VESICLE–MEMBRANE FUSION

Observation of Simultaneous Membrane Incorporation and Content Release

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ABSTRACT Vesicle fusion, the central process of neurotransmitter release and hormonal secretion, is a complex process culminating in simultaneous incorporation of vesicle membrane into the plasma membrane and release of the vesicular contents extracellularly. This report describes simulteous observation of membrane incorporation and content release using a model system composed of a planar bilayer and dye-filled vesicles.

Exocytosis, the fusion of vesicles with the plasma membrane, is central to many physiological processes, most notably quantal transmitter release at nerve terminals and secretion of prepackaged materials such as adrenalin, insulin, and other hormones. Identifying the steps involved in exocytosis and the factors that control them is a major problem of cell biology.

Artificial vesicles and planar membranes provide a simple system with which to model exocytosis. Such model systems make it possible to ask how defined sets of components facilitate fusion and the path by which fusion occurs. Previous workers have demonstrated channel insertion or vesicle content release from synthetic vesicles added to planar bilayers (1, 2). However, these experiments could not distinguish between fusion and other possible vesicle–bilayer interactions such as channel incorporation without *trans* ("extracellular") content release or content release without channel incorporation (bursting). For exocytosis to be efficient, the probability of nonfusion events must be low. Thus, it is important to ask if true fusion (channel insertion and *trans* content release) occurs in model systems and if so, with what probability.

This report describes the simultaneous observation of channel incorporation and *trans* content release in a model system. In our system fusion has a probability of 10%; most of the time vesicles burst. With different experimental conditions higher probabilities have been observed (3). The observation of true fusion in a model system demonstrates a beginning of understanding for the steps involved in exocytosis.

