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Tension in secretory granule membranes causes extensive membrane transfer through the exocytotic fusion pore

(exocytosis/membrane tension/membrane fusion/capacitance flicker/mast cells)

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ABSTRACT **For fusion to occur the repulsive forces between two interacting phospholipid bilayers must be reduced. In model systems, this can be achieved by increasing the surface tension of at least one of the membranes. However, there has so far been no evidence that the secretory granule membrane is under tension. We have been studying exocytosis by using the patch-clamp technique to measure the surface area of the plasma membrane of degranulating mast cells. When a secretory granule fuses with the plasma membrane there is a step increase in the cell surface area. Some fusion events are reversible, in which case we have found that the backstep is larger than the initial step, indicating that there is a net decrease in the area of the plasma membrane. The decrease has the following properties: (*i*) the magnitude is strongly dependent on the lifetime of the fusion event and can be extensive, representing as much as 40% of the initial granule surface area; (*ii*) the rate of decrease is independent of granule size; and (*iii*) the decrease is not dependent on swelling of the secretory granule matrix. We conclude that the granule membrane is under tension and that this tension causes a net transfer of membrane from the plasma membrane to the secretory granule, while they are connected by the fusion pore. The high membrane tension in the secretory granule may be the critical stress necessary for bringing about exocytotic fusion.**

Exocytosis occurs when a fusion pore, the connection between the lumen of a secretory granule and the extracellular space, expands irreversibly, allowing the rapid extrusion of the granule contents. Although considerable progress has been made toward understanding the regulation of exocytosis by Ca^{2+} and other intracellular messengers (1–3), the mechanism by which the secretory granule fuses with the cell membrane remains a mystery (4). On the other hand, studies of the fusion of phospholipid bilayers and vesicles have made considerable progress toward understanding the forces involved when two bilayers are brought together and fused (5). Many experimental approaches have been used to induce fusion, including the use of osmotic forces, divalent cations, electromechanical stress, and bilayer "depletion" (6–15). These strategies all increase the bilayer tension so that increased exposure of hydrocarbon at the membrane surface causes a reduction in the repulsive hydration forces. Consequently, swelling of the secretory granule core by osmotic forces has been considered a likely mechanism for exocytotic fusion (16, 17). However, several studies using the patch-clamp technique, which can measure the fusion of individual secretory granules as discrete stepwise increases in the cell membrane capacitance, have shown that fusion of secretory granules in mast cells and sea urchin eggs occurs before granule swelling and is not inhibited by hyperosmotic solutions that reduce granule swelling (18–20).

The patch-clamp technique can also be used to study properties of the fusion pore. The time course of the fusion pore conductance can easily be measured by modeling a granule fusing with the cell membrane as a conductance (the fusion pore) in series with a capacitor (the granule membrane) (20–23). The fusion pore conductance is initially ≈200 nS and normally increases, often in a rapidly fluctuating manner known as flicker, to an unmeasurably large final conductance that represents the expanded fusion pore (21–23). An earlier unexpected finding was that the fusion pore does not always expand irreversibly but instead could collapse, leaving an intact secretory granule inside the cell (21–24).

We have investigated the properties of transient fusion events to gain an insight into the mechanisms involved in exocytotic fusion. Here we report that the "off" step of a transient fusion event is larger than the initial "on" step. This corresponds to a time-dependent decrease in the cell surface membrane area, indicating that while the secretory granule and plasma membrane are connected by the fusion pore there is net movement of membrane to the secretory granule. These results suggest that the secretory granule membrane is under tension and that this may play an important role in the mechanism of exocytosis.

