

The Exocytotic Fusion Pore

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THE fusion pore is the molecular structure that transiently connects the lumens of two membrane compartments during their fusion. The fusion of membrane compartments occurs in all cells because intracellular trafficking of vesicles in both endocytic and exocytic pathways and in constitutive exocytosis are ubiquitous cellular processes. In addition, many other cells including endocrine, exocrine and neuronal cells have a specialized case where exocytosis only occurs in response to specific cellular stimuli. Despite its importance, the nature of the fusion pore is unknown. In some ways, this is surprising given how intensively the regulatory mechanisms of membrane fusion have been studied for both intracellular traffic and exocytosis (Balch, 1989; Rothman and Orci, 1992; Burgoyne, 1991; Plattner et al., 1991; Lindau and Gomperts, 1991). The principal difficulty has been the lack of techniques to directly monitor the activity of single fusion pores in isolation: the reductionist approach that has proven uniquely suited for the study of ion channels.

Recently, the application of patch clamp techniques to monitor the activity of individual fusion pores in mast cells has generated a wealth of novel and unexpected observations. Our goal here is to review these findings and speculate about their significance to our understanding of exocytotic fusion. Although the majority of the results come from mast cells, many of the observations are now being reproduced in other secretory cells suggesting that the conclusions may have wider significance in other membrane fusion reactions.

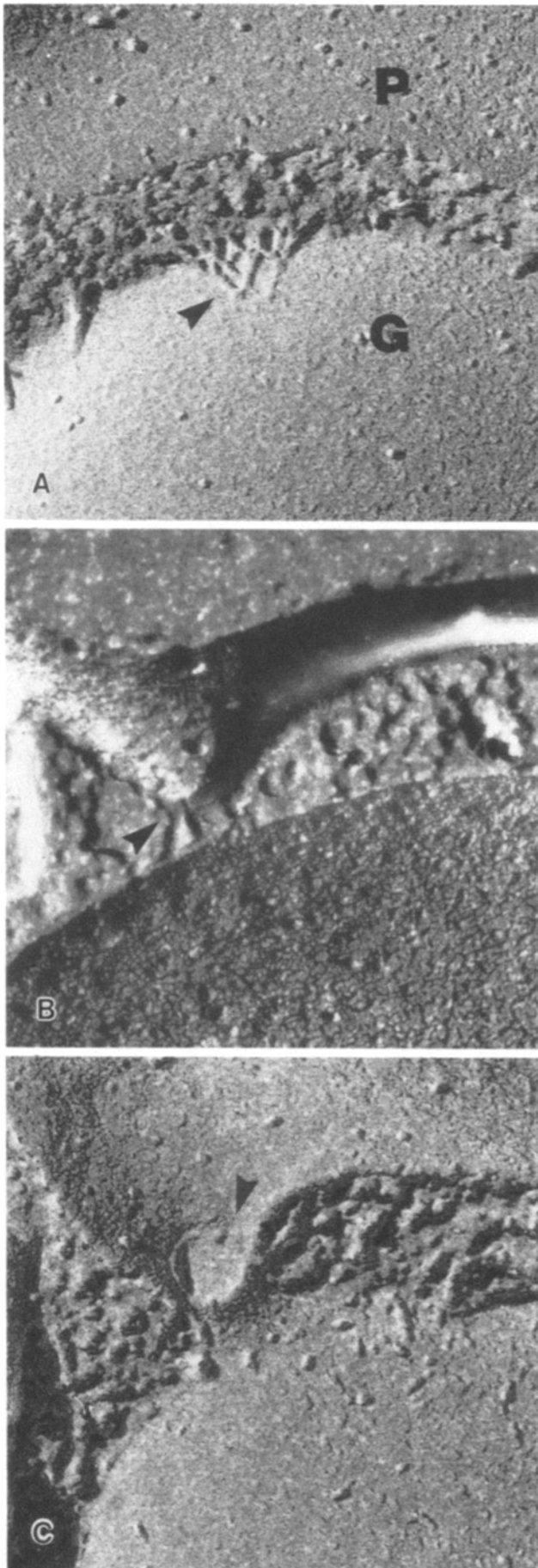
The Fusion Pore Defined by Electron Microscopy

A Lipidic Fusion Pore Spans the Gap between Fusing Membranes. Rapid freezing techniques can freeze cells in <2 ms and are thus ideal for observing the transient intermediate states associated with vesicle fusion (Chandler, 1992). In a remarkable series of micrographs, Chandler and Heuser (1980) captured the formation of exocytotic fusion pores in mast cells. Similar fusion pores have since been seen in *Limulus* amoebocytes (Ornberg and Reese, 1981), neutrophils (Chandler et al., 1983), chromaffin cells (Schmidt et al., 1983; Nakata et al., 1990) and *Paramecium* (Knoll et al., 1991), thus, providing a common picture of the exocytotic event in rapidly frozen cells. The micrographs clearly show membrane lined pores, 20–100 nm in diameter, that provide a water filled pathway between the lumen of the secretory granule and the extracellular environment. As can be seen from Fig. 1 C, these pores appear to be made of a curved bilayer which spans the granule and plasma membranes.

Membrane Dimpling Precedes Pore Formation. The

quick freeze experiments also give a hint of the events preceding the formation of a fusion pore. In unstimulated mast cells the plasma membrane and secretory granule membranes are kept at least 100 nm apart (Fig. 1 A; Chandler and Heuser, 1980). After stimulation, a new pattern is observed in which the plasma membrane invaginates to form a dimple that approaches the granule membrane (Fig. 1 B; Chandler and Heuser, 1980). This pattern of dimple formation after cell stimulation has also been observed in *Limulus* amoebocytes (Ornberg and Reese, 1981), chromaffin cells (Schmidt et al., 1983), neutrophils (Chandler et al., 1983) and *Paramecium* (Knoll et al., 1991). The secretory granules are never seen to bulge outwards towards the plasma membrane suggesting that the granule membranes are under tension and the plasma membranes relatively slack. The micrographs reveal that the dimples typically end in a tip of ~10 nm with a highly curved membrane. Because the dimple creates a focal point where the plasma and granule membranes could potentially interact at close range, it was suggested that fusion pores form at these focal points of contact. Since the plasma membrane dimples have been seen only upon cell stimulation, it is likely that the dimple forming structures are the ones that respond to the intracellular messengers generated by the stimulus-secretion coupling mechanisms.