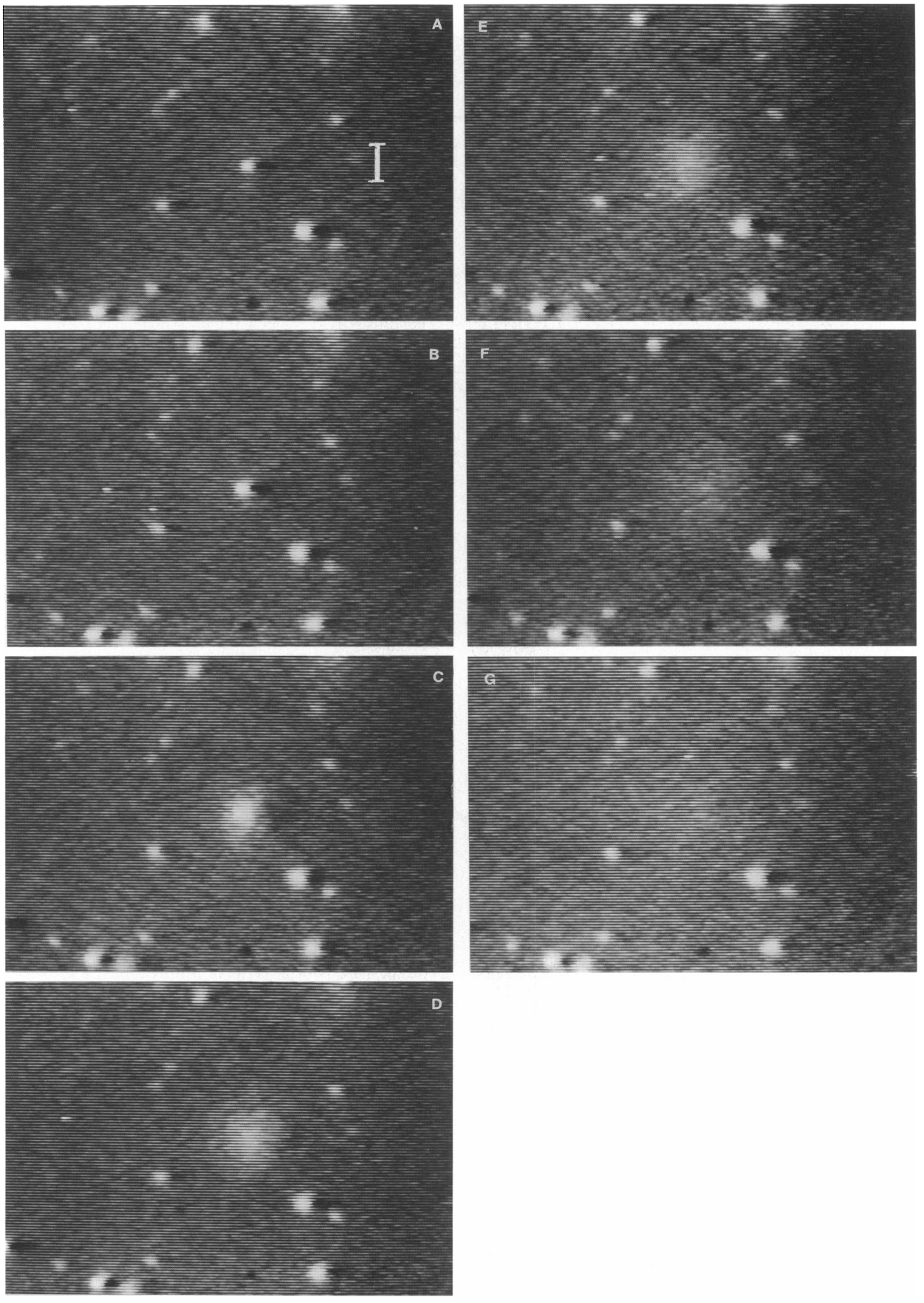
MODEL SYSTEM

The model system is composed of a planar bilayer and dye-loaded vesicles containing porin channels. Fusion is induced by forming a transmembrane osmotic gradient (2–6). Content release is detected through a microscope as release of the fluorescent dye, calcein, from large unilamellar vesicles. Vesicle–membrane incorporation is detected by the appearance of vesicle-borne porin channels in the planar membrane. Finally, *trans* content release is determined by selectively masking fluorescence due to *cis* release. (The *cis* side is the side to which the vesicles are added.) This is done by adding a divalent metal ion such as manganese (Mn^{2+}) to the *cis* side of the planar membrane. Thus it is possible to measure simultaneously vesicle–membrane fusion and release of vesicle contents to the *trans* side.

Release of calcein from vesicles appears as a sudden change in vesicle fluorescence. For vesicles that contain submillimolar concentrations of calcein release appears as a sudden disappearance of vesicle fluorescence due to release and diffusion of dye away from the original location of the vesicle. For vesicles that contain self-quenched concentrations of calcein (>6 mM, reference 6 and manuscript in preparation), release appears as a flash of fluorescent light. The flash arises because initially dye is quenched inside the confined interior of a vesicle, but when a vesicle releases its contents (either by fusion or bursting) the dye expands into the surrounding space, quenching diminishes, and the total fluorescence intensity increases. The increase is brief (tens of milliseconds) and is followed by a slow decrease in fluorescence as the dye dilutes by diffusion to submillimolar levels.

In a typical experiment the membrane was formed with neutral lipids in a chamber filled with standard (low Ca^{2+}) buffer. A pipette filled with vesicles was positioned close to the *cis* side of the membrane. Pressure was applied and vesicles were squirted at the membrane. To induce fusion, the *cis* side of the membrane was perfused with standard buffer made hyperosmotic by the addition of urea. Usually several serial perfusions with increasing concentrations of urea were performed.

Perfusion of urea-containing solutions caused a fraction of the adherent vesicles to release contents. This was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observed through the fluorescence microscope as a flash or disappearance. Fig. 1 shows a series of sequential video frames (separated by 33 ms) of a flashing vesicle recorded from the microscope images. Whether a vesicle flashes or disappears presumably depends on the internal concentration of dye at the moment it is released. If the dye concentration is large enough so that the confined dye is self-quenched (>6 mM), then release of the dye results in a flash. Otherwise, release of dye results in disappearance without a flash.

CORRELATION

Fig. 2 shows representative correlations between optical events and conductance changes in the planar membrane. The step change in conductance (top trace of each graph) at time zero records the moment of channel incorporation (membrane fusion). The estimated number of channels incorporated is written above the conductance traces. The lower trace is the fluorescence intensity of the vesicle undergoing content release. The resolution of the trace is limited to 17 ms, the response of the camera. Note that each labeled point (Fig. 2, *A–G*) on the top fluorescence trace corresponds to the video frames shown in Fig. 1. The bottom fluorescence trace records the disappearance of a vesicle (release of nonquenched calcein) correlated with channel insertion. The probability of random correlation was <0.5%. These results demonstrate conclusively that simultaneous content release and membrane incorporation occur in this model system.

Correlated optical and electrical changes are not, however, the most likely events which occur. Under normal conditions ~90% of the flashes or disappearances induced by perfusion with urea are not correlated with conductance jumps. Conductance jumps not correlated with optical changes are also detected. These latter events are expected in this model system because many porin-containing vesicles are too small to see. The former events, content release without channel incorporation, have two possible explanations. Either the vesicle did not contain any channels, or the channels were not transferred to the planar membrane.

