across the bilayer.

Fig. 10 *a* is a chart record of the current across a BLM
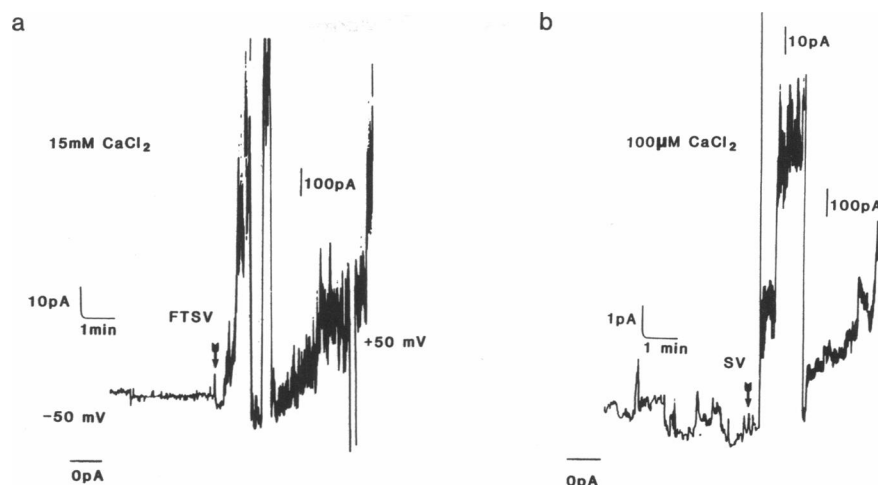
FIGURE 10 (a) Chart recording of the current across a BLM during addition of FTSVs to the BLM. Current and fluctuations in current both increase rapidly following addition of FTSVs to the membrane. The BLM was formed in 400 mM KCl, 20 mM MOPS, pH 7.4. Upon thinning, the upper chamber was perfused with MbK containing 15 mM calcium chloride. FTSVs were then added and allowed to settle onto the BLM. The voltage across the BLM was −50 mV. (b) Chart recording of the current across a BLM formed in MbK containing 100 µM calcium chloride during addition of a synaptic vesicles to the BLM. Current and fluctuations in current increase rapidly upon addition of FTSVs to the membrane even at calcium concentratins as low as 100 µM. Procedures were as above except that the voltage across the BLM was −25 mV.

that shows the consequence of addition of FTSVs in the presence of 15 mM calcium ion. As in the case of optical investigations, the BLM was a PS/BPE 1:1 squalene-based planar bilayer in a MK solution with 15 mM calcium chloride in the cis (upper) chamber. Addition of FTSVs leads, after a short delay, to a large, erratic increase in conductance which is unchanged by voltage polarity. Similar conductance increases were recorded using cis calcium concentrations as low as 100 µM.

The BLM conductance increases soon after addition of FTSVs above the BLM. Although we presume that the steps seen in the current records correspond to fusion of individual FTSVs, we were not successful in correlating steps in conductance with flashes of fluorescence because optical recording of flashes requires perfusing away unbound FTSVs, during which time the conductance has increased to the point where steps are no longer identifiable.

An increase in conductance occurred under all conditions where FTSVs were observed to adhere and fuse to BLMs. Thus, it was seen at 0.1 mM Ca^{+2} and at 25 mM Mg^{+2} but not at 5 mM Mg^{+2} or in the absence of divalent cations.

We also investigated the electrical consequences of the interaction of intact synaptic vesicles with squalene-based BLMs. Fig. 10 b shows a chart record of membrane current upon the addition of synaptic vesicles to a squalene-based BLM in the presence of 100 µM calcium chloride. After a lag, perhaps related to the time for

synaptic vesicles to sink down to the bilayer, the conductance of the bilayer increases greatly (>10X). The addition of synaptic vesicles to BLMs in 25 mM $MgCl_2$ did not produce increases in conductance like those seen with FTSVs. It may be that at 25 mM Mg^{+2}, intact synaptic vesicles adhere to, but do not fuse with, PS/PE planar membranes. We were unable to test the hypothesis that intact synaptic vesicles only fuse with planar bilayers in the presence of Ca^{+2} because we could not independently verify fusion of intact vesicles.

DISCUSSION

The unambiguous demonstration of fusion of a single pair of contacting membranes has been the goal of an important branch of membrane research for several years. By loading synaptic vesicles with calcein, the fluorescence of which is intense, self-quenched and quenched by cobalt ion as well as easily visible in the fluorescence microscope, we have been able to investigate individual fusion events. The methods we used are capable of providing quantitative information on efficiencies of fusion and although in this first application of those methods our estimates remain rather approximate, we have been able to establish limits on fusion events relative to release of vesicle contents by lysis and as a proportion of adherent vesicles.