A Scaffold of Filaments Bridges the Gap. Another important observation from the electron micrographs is that filamentous structures are seen spanning the gap between the secretory granule and cell membranes. These structures have been seen in mast cells (Fig. 1 A; Chandler and Heuser, 1980) as well as in *Limulus* amoebocytes (Ornberg and Reese, 1981). These filaments might be made of actin because filamentous networks containing actin have been seen between the cell and granule membranes in many secretory cells (Segawa and Yamashina, 1989; Nielsen, 1990; Nakata and Hirokawa, 1992). Alternatively, they could be made of annexins, which have been shown to form filamentous structures between liposomes that closely resemble the filaments between chromaffin granules and the cell membranes (Nakata et al., 1990). Because the filamentous structures are seen in regions where fusion pores can form, and given the large amount of biochemical evidence implicating cytoskeletal components in regulation of exocytosis (Burgoyne, 1987; Linstedt and Kelly, 1987), it is possible that they form part of a macromolecular structure, which can direct and regulate dimpling and fusion pore formation. We can envisage this structure as a scaffold which, by bringing about formation of the dimple, causes the two membranes to come into close proximity and promotes conditions favorable for membrane fusion.



Patch Clamp Measurements of Exocytosis

The observations from quick freeze fracture electron microscopy defined the fusion pore as a discrete entity but provided no kinetic information and are limited to observation of fairly large, water filled structures that can be etched. A glimpse of an earlier smaller fusion pore was provided from patch clamp studies of exocytosis in mast cells from the beige mouse, a mutant mouse with unusually large secretory granules. As we will see below, the patch clamp technique, by measuring the fusion pore conductance from the instant it conducts ions, has shown that the early fusion pores are much smaller than those seen by electron microscopy. These measurements have recently led to a number of important new discoveries about the nature of the fusion pore and the mechanisms underlying fusion pore formation and development. To appreciate these findings, it is at first necessary to briefly review how these measurements are made.

Patch Clamp Technique. Over the last decade, the patch clamp technique has created a revolution in biology, making it possible to directly observe the activity of single membrane proteins, namely ion channels. These studies have revealed a diverse family of ion channels, many novel regulatory mechanisms and more recently, in combination with molecular biological techniques, an insight into structure-function relationship (Neher, 1992; Sakmann, 1992). Patch clamp is elegantly simple. A small, fire polished micropipette is pushed against the plasma membrane of a cell to form an electrically tight "giga-seal" that ensures complete electrical and chemical isolation of the pipette interior from the extracellular medium. After seal formation, the patch of membrane under the pipette tip can be disrupted by gentle suction to establish continuity between the cytosol and the pipette interior (Fig. 2 A). In this "whole-cell" recording mode the cytosol is dialyzed with the solution in the pipette, allowing control of the intracellular environment.

Once intracellular perfusion is established, a patch clamp amplifier, connected between an electrode in the patch pipette and an extracellular reference electrode, is used to control the membrane potential and measure the current passed. Two types of ionic currents can be measured: transmembrane and capacitive. Transmembrane currents are due to ion permeation through ion channels. The ionic conductance of these channels is calculated from the measured current and the voltage using Ohm's Law ($I = VG$, where G is the conductance). Capacitive currents correspond to the flow of ions required to charge the cell membrane capacitance to

Figure 1. Different stages of fusion pore formation and development seen in rapidly frozen rat peritoneal mast cells. (A) Unstimulated cell showing secretory granule clearly separated from the overlying plasma membrane. *P* marks the plasma membrane, *G* the secretory granule membrane and the arrowhead indicates filamentous structures that appear to span the gap between the cell and secretory granule membranes. Magnification, 190,000 (B). Cell frozen 15 s after stimulation with 8 $\mu\text{g}/\text{ml}$ compound 48/80. A dimple, marked by the arrowhead, forms in the plasma membrane and approaches the secretory granule. Magnification, 225,000. (C) An exocytotic fusion pore (arrowhead) forms at the site of membrane dimpling. Specimen frozen 15 s after cell stimulation. Magnification, 190,000. Electron micrographs supplied by Douglas E. Chandler, Department of Zoology, Arizona State University, Tempe, Arizona.