METHODS

Cell Preparation. Mast cells were prepared from adult normal or beige ($bg^j bg^j$) mice (The Jackson Laboratory) following a procedure described in detail elsewhere (25). Briefly, cells were obtained by peritoneal lavage with a solution of the following composition: 136 mM NaCl, 9 mM Hepes, 2.5 mM KOH, 1.4 mM NaOH, 0.9 mM $MgCl_2$, 1.8 mM $CaCl_2$, 45 mM $NaHCO_3$, 6 mM glucose, 0.4 mM phosphate. The cells were incubated at 37°C under a 5% CO_2/95% air atmosphere for at least 30 min prior use. For the patch-clamp experiments, the extracellular medium was changed to one containing the following: 150 mM NaCl, 10 mM Hepes, 2.8 mM KOH, 1.5 mM NaOH, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM glucose (310 mmol/kg, pH 7.25). In some experiments, the normal extracellular medium was replaced by an acidic histamine medium (130 mM histamine hydrochloride/1 mM $CaCl_2$/1 mM $MgCl_2$/5 mM citrate; 310 mmol/kg, pH 4.2–4.5), which inhibited the swelling of the secretory granule matrix (44).

Cell Capacitance Measurements. The cell membrane capacitance was measured by using the whole cell mode of the patch-clamp technique. The pipette solution contained the following: 140 mM potassium-glutamate, 10 mM Hepes, 7 mM $MgCl_2$, 3 mM KOH, 0.2 mM ATP, 1 mM $CaCl_2$, 10 mM EGTA, and various concentrations of GTP[γS] (1–40 μM) to induce degranulation. The free Ca^{2+} concentration in the pipette solution was 30 nM. The cell membrane capacitance

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was determined with a digital phase detector implemented on a system comprising an Indec System (Sunnyvale, CA) data acquisition interface and a microcomputer (Digital PDP11/73, Beltron 286, or Compaq 386/25) (26). After applying a sinusoidal voltage (833 Hz, 54 mV; peak to peak) to the stimulus input of the patch-clamp amplifier (EPC-7, List Electronics, Darmstadt, F.R.G.), the magnitude of the current was measured at two different phase angles—ϕ and ϕ-90. The phase detector was aligned so that one output (at ϕ-90) reflected the real part of the changes in the cell admittance [Re(ΔY)] and the second output reflected the imaginary part of the admittance [Im(ΔY)]. During the experiments, the angle of the phase detector was periodically adjusted by using the phase tracking technique (27). In the figures, the traces labeled C and G_{ac} correspond to Im(ΔY) and Re(ΔY), respectively. In Figs. 1 and 2, the C and G_{ac} traces were filtered with a digital low-pass filter [$x_i = (x_{i-1}/4) + (x_i/2) + (x_{i+1}/4)$, where x_i is the value of the ith point] and corrected for slow drifts in the baseline by subtracting a linear slope determined from the baseline prior to the fusion event. A calibration signal for the C trace was obtained by unbalancing the C slow potentiometer of the compensation circuitry of the patch-clamp amplifier by 100 fF. The capacitance of the cell membrane can be used to estimate the cell surface area by using a conversion factor of 10 fF/μm^2.

RESULTS AND DISCUSSION

The fusion of single secretory granules with the cell membrane was recorded by measuring the cell membrane capacitance in mast cells from normal mice and from beige mice, a mutant with giant secretory granules. Exocytosis was stimulated by including guanine nucleotides in the patch pipette. The capacitance recordings for four secretory granules undergoing transient fusions in a mast cell are shown in Fig. 1. We have found that a striking feature of the transient fusion events is that the initial step increase in capacitance is smaller than the final backstep. The fluctuations observed in both the capacitance (C) and conductance (G_{ac}) traces shown in Fig. 1*B* result from wide variations in the resistance of the fusion pore (21–23). However, the conductance before and after the transient fusion event is the same (G_{ac}; Fig. 1*B*), indicating that there is a genuine decrease in capacitance. These capacitance differences correspond to a decrease in the surface area of the plasma membrane. The magnitude of the difference can be quite large compared to the surface area of the granule. For example, the step in Fig. 1*B* has an initial step of 2.6 μm^2 and a backstep of 3.4 μm^2. The difference is equivalent to 30% of the granule surface area.