The size of the flash that should be visible when the contents of a vesicle are abruptly released can, within

broad limits, be estimated. The concentration of calcein that is readily detected is of the order of 10^{-7} M; giant vesicles containing 10^{-7} M calcein are visible to dark-adapted eyes and can also be seen by video microscopy when the SIT camera is used. Because the concentration of calcein in FTSVs is 10^6 times higher than the visible concentration, it is evident that the contents released from the vesicle can be diluted by a factor of 10^6 before the fluorescence thereof becomes too dim to be seen. This will occur when the cloud of diffusing calcein has increased in diameter by the cube root of 10^6, i.e., 10^2. Because freeze-thawing produces vesicles several times larger than the initial vesicle diameter of ~0.1 μm, the expected visible diameter of unquenched flashes is several tens of μm. Such diameters are at the upper limit of flashes observed by video microscopy which, given the fact that the video camera is somewhat less sensitive than the dark-adapted eye, is close to the expected size. The effect of Co^{+2} on flash diameters can also be calculated approximately. Calcein concentrations must exceed Co^{+2} concentration to be visible. Thus, at 100 μM Co^{+2}, the threshold of visibility of calcein is raised by ~1,000X, and flash diameters are then reduced by the cube root of 1,000, or ~10X. Hence flashes in the presence of 100 μM Co^{+2} should be only ~$\frac{1}{10}$ as large as in its absence. Indeed, flashes in the presence of Co^{+2} do not exceed 10 μm and most are smaller than 5 μm (Fig. 7).

The effect of Co^{+2} on the size of the light flash that accompanies the release of calcein from FTSVs is predicted to be large enough to allow one to distinguish between lysis and fusion of the vesicles to the planar membrane. Although the analysis is rendered approximate by the heterogeneity of the vesicle population, it is possible to provide a minimum estimate of fushion probability. It is evident from Fig. 7 that what differentiates cis-Co^{+2} from cis and trans Co^{+2} is that the 10–30 μm diam flashes are absent under the latter conditions. Because those are the conditions under which all release must be quenched, it is reasonable to conclude that the large flashes seen when Ca^{+2} is not present in the trans compartment represent release to that compartment of the contents of vesicles that fused with the planar bilayer. On this basis, it is found that ~60% of the events of Fig. 7 represent fusion. Presumably this is a minimum estimate because it does not include fusion of very small vesicles that may produce flashes <10 μm in diam. It is very likely that some of the larger flashes in this category represent fusion. This estimate also assumes that the likelihood of fusion over lysis is independent of vesicle size. If, as seems likely, the probability of fusion over lysis increases with decreasing diameter, the percentage fusion is again underestimated.

In a similar way, it can be demonstrated that some of the flashes represent lysis. In experiments where the trans compartment contained Co^{+2} and the cis-compartment did not, 12% of the flashes were larger than 10 μm in diam and thus can be presumed to represent lysis. This is again a lower limit, because under these conditions vesicle leakage renders the cis compartment noticeably fluorescent, and against this relatively bright background the size of the flashes is underestimated.

In addition to placing limits on fusion of between ~60 and 90% of the release events, we can make minimum estimates of the fraction of bound vesicles that fuse. In the presence of MbK, $CaCl_2$ (15 mM) and $CoCl_2$ (100 μM), the average number of vesicles bound per 1,000 $μm^2$ is nine. The number of release events under the same conditions is ~1 per 30 s. Although the frequency of release events falls as the residence time of vesicles on the bilayer increases, it is evident that a minimum of 20% of the visible vesicles eventually release their contents. Since at least half of those events represent fusion, it is clear that fusion is by no means a rare event after a FTSV has adhered to a planar bilayer. This estimate is definitely an underestimate because flashes cannot be observed immediately after vesicles contact the BLM, at which time the flash frequency is evidently at a maximum.

It should be noted that because Co^{+2} is an essential component, albeit at low concentration, on the cis side of the membrane, we cannot rule out a possible effect of Co^{+2} on membrane fusion. If there is such an effect, however, it is unlikely to be large, for the ion is not required for adhesion of FTSVs and release of calcein (direction indeterminate), nor is it prerequisite to the vesicle-induced conductance change.

Estimates of the frequency of fusion and lysis of simple lipid bilayer vesicles bound to a BLM have been recently made by Niles and Cohen (8) and it is of interest to compare their results with ours for synaptic vesicles. The method they used is similar to ours, the principal difference being in calcein detectability. Based on the effect magnification and numerical aperture have on illumination intensity and collection efficiency, our sensitivity exceeds theirs by ~5–10×. The consequence of this is that we could use lower (by 50×) concentrations of Co^{+2} and still detect large effects of Co^{+2} quenching on flash diameters. Niles and Cohen carried out an extensive analysis of their data and concluded that at 20 mM Ca^{+2}, 12–37% (depending upon osmotic conditions) of bound vesicles ruptured and of those events, 50% were due to fusion. Our minimum estimate for 15 mM Ca^{+2} is close to this range. With respect to the ratio to fusion of lytic events, synaptic vesicles may be slightly more prone to fuse rather than lyse, but the difference is small. It thus appears that at the high calcium concentrations required to induce either lipid or synaptic vesicles to bind to planar

membranes, the natural membranes exhibit no significantly greater propensity toward fusion than the purely artificial membrane. As discussed below, however, a portion of the synaptic vesicle population does seem able to fuse at Ca^{+2} concentrations of 100 μM. Purely lipid vesicles are quite inactive at these concentrations, and it may be that the activity of this population of synaptic vesicles is a manifestation of their physiological fusion capacity.