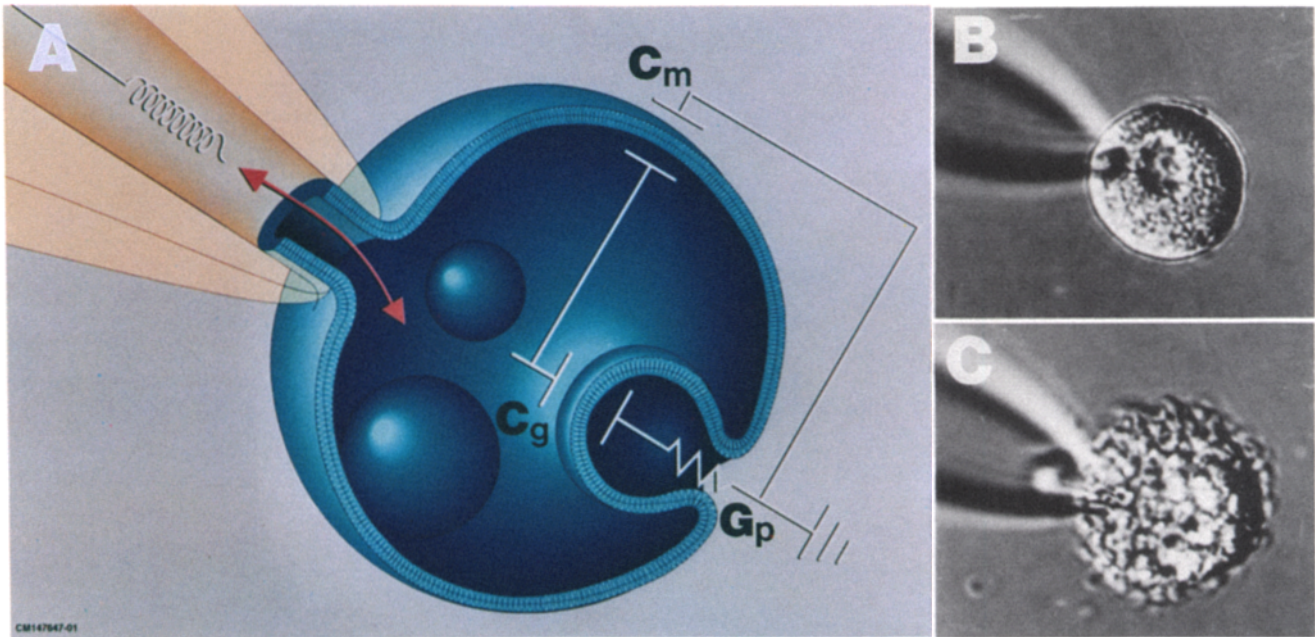


Figure 2. Patch clamp of a mast cell. (A) Schematic representation of whole cell configuration of the patch clamp technique showing the electrical equivalent elements for the plasma membrane capacitance (C_m), the secretory granule membrane capacitance (C_g) and the fusion pore conductance (G_p). (B) Photograph of a patch clamped mast cell in whole cell configuration prior to degranulation. (C) The same cell after a complete degranulation induced by intracellular perfusion of the cell with GTP γ S in the patch pipette solution.

the desired membrane potential. The total charge needed depends linearly on the cell surface area because biological membranes have a constant specific membrane capacitance. Therefore, the membrane capacitance can be used to measure the increase in cell surface area as secretory granules fuse with the cell membrane during exocytosis.

Measurements of capacitive currents are not as straightforward as measurements of ion channel currents. Because no current passes through a capacitor when it is fully charged, the voltage must be changed to measure capacitance. A simple approach is to apply a sinusoidal voltage and determine the cell admittance, the alternating current equivalent of conductance. As with transmembrane ion currents, we determine the admittance from the measured current using Ohm's Law for alternating currents: $I(\omega) = V(\omega) \cdot Y(\omega)$, where $Y(\omega)$ is the admittance, $\omega = 2\pi f$ and f is the frequency of the sine wave. The admittance of a patch clamped cell has two components, one in phase and one 90° out of phase with the membrane potential, from which we can determine the membrane capacitance as well as the other circuit elements shown in Fig. 2 A. Continuous measurements of cell admittance from a patch-clamped cell undergoing exocytosis can be made using a phase sensitive detector, either a lock-in amplifier (Neher and Marty, 1982; Lindau and Neher, 1988) or a digital phase detector (Joshi and Fernandez, 1988; Fidler and Fernandez, 1989).

Measurement of the Fusion Pore Conductance. When a secretory granule fuses with the plasma membrane, a water filled fusion pore forms an electrical connection between the lumen of the secretory vesicle and the extracellular environment. As illustrated in Fig. 2 A, the membrane capacitance of the secretory granule (C_g) is in series with the conductance of the fusion pore (G_p). Assuming that the secretory granule matrix is freely conducting, this series combination creates a well defined signature in the electrical admittance

of the patch clamped cell, which can be used to make time resolved measurements of the conductance of the fusion pore (Breckenridge and Almers, 1987a; Alvarez de Toledo and Fernandez, 1988). The methods for making the measurements and determining the fusion pore conductance have recently been reviewed in detail (Lindau, 1991).

Although the admittance techniques are well suited for continuous measurements of the fusion pore conductance, the time resolution (10 ms at best) is insufficient to measure the fusion pore conductance in the first few milliseconds of its lifetime. Fortunately, an alternative method, developed by Almers and co-workers (Breckenridge and Almers, 1987a; Spruce et al., 1990), allows us to do just this. When the pore forms, there is brief current transient, which corresponds to the current necessary to charge the vesicle membrane to the plasma membrane potential. Analysis of the magnitude and time course of these current transients can be used to determine the conductance of the fusion pore with submillisecond resolution, but only for the few milliseconds that the transient lasts. In contrast, the admittance measurements can be used to follow the pore for hundreds of milliseconds or seconds. By combining the charging transient measurements with admittance measurements, it is possible to study the formation and fast evolution of exocytotic fusion pores from their initial structure to the final irreversible expansion (see Spruce et al., 1990).

Opening of a Fusion Pore Causes a Step Increase in Membrane Capacitance. As predicted by Cole (1972), long before the patch-clamp techniques were available, the capacitance of a secretory cell should reflect increases in surface membrane area due to the addition of membrane upon fusion of a secretory granule. Indeed, with the improved resolution afforded by patch clamp measurements, stepwise increases in the cell membrane capacitance as single secretory granules fuse with the plasma membrane have been demonstrated