A simple explanation for the backstep being larger than the initial step in capacitance is that a small piece of membrane is transferred from the plasma membrane to the secretory granule, as depicted in Fig. 1*C*. This explains why the net decrease in plasma membrane area is not observable while the granule and plasma membrane are fused together, as shown by the relatively constant capacitance during the transient fusion event (Fig. 1 *A* and *B*), since the capacitance measures the total area of the plasma membrane and the granule membrane (Fig. 1*C* II). The decreased plasma membrane area becomes visible only after the granule membrane, along with the transferred membrane, pinch off (Fig. 1*C* III). Another explanation for the cell membrane capacitance difference is that there is a decrease in capacitance per unit area, which might occur if the secretory granule membrane became closely juxtaposed with the plasma membrane (28); the capacitance would become that of three capacitors (one for each bilayer) in series, or one-third of the capacitance of a single bilayer. If the area of contact between the secretory granule and plasma membranes was one-half the total granule area, the capacitance could be reduced by an amount equivalent to one-third the granule capacitance, assuming that the contact was electrically tight. We have observed several transient fusion events in which the capacitance difference was 35–40% of the initial granule capacitance, which would require even more extensive areas of contact between the two membranes. However, large areas of contact between secretory granules and the plasma membrane are not seen in freeze-fracture electron micrographs of degranulating mast cells; extensive areas of contact seen in earlier studies turned out to be artifacts of the fixation technique (29).

Fig. 2 shows the transient fusion of two giant secretory granules in a mast cell from a mutant beige mouse. As in Fig.

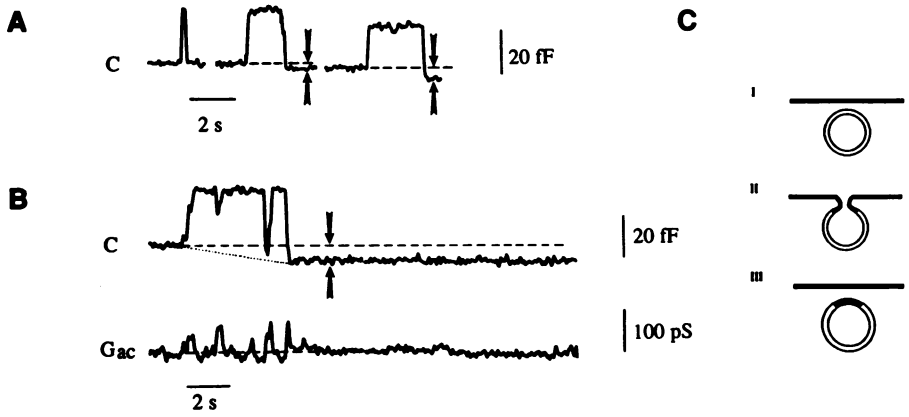

FIG. 1. The area of the plasma membrane is decreased after a transient fusion event. (*A*) Three granules that underwent transient fusions of different durations recorded in a mast cell from a wild-type mouse. Note that the magnitude of the backstep is larger than the initial step, indicating that the cell surface area is reduced after a transient fusion. (*B*) The C and G_{ac} components of the ac admittance contributed by a secretory granule during a transient fusion event. This event was recorded from a ruby eye (*Ru/Ru*) mouse, which has normal-sized secretory granules. Upon fusion there is a step increase in the C trace of 26 fF. Fluctuations in both the C and G_{ac} traces occur throughout the event; these fluctuations are due to changes in the fusion pore resistance, which can be calculated from the C and G_{ac} traces (20–23). After 5 s, the fusion pore collapses and there is a backstep of 34 fF, indicating a net loss of 8 fF from the plasma membrane capacitance measured prior to fusion. (*C*) Scheme showing a possible interpretation for the decreased plasma membrane area after a transient fusion event. Before fusion the capacitance gives a measure of the cell surface area (I). Upon fusion the granule is connected to the plasma membrane by a narrow-necked fusion pore (II) and the extra membrane comprising the granule membrane contributes to the measured capacitance. At this stage, some of the membrane from the plasma membrane is drawn into the granule because the granule has a higher membrane tension (see text for discussion). When the pore is disrupted at the end of the transient fusion event, the membrane that has been incorporated into the granule membrane remains there and the cell surface area is decreased (III).