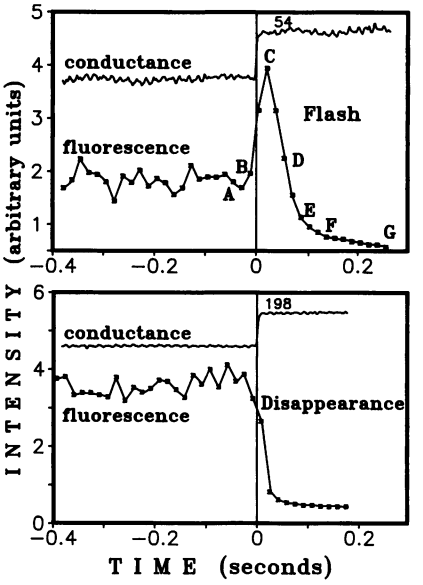

FIGURE 2 Representative correlations of optical and electrical events. Both a flash (*top*) and a disappearance (*bottom*) are shown. The bottom trace in each graph shows the optical signal from a vesicle adhering to the membrane. The video image corresponding to each labeled point in the top graph (*A–G*) is shown in Fig. 1. The membrane conductance is plotted in the top trace of each graph. The number above the trace is the size of the conductance jump expressed in number of channels, based on the measured single-channel conductance for porin of 720 pS. The average number of channels incorporated per step was 15 (n = 74). *Method:* The conductance was measured by applying a known voltage and monitoring bilayer current with an operational amplifier in the standard current-measuring configuration. (The amplifier was a Burr-Brown OPA 111 with a 100-MΩ feedback resistor.) Voltage was applied either by a triangle wave generator or a battery-driven potentiometer. Voltage and current were recorded and analyzed by a Data 6000 (Data Precision, Danvers, MA) digital signal analyzer under the control of a Kaypro II (Kaypro Computers, Solana Beach, CA) programmed in Pascal. The microscope was fitted with an RCA image-intensified television camera whose output was recorded on video tape using a Panasonic AG-6200 video recorder. Optical events were measured from the screen of a television monitor using a photodiode. One of the audio channels of the VCR was used to record a voice description of the experiment, and the other was used to record the FM modulated value of the membrane current at known voltage.

FIGURE 1 Video images of a planar bilayer with adhering vesicles. The sequence (*A–G*) shows the release of contents from a fusing vesicle at the center of the image (the time between each picture is usually 33 ms and is shown in Fig. 2). The vesicle is about 2 μm in diameter. Scale bar in *A* is 10 μm. *Method:* The bilayer chamber was mounted on a Nikon inverted microscope modified for fluorescence and capable of viewing the vertical bilayer face on. The chamber was filled with standard buffer: 400 mM KCl, 0.5 mM EDTA (ethylenediamine tetraacetic acid), and 15 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.0. The membrane was painted from a 15 mg/ml decane solution of phosphatidylethanolamine/phosphatidylcholine (2:1). Large unilamella vesicles (0.5–3 μm in diameter) were prepared from pure lipids using a modification of the procedure of Kim and Martin (6, 15). Vesicles were filled with purified (16) calcein (Sigma Chemical Co., St. Louis, MO) by soaking them in a concentrated dye solution (for 10 min at 22°C for porin-containing vesicles, for 30 min at 30°C for porin-free vesicles). The calcein solution is dense and calcein-filled vesicles were isolated by centrifugation after the soak solution was diluted with the less dense standard buffer. The vesicle pellet, which retains the color of the concentrated dye solution, is resuspended in standard buffer. To obtain a membrane decorated with 5–50 adhering vesicles, a micropipette (tip diameter, 4–6 μm), filled with vesicle-containing solution was moved with a micromanipulator near the membrane. With the mouth of the pipette ~40 μm from the membrane, pressure was applied to the pipette and a stream of vesicles squirted at the membrane. Vesicles adhering to the membrane appeared as fluorescent disks in sharp focus when the membrane lay at the plane of focus. To remove nonadherent vesicles, the aqueous compartment was stirred vigorously. Adherent vesicles swirled about on the surface of the membrane, but remained in focus. Nonadherent vesicles were carried off into the large volume of the rear compartment (~1 ml) never to be seen again.

It is clear that most of the vesicles made in the presence of porin actually contain porin channels. Porin enhances the filling of vesicles with calcein and only filled vesicles are isolated in the final preparation of vesicles. More compelling evidence is shown in Fig. 3 which is a comparison of urea-induced content release from porin-containing and porin-free vesicles. Only with porin-containing vesicles is there significant content release after perfusion of urea on the *cis* side. This vast difference in content-release properties indicates that >90% of the optical events observed under normal fusion conditions (perfusion of hyperosmotic solution) are from porin-containing vesicles. Thus, optical events not correlated with a conductance jump arise not from fusion of porin-free vesicles, but from a release process like bursting.