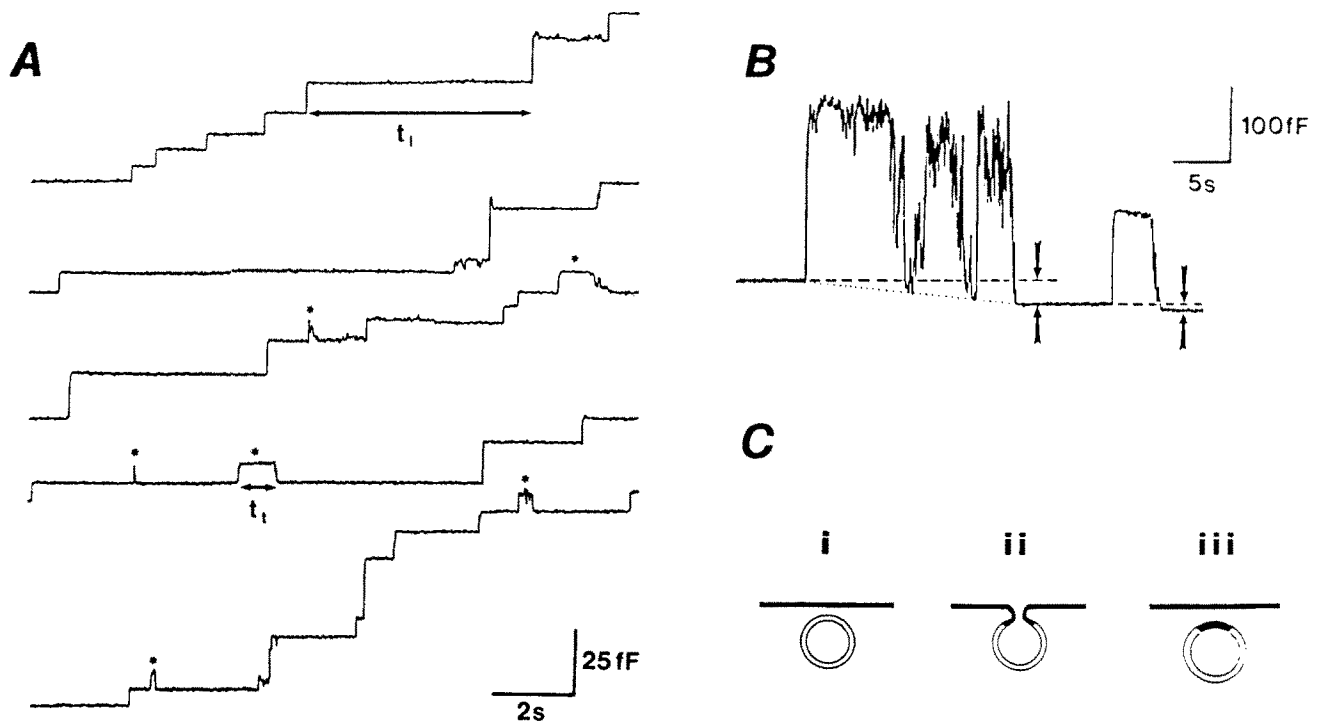


Figure 3. Exocytotic fusion events captured by capacitance measurements from a degranulating mast cell stimulated with GTP γ S. (A) A typical capacitance staircase resulting from sequential fusion of many secretory granules. Two types of fusion event are discernible: irreversible step increases in capacitance upon membrane fusion and transient fusion events (e.g., *asterisks*) where the fusion pore closes. Note that the interval between successive steps (t_i) and the dwell time of transient fusion pores (t_d) can be used for kinetic analysis of opening and closing of fusion pores (Oberhauser et al., 1992a). (B) An example of a transient fusion of a giant secretory granule from a beige mouse mast cell. Note the magnitude of the backstep is larger than the initial step indicating that the area of the plasma membrane is decreased after a transient fusion event suggesting that there is a large lipid flux through the fusion pore. (C) A simplified scheme of a transient fusion event showing membrane transfer (see Monck et al., 1990).

in a variety of secretory cells undergoing exocytosis (Neher and Marty, 1982; Fernandez et al., 1984; Nusse and Lindau, 1988; Nusse et al., 1990). Because fusion of a secretory vesicle with the plasma membrane increases the surface area of the cell membrane by an amount equal to the vesicle membrane area, the steps have different amplitudes corresponding to different granule sizes (Fig. 3 A). For example, a 10-fF capacitance step corresponds to the addition of $1 \mu\text{m}^2$ of membrane, equivalent to a spherical granule with a diameter of $\sim 0.5 \mu\text{m}$. The size distribution of secretory granules measured from the capacitance are in good agreement with the size distributions determined from electron micrographs, not only for mast cells (Fernandez et al., 1984; Alvarez de Toledo and Fernandez, 1990), but also for neutrophils (Nusse and Lindau, 1988) and eosinophils (Nusse et al., 1990).

Pipette Biochemistry. A patch pipette typically accesses the cytosol of the cell through a large, micrometer sized opening in the cell membrane through which there is rapid diffusional exchange of soluble molecules between the pipette and the cytosol. Because the volume of the patch pipette ($>10 \mu\text{l}$) dwarfs that of the cell ($\sim 1 \text{pl}$), the freely soluble contents of the cytosol, including proteins, nucleotides, metabolites and ions, will be lost into the patch pipette and rapidly replaced by the pipette solution. Calculations from the rates of diffusional exchange between cells and a patch pipette predict that ions and nucleotides will be lost within a few minutes and small soluble proteins within $\sim 15 \text{min}$

(Pusch and Neher, 1988). For example, 99% washout from a small cell will take 1.3 min for K^+ (39 D), 3.2 min for GTP (530 D), 11.7 min for rab3a (25 kD) and 19.1 min for βCOP (110 kD). It has been demonstrated that, in some cells, this "washout" uncouples receptor mediated responses from the secretory response.

The washout of the cytosol is a blessing in disguise because it challenges the investigator to identify the soluble components mediating the sought response. These manipulations, collectively named "pipette biochemistry" have led to several unanticipated discoveries. For example, a long standing paradigm of the stimulus secretion field was that an elevation of cytosolic calcium into the micromolar range was sufficient and necessary to elicit exocytotic fusion. Surprisingly, patch clamped mast cells failed to undergo exocytotic degranulation in response to perfusion with pipette solutions that contained an elevated calcium concentration. In contrast, mast cells undergo a complete and spontaneous degranulation in response to the hydrolysis-resistant GTP analog, GTP γ S (0.1–50 μM), when the Ca^{2+} concentration is clamped below 50 nM with strong calcium buffers (Fernandez et al., 1984; Neher, 1988). These results demonstrated that an elevated concentration of cytosolic calcium was neither sufficient nor necessary under the conditions created in a patch clamped cell, and directly implicated guanine nucleotide binding proteins in the regulation of exocytotic fusion. Similar results have since been seen in patch clamped neutrophils (Nusse and Lindau, 1988) and eosinophils