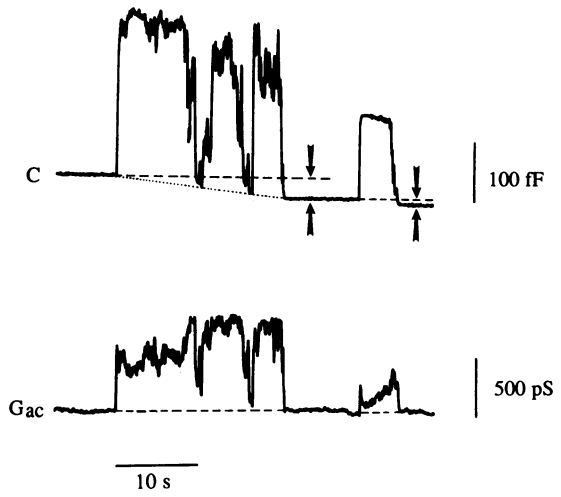

FIG. 2. C and G_{ac} components of the ac admittance contributed by giant secretory granules during two consecutive transient fusion events recorded from a beige mouse mast cell. Fusion of the granules with the plasma membrane is seen as an abrupt increase in both C and G_{ac} traces. The fusion pore undergoes large fluctuations in conductance and, in the first granule, two brief closures. The fluctuations in the fusion pore conductance result in much larger changes in the C trace for large granules than for small granules (compare with Fig. 1) because a proportionally larger fraction of the ac voltage drop will occur across the fusion pore (resistance) when the granule capacitance is large (see refs. 20–23 for equations and explanations). Closure of the fusion pore results in abrupt decreases in both C and G_{ac} traces. Note that the C trace returns to a new baseline well below the original baseline prior to fusion. The second transient fusion event reduces the baseline capacitance further. In these recordings the total granule capacitance cannot be determined with certitude because of the fluctuations in the fusion pore resistance (contrast with the smaller granules in Fig. 1). Thus, we cannot confirm if the sum of the vesicle and plasma membrane capacitances remains constant during the giant granule fusion events. It is clear, however, that after completion of the transient fusion event there is a decrease in cell membrane capacitance despite no significant change in G_{ac}, indicating a decreased plasma membrane area.

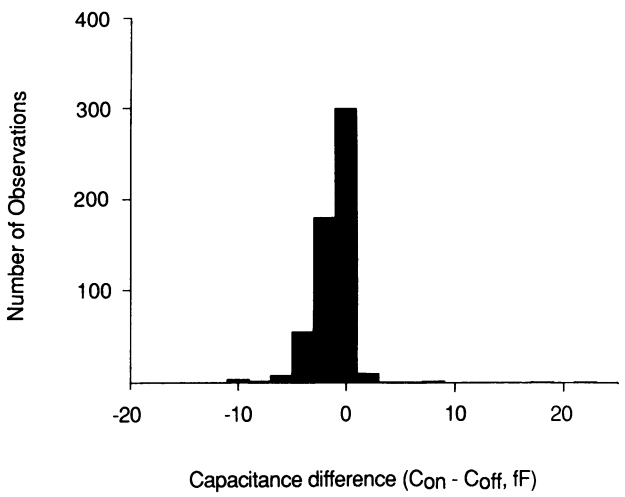

FIG. 3. Histogram showing the size distribution of the capacitance difference measured after transient fusion events in normal mast cells. The capacitance difference is the difference between the magnitude of the initial capacitance step (C_{on}) minus the backstep (C_{off}). The histogram, comprising measurements from 564 transient fusion events, is skewed to the left, showing that approximately one-half of the transient fusion events caused a decrease in the cell surface area. The remaining events were too short lived to produce membrane uptake (see Fig. 4). Only a few events showed a positive $C_{on} - C_{off}$ difference. Some of the events with a positive $C_{on} - C_{off}$ difference >10 f F could be explained by the irreversible fusion of an unrelated secretory granule during the transient fusion event.