It should be recognized that our experiments do not demonstrate that the vesicles that fuse (or lyse) represent a random selection from the population of vesicles presented to the bilayer. The vesicles are heterogeneous with respect to size and, although we have used a standard preparation method that gives one of the highest yields of a single type of synaptic vesicle, it is by no means pure. We have no way of comparing the properties of the vesicles that bind tightly enough to resist removal under perfusion from those which do not, and it may be that there are differences between them.

As shown in Fig. 2, calcein is slowly released from FTSVs. Some of this loss is due to lysis and abrupt release of calcein, for flashes are readily observed among FTSVs in suspension under the light microscope. It is thus evident that FTSVs are unstable in isotonic KCl. The most plausible explanation of this behavior is that FTSVs are more permeable to KCl than to calcein and the water accompanying KCl entry causes the vesicle to burst. FTSVs must be under osmotic stress by the time they adhere to the planar membrane and osmotic stress may, at least in part, provide the driving force for fusion, as has been suggested for the case of fusion of lipid vesicles to planar bilayers (5).

Experiments in which KCl was replaced with other hypertonic and isotonic solutions (Fig. 8) are consistent with the suggestion that osmotic stress supports fusion. Choline and MOPS were tested because they are large ions and would not be expected to penetrate as rapidly as K^+. Indeed, release rate is significantly reduced in these solutions. Glucose would appear to penetrate FTSVs very slowly, at least in the presence 15 mM $CaCl_2$, for flash frequencies in iso- and hypertonic glucose solutions are drastically reduced. This effect is not due to an inhibition of the release process per se, because inclusion of urea, a membrane permeant ion, leads to a significant increase in rates of flashes. In addition, 15 mM Ca^{+2} is required for inhibition of release by glucose. In the absence of Ca^{+2} a high rate of release is observed. Given the common effect of divalent ions in reducing natural membrane permeability (15), it is not unexpected that Ca^{+2} would depress the permeability of FTSVs to glucose.

It is evident from the data on vesicle binding in Fig. 6

that Ca^{+2} is a far more effective adhesion agent than is Mg^{+2}. Of particular interest is the fact that some vesicles bind at concentrations as low as 0.1 mM Ca^{+2} and possibly even lower. The FTSVs that bind at such calcium concentrations are very small. Because of this small size and rapidity of bleaching it was difficult to examine their fusion to planar bilayers. On the other hand, because the binding process occurs at a concentration below that at which acidic lipid vesicles aggregate, it was possible to demonstrate fusion of FTSVs to LUVs. The frequency of fusion was low, relative to the planar bilayer, perhaps because of the larger area presented by LUVs, but the occurrence of fusion is unambiguous. Through the microscope one observes the sudden appearance of a fluorescent LUV, an event that cannot be interpreted other than as fusion between the FTSV and the LUV.

Whether or not the FTSVs that interact with PS-PE bilayers at 0.1 mM Ca^{+2} represent vesicles that differ from the general population in respects other than size has not been determined. Different stages of maturity of synaptic vesicles exist, and it is tempting to suggest that the vesicles sensitive to 0.1 mM Ca^{+2} may be those that were ready for release at the time the tissue was processed (30, 31). Concentrations of calcium could reach 100 μM during neurotransmitter release, but exocytosis in general is triggered at even lower concentrations (32, 33). In view of the fact that the system described here represents only a partial reconstitution of the in vivo situation and as yet we have no appreciation of what components of the presynaptic membrane are required for efficient fusion without leakage, we cannot judge whether fusion events at 100 μM Ca^{+2} represent processes that are closer to the physiological process than are those that occur at Ca^{+2} concentrations that are clearly much higher than possible in the nerve cell.

The changes in bilayer conductance induced by the addition of FTSVs to planar bilayers under conditions where their fusion was observed is consistent with our observations that FTSVs are relatively permeable to KCl and calcein. An increase in conductance was also observed when SVs were used. A 25 pA step such as seen in Fig. 10 corresponds to the conductance of ~10–100 ion channels, depending upon channel conductance. An alternative to incorporation of channels is that fusion produces a leak.

The study presented here has documented the fusion of a natural membrane vesicle with a simple lipid bilayer. In most instances the fusion event was such that vesicle contents were delivered to the far side of the bilayer. The kind of physical process that occurs during the neurotransmitter release event may thus have been reconstituted, but the extent to which the molecular details of the

reconstituted process correspond to those that obtain at the synapse remains to be determined.

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