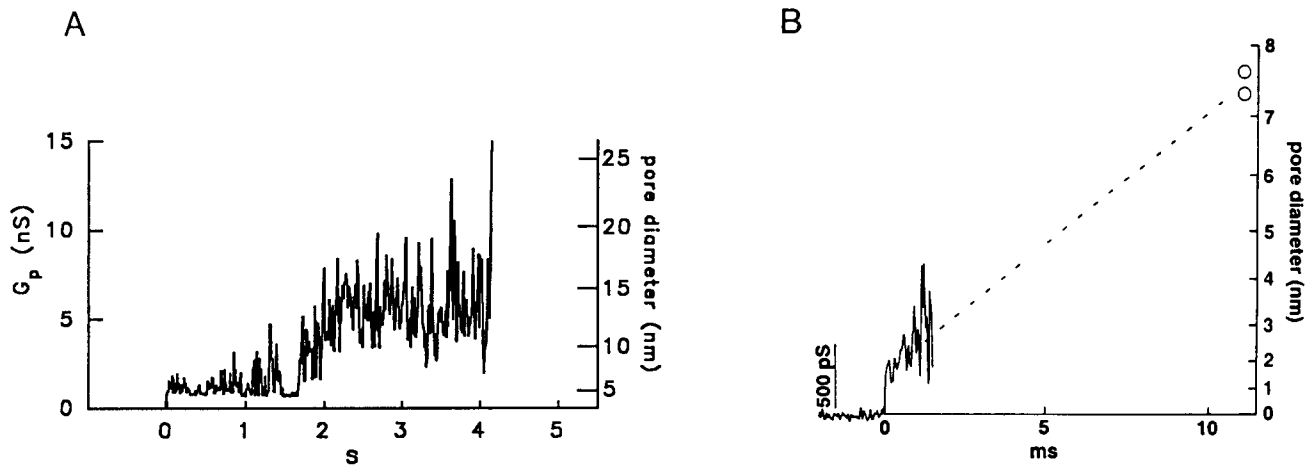


Figure 4. Time-course of fusion pore conductance measured in beige mouse mast cells. (A) Pore conductance calculated from the real and imaginary components of the cell admittance. See Nanavati et al. (1992) for details. (B) Pore conductance calculated from the brief current transient that occurs while the membrane of the secretory granule is charged to the cell membrane potential (see Breckenridge and Almers (1987a), from where B is taken, for details). Note that the fusion pores shown opened abruptly with initial diameters of 5 and 2 nm, respectively. In A, the pore remained in a semistable state for more than a second before expanding.

(Nusse et al., 1990), as well as in various permeabilized cells (Vallar et al., 1987; Lindau and Gomperts, 1991). The measurement of capacitance in patch clamped cells has become a productive approach for the study of stimulus-secretion coupling (Neher, 1988; Penner, 1988; Mason et al., 1988; Penner et al., 1988; Zorec et al., 1991).

The Fusion Pore Evolves in Distinct Stages

Patch clamp measurements of secretory cells undergoing exocytosis have revealed the exocytotic fusion pore as a remarkably dynamic entity. The existence of the fusion pore can be divided into several distinct phases. The fusion pore is first detected as an abrupt increase in pore conductance. This is followed by an expansion phase characterized by the wide fluctuations in fusion pore conductance. These fluctuations, termed flicker, can be brief (ms) or prolonged (s). Flicker usually terminates with a rapidly increasing phase as the pore irreversibly expands to a structure akin to that seen in electron micrographs, but sometimes the pore can close again completely (Fernandez et al., 1984; Spruce et al., 1990). The properties of these different phases will be discussed in turn.

Opening of the Fusion Pore. The fusion pore opens abruptly with a conductance similar to that of a large ion channel (Breckenridge and Almers, 1987a; Spruce et al., 1990). This observation is interesting because several secretory granule membrane proteins, including synexins and synaptophysin, have been shown to form ion channels in vitro (Thomas et al., 1988; Pollard et al., 1990; Sudhof and Jahn, 1991). This has led to the hypothesis that the opening of the fusion pore corresponds to the opening of an ion channel (Almers, 1990; Almers and Tse, 1990). Analysis of the transient current discharges through the fusion pores of beige mice mast cells showed that the median initial conductance is ~ 300 pS, but varies between 80 and 1,000 pS (Fig. 4 B; Breckenridge et al., 1987a; Spruce et al., 1990). Admittance measurements give similar values for initial fusion pore conductance (Fig. 4 A; Spruce et al., 1990; Nanavati et al., 1992).

The smallest fusion pores seen in the electron micrographs

are approximately 20 nm across and 50 nm long. By modeling the pore as a water-filled tube, we can estimate that this pore would have a conductance of ~ 10 nS. Thus, the fusion pore when it first opens is considerably smaller and must represent an earlier, more transient stage in the development of the fusion pore. If we assume that this early fusion pore is merely a smaller version of those seen in electron micrographs, a lipidic tube with a length of ~ 15 nm, slightly larger than the width of two bilayers, then an initial conductance of 80 pS corresponds to a diameter of 1 nm.

Expansion of the Fusion Pore. After fusion pore opening the pore begins to expand. This expansion phase can be fast or slow and is characterized by large, rapid fluctuations in pore conductance. Hundredfold changes in conductance (0.1–10 nS) can occur within tens of milliseconds (Alvarez de Toledo and Fernandez, 1988; Spruce et al., 1990; Monck et al., 1991a). These changes usually appear to be continuous (Breckenridge and Almers, 1987a; Spruce et al., 1990; Nanavati et al., 1992), rather than as discrete levels typical of ion channels, although on occasion several semi-stable conductance states can be seen (Spruce et al., 1990).

Flicker usually ends with the irreversible expansion of the fusion pore to a large conductance, which is beyond the detectable limit of the admittance measurements. The limiting value is typically ~ 10 nS, corresponding to a pore with a diameter of more than 20 nm. Once the pore expands beyond this size the pore rarely or never closes, suggesting the possibility of a structural change from an early fusion pore which can close to a larger pore that is unable to close. The lifetime of the early fusion pore can be measured from the length of flicker because, in mast cells from the beige mouse, flicker begins abruptly when the fusion pore opens and terminates equally abruptly when the pore expands. The distribution of flicker length is exponential, allowing extraction of a time constant for pore expansion of ~ 200 ms (Monck et al., 1991a). Although some fusion pores flicker for hundreds of milliseconds or even several seconds, most pores expand in less than 50 ms. It is quite possible that the pore expansion is faster in cells with smaller, more highly curved secretory granules such as synaptic vesicles.

The mechanism of fusion pore expansion is unknown. The fusion pore provides a path for water entry into the secretory granule matrix, which causes decondensation of the contents and matrix swelling (Verdugo, 1991). However, inhibition of this swelling does not change the fusion pore expansion in mast cells, suggesting that the initial phase of pore expansion seen with the patch clamp reflects some other property of the fusion pore (Monck et al., 1991a). The fusion pores seen in electron micrographs are still relatively small structures, so it seems likely that matrix swelling will provide the force for a second phase of pore expansion leading to complete incorporation of the secretory granule membrane into the cell membrane and expulsion of the secretory granule contents into the extracellular environment.

Closure of the Fusion Pore. The first new finding provided by patch clamp measurements of rat mast cells was that a fusion pore does not always expand irreversibly but sometimes closes leaving an intact secretory vesicle inside the cell (Fernandez et al., 1984). The ability of fusion pores to close was quite unexpected. Transient fusion events have since been observed in normal and beige mouse mast cells (Zimmerberg et al., 1987; Breckenridge and Almers, 1987b; Spruce et al., 1990; Monck et al., 1990). Several examples of transient fusion events are shown in Fig. 3 (A and B). Sometimes a vesicle can undergo many transient fusions before a final irreversible fusion occurs (Breckenridge and Almers, 1987b; Alvarez de Toledo and Fernandez, 1988; Spruce et al., 1990).