1*B*, the fluctuations in the measured capacitance result from wide variations in the conductance of the fusion pore. However, for the same range of fusion pore conductances the effect of these variations on the capacitance trace is more pronounced in beige mast cells due to the much larger granule size (see Fig. 2 legend). Therefore, in experiments with beige mouse mast cells, the C trace does not reflect the full capacitance of the granule. For example, the slow decrease in the C trace during the transient fusion of the smaller granule in Fig. 2 is due to a gradual increase in the fusion pore resistance (data not shown), which results in a concomitant increase in the G_{ac} trace. However, it is evident from Fig. 2 that following the fusion of giant secretory granules there is a net decrease in plasma membrane area, similar to that observed in wild-type mast cells (Fig. 1).

The reduction in plasma membrane surface area observed after a transient fusion is not a rare event. As shown in Fig. 3, the frequency distribution histogram for the capacitance differences measured from 564 transient fusion events in normal mast cells shows a clear asymmetry. Although not all the transient fusion events show a change, a decrease in plasma membrane area is much more probable than an increase when a change occurs.

There is considerable variation in the amount of membrane transferred during a transient fusion event (Fig. 3). This is because the size of the capacitance difference (between the on and off steps) is proportional to the duration of the transient fusion event (Fig. 4). The time dependence can be seen clearly in the examples in Figs. 1 and 2. The slopes of Fig. 4 can be used to estimate that, for each second that the fusion pore exists, the cell surface area is reduced at rates of 0.16 $\mu m^2/s$ (n = 206; r = 0.87) and 0.17 $\mu m^2/s$ (n = 36; r = 0.98) for transient fusion events in cells from normal and beige mice, respectively. Given that an average phospholipid head group occupies an area of 0.5 nm^2 and counting both sides of the bilayer, we can calculate a rate of 6×10^5 phospholipid molecules per second for the membrane transfer. Surprisingly, the slopes in wild-type and beige mouse mast cells (Fig. 4) are almost identical. Since the beige mast cell granules are, on average, an order of magnitude larger than the granules of normal mast cells, the rate of decrease in plasma membrane area must be independent of the granule size.

The data presented above show that there is a time-dependent decrease in the cell surface area that occurs while secretory granules are transiently fused with the plasma membrane. The membrane removed from the cell surface is being transferred to the secretory granule, because in some events (for example, the granule in Fig. 1*B*) the fusion pore of a flickering granule collapses, revealing a decreased plasma membrane area, and then reopens to show the same total area for the cell surface plus the granule. Therefore, it is clear that during transient fusions there must be a connection between cell and granule membranes that allows movement of membrane to the secretory granule. Thus, it is likely that the fusion pore is partially or completely lipidic and that a lipidic fusion pore can close.

A straightforward explanation for the membrane transfer is that the secretory granule membrane is under tension. Then upon fusion the higher membrane tension of the secretory granule would make movement of phospholipid to the granule energetically favorable, as the surface pressure of the granule membrane is lower than that of the plasma membrane. A possible mechanism for generating the tension is osmotic swelling of the secretory granule matrix, a process that occurs after fusion pore formation (18, 20, 30, 31). Swelling of the granule matrix is due to water entry through the fusion pore and is thought to play an important role in dispersal of