SIDEDNESS

The experiments described above are sensitive only to content release, not to the side of the membrane on which release occurs. In principle, it should be possible to distinguish *cis* release from *trans* release by using the divalent metal ion Mn^{2+} to selectively quench calcein fluorescence due to release on one side of the membrane. In reality this experiment is not straightforward because millimolar concentrations of Mn^{2+} are required to quench a fluorescence flash and Mn^{2+} can easily enter vesicles through porin channels, rendering the vesicles invisible. Experimentation revealed reasonable compromise conditions under which *trans* release can be independently detected. With 10 mM Mn^{2+} *cis* and 20 mM EDTA *trans*, 47 ± 18% (95% confidence limits) of the observed optical events were correlated with electrical events. This is a significant increase from the 10 ± 3% correlation measured from experiments without Mn^{2+} and EDTA. Presumably, Mn^{2+} on the *cis* side of the membrane reduces the number of visible flashes due to bursting, and EDTA on the *trans* side rescues flashes from invisible vesicles that release to the *trans* side (7). The increase in correlation is not due to an increase in divalent cation concentration since millimolar Ca^{2+} had no effect.

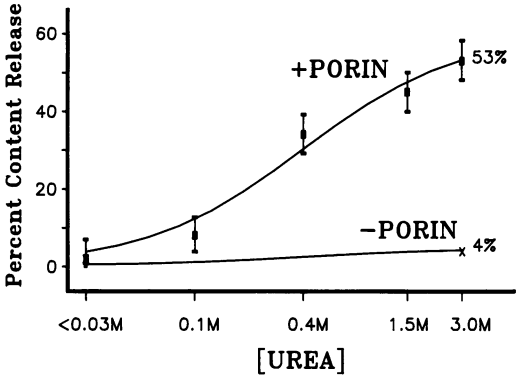

FIGURE 3 The extent of urea-induced content release is shown for vesicles made with and without the ion channel porin. Sequential perfusion with 0 mM, 100 mM, 400 mM, 1.5 M, and 3.0 M urea (all in standard buffer) causes ~53% of all the "porin-containing" vesicles to release their contents. A final perfusion with urea-free buffer produces maximal release (100%). The same perfusion sequence performed with porin-free vesicles shows that perfusion up to 3.0 M urea alone causes only 4% of the vesicles to release their contents. Thus almost all of the 53% observed events with "porin-containing" vesicles are from vesicles that actually contain porin channels. Maximal release means the total number of vesicles that can be induced to release their contents by perfusion and washout of 3 M urea. Maximal release represents release from ~90% of the visible vesicles (both porin-containing and porin-free). There was no visible size difference between porin-containing and porin-free vesicles. Each point is the average from 5–15 different experiments. Error bars show 95% confidence limits.

The above interpretation predicts a slower time course for correlated flashes observed in the presence of quencher and EDTA than for those observed in their absence. This is due to the longer time required for EDTA to bind Mn^{2+} released from the dye. A slower time course is in fact observed (6).

DISCUSSION

The fact that increasing urea in the perfusate induces only porin-containing vesicles to release their contents suggests that urea acts osmotically in a channel-dependent way. A simple hydrodynamic model predicts that porin channels promote the development of a hydrostatic pressure across the membrane of adherent vesicles (6 and manuscript in preparation). The essential assumption of this model is that porin channels markedly increase the permeability of the membrane to urea, but do not contribute a significant increase in water permeability. The differential in permeabilities leads to an increased hydrostatic pressure which acts by the law of Laplace ($\gamma = rp/2$, where γ is the surface tension, r the vesicle radius, and p the hydrostatic pressure across the vesicle membrane) to cause an increase in the surface tension of the vesicle. If large enough, the increased surface tension then causes the vesicle either to burst or fuse. The magnitude of the surface tension increase and the factors determining whether bursting or fusion occurs are at present unknown.

The above argument and the data of Fig. 3 suggest that an increase in vesicle surface tension, brought about by an osmotically induced increase in hydrostatic pressure in the vesicle–bilayer system, may drive fusion in biological systems. In fact, osmotic swelling of secretory vesicles has been linked with exocytosis in many systems (8–12, but see 13 and 14 for exceptions).

These results demonstrate that simultaneous content release and membrane incorporation occur in the vesicle–bilayer system. The rarity of fusion (defined as *trans* content release correlated with channel incorporation) compared with bursting in this model system is not surprising considering its simplicity. What is interesting is that some artificial vesicles do fuse with the planar bilayer in a manner characteristic of biological exocytosis. Advances in

the understanding of the components that facilitate exocytosis should lead to the design of a more efficient model.

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