Ceccarelli and colleagues have proposed that contrary to the conventional view of neurotransmission, a fusion pore might open, allow discharge of the vesicle contents and then close, obviating need for membrane merging and subsequent recycling (Valtorta et al., 1990). Recent advances in measuring low concentrations of biogenic amines using voltammetric techniques have allowed measurement of catecholamine release from single chromaffin granules (Leszczyszyn et al., 1990; Chow et al., 1992) and serotonin release from single mast cell granules (Alvarez de Toledo et al., 1993). Interestingly, there was a small amount of release before the main phase of release (Chow et al., 1992; Alvarez de Toledo et al., 1993). In mast cells this leak also occurred during transient fusion events, providing support for Ceccarelli's proposal.

When a fusion pore connects a secretory granule to the plasma membrane, there is a net flow of lipid into the secretory granule (Monck et al., 1990). Analysis of transient fusion events revealed that the backsteps were larger than the onsteps (Fig. 3 B), indicating a net movement of phospholipid membrane from the cell membrane to the secretory granule during flicker, as illustrated in Fig. 3 C. The quantities of the phospholipids moved are large, equivalent to almost a million molecules per second—enough to replace all the phospholipids in a small lipidic fusion pore every millisecond. Because biological membranes contain a mixture of lipids, the composition of the pore could change dramatically over short time intervals and could provide an explanation for the rapid fusion pore conductance changes if, as seems likely from energetic considerations, some lipids favor pore expansion and others promote closure (Kozlov et al., 1989; Nanavati et al., 1992). The flux of lipid through the fusion pore also indicates that the pore, when it closes, is partially or totally lipidic, an observation that limits the

types of molecular models that can be proposed for the structure of the fusion pore, but also raises the question as to how lipidic pores might close.

Fusion pore closure has an unusual temperature dependency. The rate of pore closure, which was determined from the dwell time of transient fusions using kinetic analysis similar to that used for single ion channels (Oberhauser et al., 1992a), undergoes a large and sharp discontinuity in the Arrhenius plot at 13°C. Such discontinuous temperature dependencies had never been seen in biological membranes, but are typical of diffusional processes in homogeneous phospholipid bilayers (Krasne et al., 1971; Hoffmann et al., 1980). Therefore, to explain this unusual observation, it was suggested that below the transition temperature, a class of lipids with a tendency to close the pore could be frozen out into a crystalline microdomain and be unavailable to induce closure of the fusion pore (Oberhauser et al., 1992a).

The rate of movement of membrane through the fusion pore is surprisingly constant throughout the lifetime of the early fusion pore, and the same rate occurs regardless of stimulus strength or a number of other chemical and physical perturbations (Monck et al., 1990, 1991b). It seems that when the fusion pore opens, there is a constant driving force for lipid flow. We proposed that a difference in lateral membrane tension between the secretory granule membrane and cell membrane is the driving force for the membrane flow. Because this tension difference is always the same when membranes fuse, we further suggested that there is a critical tension for membrane fusion. Such a mechanism is very compelling because many of the perturbations used to fuse phospholipid bilayers and vesicles in model fusion systems, are conditions that have been shown to increase the membrane tension (see below).

Molecular Structure of the Fusion Pore

Fusion pores must exist in all cases where two membrane compartments fuse, whether in artificial bilayer systems, in virus-mediated fusion, intracellular traffic, fertilization or exocytosis. These fusion pores are likely to share certain physical principles and molecular structures. If this is true, then information learned from study of other fusion pores should aid our understanding of the exocytotic fusion pore.

Lipidic Fusion Pores in Artificial Bilayer Systems. The structure of the fusion pore and the mechanism of fusion are interrelated problems. The structure can tell us something about the mechanism and vice versa. The fusion of two planar bilayers or of a vesicle with a bilayer has been extensively studied in model lipid systems. It is well established that when two bilayers are brought together, as they must be for membrane fusion to occur, there are a number of attractive and repulsive forces that determine whether the membranes are kept apart, adhere or fuse (Rand, 1981; Israelachvili and McGuiggan, 1988). At large separations, the balance between the attractive van der Waals forces, repulsive electrostatic double layer forces, and fluctuation (or undulation) forces describes the interaction between two membranes. However, as the separation is reduced below 3 nm, repulsive hydration and steric forces begin to dominate and act to keep the bilayers apart. For fusion pore formation these short range repulsive forces must be overcome.

Recently, Israelachvili and co-workers (Helm et al., 1989, 1992) have argued that reducing the repulsive forces is insuf-

ficient for membrane fusion and that exposure of a strongly attractive hydrophobic force is necessary for membrane fusion. It was shown that phospholipid bilayers applied to mica surfaces could be induced to spontaneously fuse at a separation of 1–2 nm if they were “depleted” by a technique that reduces the density of phospholipid head groups per unit area of membrane, equivalent to increasing the lateral tension. The fusion occurs as the result of localized exposure of the hydrophobic interior which allows a strong attractive hydrophobic force to bypass the repulsive hydration forces. This conclusion fits well with the fact that protocols designed to induce fusion in artificial membrane systems—osmotic and hydrostatic pressure, electric fields, temperature, Ca^{2+} and other divalent cations, pH, and possibly polyethylene glycols—also increase lateral membrane tension (Finkelstein et al., 1986; Zimmermann, 1986; Ohki, 1987; Woodbury and Hall, 1988). Thus, exposure of the hydrophobic force will cause hemifusion of two bilayers if the separation is less than 2 nm. Once the hemifusion breakthrough has occurred, increased local stresses in the common bilayer favor pore formation and subsequent full fusion (Helm et al., 1992).