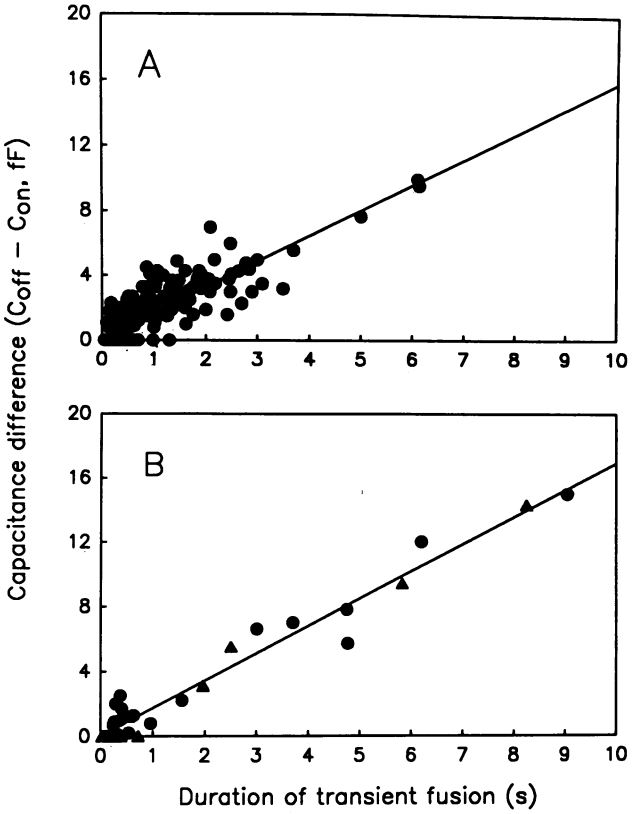

FIG. 4. Time dependence of the decrease in cell surface capacitance following a transient fusion event. (*A*) The C_{off} − C_{on} difference for 206 transient fusion events measured in mast cells with normal-sized secretory granules plotted against duration of the event. Only step sizes between 15 and 30 f F were used in this plot. (*B*) The C_{off} − C_{on} difference for transient fusions of 36 giant secretory granules from mast cells of the beige mouse. ●, Events from experiments in normal extracellular medium; ▲, events from experiments in an acidic histamine medium, which inhibits the rate of swelling of the secretory granule matrix after fusion by >95%. The rare transient fusion events in which the on step was larger than the off step (see Fig. 3) have not been included in this figure. The straight lines represent the linear regression line through the points. The slopes were 1.58 (n = 206; r = 0.860) and 1.70 (n = 36; r = 0.981) f F/s for the normal and beige mouse data, respectively.

secretory granule contents (30, 31). It has recently been shown that granule swelling can be reversed by acidic histamine solutions (32, 33). By using an isotonic acidic histamine solution, we can inhibit the extent and rate of granule swelling by 10- and 20-fold, respectively (44). Under these conditions, the rate of membrane uptake is unchanged (Fig. 4*B*, triangles). Therefore, since reducing the rate of swelling 20-fold does not change the rate of membrane uptake, swelling of the secretory granule matrix due to water entry through the fusion pore cannot be the driving force for the membrane transfer.

The uptake of membrane by the secretory granule is linear with time, which suggests that the membrane tension difference is constant throughout the transient fusion event. However, a bilayer can be stretched only by 3–5% without being ruptured (34). Since the amount of membrane transferred exceeds this value, the granule membrane cannot be stretched sufficiently prior to fusion to account for the observed membrane uptake, unless there are other elastic elements in parallel with the granule membrane. These would need a high modulus of elasticity to explain the linearity observed in Fig. 4. Alternatively, an early event during exocytosis might be stimulation of a mechanism that increases the membrane tension of the secretory granule prior to fusion and then maintains it at this value so as to produce the constant rate of membrane uptake.