The fusion pore formed according to this mechanism is a pore through a single bilayer and has a purely lipidic structure. But can a purely lipidic pore show the dynamic properties of the exocytotic fusion pore, namely multiple conductance levels, rapid fluctuations and reversibility? A lipidic fusion pore can be modeled by assuming that the free energy of the pore is determined by the elastic energy associated with the bilayer curvature and the isotropic tension of the fusing membranes (Markin et al., 1984; Chernomordik et al., 1987; Kozlov et al., 1989; Nanavati et al., 1992). When experimentally determined values for the spontaneous curvature of biological membranes and estimates of the membrane tension present in the secretory granule were used in such models, fusion pores that can expand irreversibly or open for several seconds and then close, were predicted (Nanavati et al., 1992). Strikingly, the range of conductances predicted for such lipidic pores was indistinguishable not only from that measured for exocytotic fusion pores in the beige mouse mast cell, but also for electromechanically induced pores in the secretory granule membrane (Oberhauser and Fernandez, 1992). Further evidence that such pores through a single bilayer can occur in biological membranes is provided by the observation that electrically-induced fusion of erythrocytes proceeds through hemifusion (Song et al., 1991).

Bilayer fusion, pore formation and pore expansion can be very fast. For example, fusion of vesicles with planar bilayers can be measured as incorporation of ion channels into the bilayer. The full step in single channel conductance occurs in less than 200 μs (Cohen et al., 1980) indicating that, within this time interval, the fusion pore has both formed and expanded to a size where the resistance is negligible, equivalent to a cylindrical pore 10 nm long and 30 nm in diameter (Zimmerberg, 1987). There is evidence that pores in a single bilayer induced by electroporation form at sub-microsecond time-scales (Hibino et al., 1991). Therefore, such a mechanism is fast enough to account for even the most rapid exocytotic responses such as those occurring during neurotransmission.

Virus-induced Fusion Pores. The ability of viruses to induce fusion pores is particularly instructive because this fu-

sion reaction is the only one where the proteins that induce fusion have been identified (reviewed Hoekstra, 1990; Bentz et al., 1990; White, 1990, 1992). The fusogenic activity resides in virally encoded membrane glycoproteins, of which the influenza virus hemagglutinin is the best studied. The hemagglutinin fusion proteins have a transmembrane anchoring segment, which holds the two membranes together, and a fusogenic domain, which forms an amphiphilic peptide, with bulky hydrophobic residues on one side and acidic groups on the other. Putative fusion peptides for other viruses have been assigned, but fusogenic activity has only been demonstrated for some peptides, such as the hemagglutinin peptides. The fusogenic domain of most viral fusion proteins forms an α helix, with most of the hydrophobic residues on one face (White, 1990).

Recent models for viral fusion propose that several hemagglutinin trimers assemble to form a “collar” within which the fusion pore forms (Bentz et al., 1990; White, 1990). The membrane region within this collar is ~ 5 nm in diameter. The hydrophobic side of the fusion domain, on the inside of the collar, is proposed to form a bridge that allows lipids from the two membranes to come into contact. However, given that two closely opposed bilayers fuse when under tension, or when a local perturbation of the bilayer structure occurs, it is possible that the arrangement of the viral proteins could draw the two membranes into close apposition within the collar and induce fusion by either increasing the membrane tension, or inducing appropriate local perturbations in the physical structure of the bilayer.

The viral fusion pore has also been examined using electron microscopy and patch clamp techniques. The lipid pores seen in the electron micrographs appear identical to the exocytotic fusion pore with a diameter of ~ 50 nm (Knoll et al., 1988). Patch clamp measurements of the fusion between two cell membranes induced by influenza virus hemagglutinin, that has been transfected into one of the cells, reveal a fusion pore that opens abruptly and undergoes fluctuations in pore conductance, much as the exocytotic fusion pore, but expands relatively slowly (Spruce et al., 1989, 1991). These similarities between the properties of the viral and exocytotic fusion pores suggest similar structures and mechanisms of formation. Recently, a sperm glycoprotein implicated in sperm-egg fusion during fertilization has been proposed to contain a sided helical domain, like the viral fusion peptides (Blobel et al., 1992), suggesting that this mechanism might be more widespread.

Models for Exocytotic Fusion Pores. Many models for the exocytotic fusion pore have been proposed. One model proposes that the initial pore is a proteinaceous ion channel-like structure traversing the bilayers of the secretory granule and plasma membranes (Almers, 1990; Almers and Tse, 1990). According to this hypothesis, the exocytotic fusion pore results from a preassembled, ion channel-like structure that opens in response to a cellular messenger. The rationale for this model is that opening of an ion channel would be readily subject to the rapid regulation necessary, for example, in neurosecretion. In addition, it is easy to explain how such a fusion pore would close. Drawing analogy with the gap junction, the only known channel to span two bilayers, it was proposed that the initial pore (conductance, 80 pS) corresponds to an oligomeric channel with an internal diameter of ~ 1 nm (assuming the 15-nm length of the gap junction).

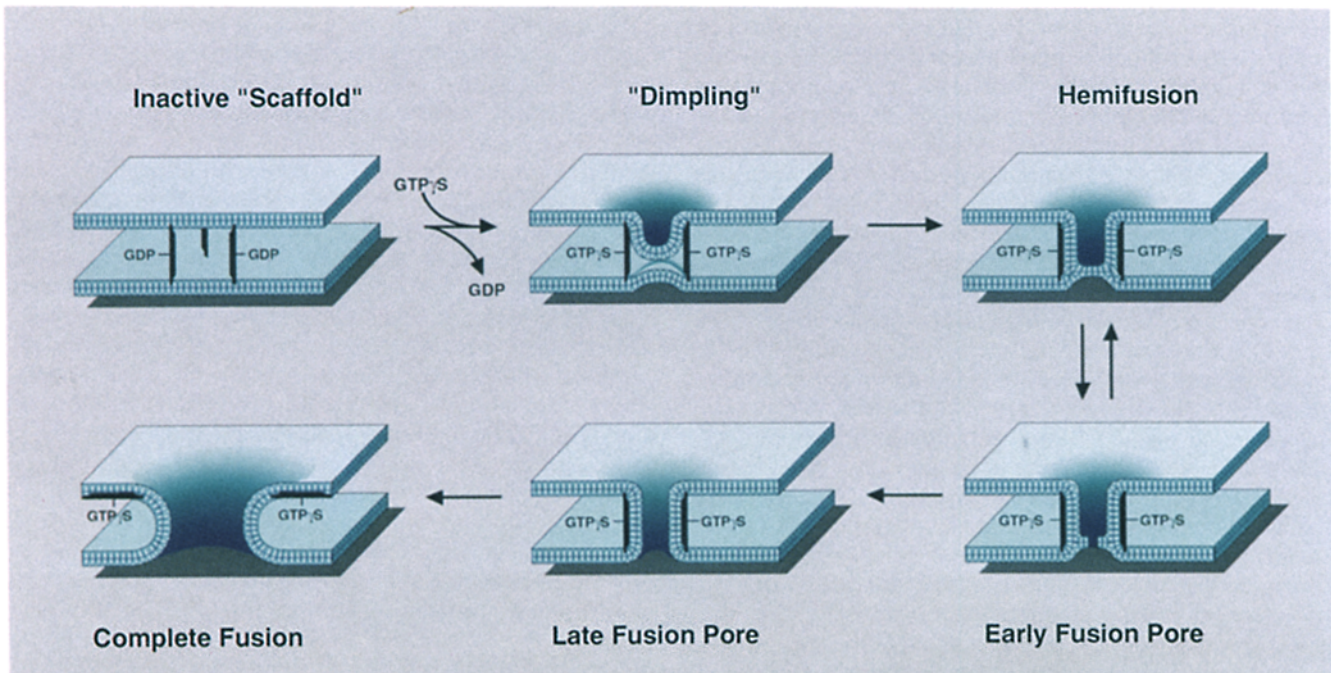


Figure 5. Model describing exocytotic fusion as a lipidic fusion event directed by a protein scaffold. (Top left) Membranes separated by a protein scaffold. Because $GTP\gamma S$ is the trigger for exocytosis in most fusion pore experiments, GDP is depicted as bound to the scaffold to represent the inactive state of a GTP-binding protein, which is the cellular switch for fusion pore formation and forms part of the scaffold. (Top center) In response to exchange of GDP for $GTP\gamma S$ on the GTP-binding protein, the scaffold directs a dimple in the plasma membrane towards the secretory granule. (Top right) Hemifusion occurs spontaneously due to local tension in the two membranes. (Bottom right) A small pore forms in the stressed common bilayer of the hemifusion structure. This pore can either close or expand. (Bottom center) The fusion pore develops into the hour-glass structure seen in the electron micrographs. (Bottom left) The pore further expands to allow release of secretory products.

The ion channel would show multiple initial conductance levels, like the gap junction, and expand by intercalation of lipid molecules between the subunits of the structure leading to a mostly lipidic fusion pore that expands irreversibly, completing exocytosis (Almers, 1990; Almes and Tse, 1990).

Several models have proposed a fusion pore which comprises both proteins and lipids from its earliest moments and where the proteins serve as a hydrophobic bridge between the membranes. The "hydrophobic bridge" model has recently been reviewed in two different flavors (Pollard et al., 1991; Zimmerberg et al., 1991). According to this model, proteins form a bridge across which phospholipids can cross with their hydrophobic tails interacting with the hydrophobic surface of the proteins. The rationale of this model is that the repulsive hydration forces do not have to be overcome en masse. In one form, synexins are the bridge proteins (Pollard et al., 1991). These models have many similarities with models for viral fusion (see above). The fusion pore would quickly become mainly lipidic as otherwise the model could not readily explain the large fluctuations in pore conductance.

Alternatively, the initial fusion pore could be an entirely lipidic structure. As discussed above, the lipid flux through the fusion pore, the unusual temperature dependency and the comparison between the exocytotic fusion pore and a theoretical lipidic pore suggest that the fusion pore has a strong lipid character at an early stage. Given that two bilayers will fuse spontaneously at separations below 2 nm when the membranes are under tension, we favor this latter mecha-

nism for exocytotic membrane fusion. It is not unreasonable that the cell will utilize intrinsic properties of phospholipid bilayers as the fusion mechanism. We propose that the role of proteins is to respond to intracellular signals, to draw the plasma membrane into close proximity with the secretory granule membranes, and to favor fusion by increasing membrane tension or inducing local perturbations in the bilayer structure.

A Protein Scaffold Directs Exocytotic Membrane Fusion

The electron micrographs show that the secretory granule and cell membranes are normally kept apart until, after cell stimulation, a dimple in the plasma membrane approaches the granule membrane. According to our model, the macromolecular scaffold of proteins is responsible for directing the dimpling of the cell membrane in response to intracellular signals (Fig. 5; Nanavati et al., 1992). The filamentous structures seen between the two membranes are likely to be part of this scaffold. The dimple is seen to end in a small (10 nm), highly curved tip. The high curvature of this tip will provide a localized region of high tension. When the tip of the dimple approaches the granule membrane, which in the stimulated cell has a high tension, hemifusion of the two bilayers will occur spontaneously.

Pore formation will follow because stresses in the common bilayer, comprising the intragranular leaflet of the granule membrane and the extracellular leaflet of the plasma membranes, will induce membrane breakdown. Thus, the initial

fusion pore is a pore in a single bilayer. As discussed earlier, models of the free energy profiles of such pores predict that they will behave similarly to the measured exocytotic fusion pore. These pores should fluctuate around a certain size before either expanding irreversibly or closing. An advantage of progressing through hemifusion, is that potentially injurious secretory products cannot leak into the cytosol. It is also easy to explain pore closure by changes in the spontaneous curvature of the lipid that make up the fusion pore (Nanavati et al., 1992). As the pore expands the trilaminar structure retreats and the full fusion pore typical of the electron micrographs is irreversibly formed.

There is precedent for macromolecular scaffolds in processes that regulate membrane organization and shaping of organelles: clathrin scaffolds for endocytosis, small GTP-binding proteins and β COPs for maintenance of the shape of transport vesicles, a complex of several proteins including SNAPS and NSF for regulating intracellular fusion in the Golgi, and the association of several hemagglutinin trimers for viral fusion (Rothman and Orci, 1992; Wilson et al., 1992; White, 1990; Bentz et al., 1990). The exocytotic scaffold is likely to contain GTP and Ca^{2+} binding proteins to detect and respond to intracellular signals, and share certain regulatory and structural components with other membrane fusion reactions like those of intracellular traffic (Oberhauser et al., 1992; White, 1992). Although the scaffold proteins have not yet been identified, many proteins that may play important structural and regulatory roles in the scaffold are being discovered (Sudhof et al., 1989; Burgoyne and Geisow, 1989; Sudhof and Jahn, 1991; Bennett et al., 1992). Identification of the scaffold proteins and characterization of how they interact to promote fusion will provide many challenges in the years ahead. In combination with molecular biological techniques, biophysical measurements of the fusion pore will define structure-function relationships between the proteins of the scaffold and the fusion pore and provide the molecular basis for understanding regulated membrane fusion.

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