Assuming that the secretory granule membrane is under tension, it is interesting to speculate as to the role of the tension. One possibility is that only granules that undergo transient fusions are under tension and that granules that fuse irreversibly are not under tension. The tension might be the mechanism responsible for terminating the fusion event. However, a more compelling role of tension in the mechanism of fusion is suggested by studies of bilayer fusion with model membranes, which have led to the proposal that an increase in membrane tension—i.e., an increased separation of the phospholipid head groups—results in an increased hydrophobicity of the membrane and reduces the strongly repulsive hydration forces that normally act to keep bilayer membranes apart (9–15, 35–37). Osmotic forces have been widely used to induce fusion of phospholipid vesicles and bilayers, secretory granules from chromaffin cells, and erythrocytes (6–11). Significantly, the osmotic gradients promote fusion of phospholipid vesicles with planar bilayers only under conditions in which the surface tension of the vesicles is increased due to osmotic or hydrostatic pressure (9–11). Likewise, other perturbations that induce fusion, such as raising the temperature or binding of divalent cations, have been shown to increase the membrane tension (12–15). Recently, it was shown that phospholipid bilayers applied to mica surfaces could be induced to fuse spontaneously 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 and increases the surface tension (35). The fusion induced by electric fields can be explained in a similar way since the electromechanical stress thins the membrane and increases the hydrophobicity (36, 37). Thus, it appears that protocols designed to induce membrane fusion, whether induced osmotically, electromechanically, or by membrane depletion, increase membrane tension and expose more hydrocarbon at the membrane surface. The entropy of the water in the intermembrane space is decreased, causing an increased attraction between the two bilayers leading to hemifusion and subsequently full bilayer fusion.

The evidence implicating tension in the mechanism of fusion of phospholipid bilayers along with the evidence shown here suggesting that the secretory granule membrane is under tension raise the intriguing possibility that an increased secretory granule membrane tension is a necessary, or facilitatory, factor for fusion. Thus, one can envisage a purely lipidic mechanism for exocytotic fusion, with the role of proteins restricted to that of orienting a tense secretory granule and the plasma membrane so as to favor fusion, although a further role for "fusion proteins" has to be considered likely. One proposal suggests that an early event in exocytotic fusion is the formation of a gap junction-like ion channel (4, 21, 23). Such a mechanism appears, at first, inconsistent with the membrane uptake phenomenon. However, the finding that alamethicin, a multisubunit ion channel, can support a high rate of phospholipid exchange between the leaflets of a planar bilayer when it is in the open state, but not when closed, led Hall (38) to propose that the alamethicin subunits are interspersed with phospholipid in the open state, thus providing a pathway for phospholipid exchange. Furthermore, formation and activation of the alamethicin channel are greatly enhanced by increases in the membrane tension (39). From the data given by Hall (38), the rate of phospholipid exchange can be calculated as 10^6 molecules per second per alamethicin channel (James Hall, personal communication), which is similar to the rate of 6×10^5 molecules per second for the membrane uptake calculated earlier. Thus, an alamethicin-like channel as the fusion pore could provide a mechanism for transfer of the membrane. A similar model

has recently been proposed with synexin, a Ca^{2+} binding protein that promotes fusion, as the ion channel (40, 41).

We have presented evidence that suggests that the secretory granule membrane is under tension. A possible mechanism for generating this tension is osmotic swelling of the proteoglycan core of the secretory granule. However, fusion of secretory granules has been demonstrated in high osmotic strength media in which the granules were presumably rendered flaccid prior to stimulation, indicating that membrane tension induced by osmotic forces does not participate in exocytotic fusion (18, 20). Moreover, the experiments with beige mouse mast cells in an acidic histamine extracellular medium, which reduces the rate of granule swelling by >20-fold, showed an identical rate of membrane uptake during the transient fusion events (Fig. 4*B*). Therefore, secretory granule swelling due to water entry through the fusion pore is not the origin of the membrane tension. Tension in the secretory granule membrane could be produced by a mechanical force acting externally upon the bilayer, even if the granules are initially flaccid. Such a force would have to be able to maintain the membrane tension at a constant value to explain the linearity of the membrane uptake during the reversible fusion events. The association of secretory granules with components of the cytoskeleton has been widely documented (42, 43). It is an interesting possibility that an early event in exocytosis is the stimulation of a sustained interaction of the secretory granule with cytoskeletal elements and that this interaction increases the membrane tension of the granule to a critical level necessary for fusion.

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