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Patch Clamp Studies of Single Intact Secretory Granules

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ABSTRACT The membrane of secretory granules is involved in the molecular events that cause exocytotic fusion. Several of the proteins that have been purified from the membrane of secretory granules form ion channels when they are reconstituted in lipid bilayers and, therefore, have been thought to form part of the molecular structure of the exocytotic fusion pore. We have used the patch clamp technique to study ion conductances in single isolated secretory granules from beige mouse mast cells. We found that the membrane of the intact granule had a conductance of <50 pS. No abrupt changes in current corresponding to the opening and closing of ion channels were observed, even under conditions where exocytotic fusion occurred. However, mechanical tension or a large voltage pulse caused the breakdown of the granule membrane resulting in the abrupt opening of a pore with an ion conductance of about 1 nS that fluctuated rapidly and could expand to an immeasurably large conductance or close completely. Surprisingly, the behavior of these pores resembled the pattern of conductance changes of exocytotic fusion pores observed in degranulating beige mast cells. This similarity supports the view that the earliest fusion pore is formed upon the breakdown of a bilayer such as that formed during hemifusion.

INTRODUCTION

Morphological and electrophysiological studies in mast cells have suggested that the earliest event in exocytosis is the formation of a small ion conducting pore, the fusion pore, that connects the plasma and vesicle membranes, and that leads to full fusion (Chandler and Heuser, 1980; Breckenridge and Almers, 1987a, b; Monck and Fernandez, 1992). The exocytotic fusion pore forms abruptly with an initial conductance that ranges between 50 and 1500 pS. After opening, the fusion pore can expand in a fluctuating manner to a conductance that exceeds 10 nS, leading to full fusion. Alternatively, the fusion pore can close completely after fluctuating in the open state over a wide range of conductances. The abrupt opening of the fusion pore, its initial conductance range, and its ability to close led to the proposal that, in its initial stages, the fusion pore was a proteinaceous ion channel composed of two halves, one in each membrane, similar to the gap junction protein connexin (Breckenridge and Almers, 1987a, 1987b; Spruce et al., 1990; Almers and Tse, 1990). However, the finding of a large flow of lipids through the open fusion pore and a rate of pore closure that had a discontinuous temperature dependence led to the suggestion that the fusion pore was entirely lipidic and formed through a hemifusion bilayer (Monck et al., 1990; Oberhauser et al., 1992a; Nanavati et al., 1992; Monck and Fernandez, 1992).

Biochemical studies have revealed many proteins associated with the membrane of secretory vesicles (Sudhof and Jahn, 1991). It has been proposed that some of these proteins form part of the fusion pore structure (Thomas et al., 1988; Knaus et al, 1990). Synaptophysin, for example, an abundant synaptic vesicle membrane component, exhibits a hydrophobic profile similar to the protein that forms the gap junction channel. Synaptophysin may form an ionic pore between the synaptic vesicle membrane and plasma membrane. Indeed, reconstitution studies have suggested that synaptophysin forms voltage-dependent cation channels (Thomas et al., 1988), similar to cation channels found in patch-clamped fused synaptic vesicles from *Torpedo* (Rahamimoff et al., 1989). Furthermore, ion channels have been found in secretory vesicle and granule membranes indirectly through reconstitution in artificial lipid bilayers. These include anion selective channels from bovine neurohypophysis granules (Stanley et al., 1988; Lemos et al., 1989) and *Torpedo* electric organ synaptic vesicles (Rahamimoff et al., 1988), and cation selective channels from bovine neurohypophysis granules (Lee et al., 1992), *Torpedo* electric organ synaptic vesicles (Rahamimoff et al., 1989), rat adenohypophysis secretory granules (Balden et al., 1991), adrenal chromaffin granules (Arsipe et al., 1992), and rat peritoneal mast cell granules (Hirashima and Kirino, 1992). It was proposed that some of the ion channels found in the secretory vesicles may be part of the fusion pore structure (Rahamimoff et al., 1989; Lemos et al., 1989; Lee et al., 1992). Even if these ion channels do not form an integral part of the fusion pore it has been proposed that they may promote bilayer fusion by increasing the osmolarity of the vesicle and, thus, its membrane tension (Pollard et al., 1979; Stanley and Ehrenstein, 1985; Uvnas and Aborg, 1989; Lemos et al., 1989).

In this work, we have used the patch clamp technique to study the ionic conductances present in isolated intact mast cell granules in an attempt to identify components and modulators of the exocytotic fusion pore. For these experiments we have used peritoneal mast cells from beige (bg^j/bg^j) mice that have giant secretory granules (≤ 5 μm diameter) and where all of the measurements of the exocytotic fusion pore have been made. In early experiments, upon establishing a connection between the patch pipette interior and the lumen of an isolated secretory granule ("whole-granule configuration"), exposure of the granule matrix to the Na^+- or K^+-containing solution resulted in an explosive swelling of the

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granule core, breaking the seal between the membrane and the glass pipette. We have recently shown that acidic histamine solutions prevented the swelling of the secretory granule matrix without any effect on the formation and development of fusion pores (Monck et al., 1991). We have used patch pipettes filled with a histamine solution at acidic pH allowing continuity between the pipette solution and the granule interior without any measurable swelling, allowing sustained recordings in the "whole-granule" configuration. We found that the membrane of the intact granule did not have spontaneously active ion channels under conditions where exocytotic fusion is observed. We also observed that an electrical or mechanically induced breakdown of the granule membrane caused the abrupt opening of a pore with an ion conductance of about 1 nS that expanded in a fluctuating manner and that could close. Surprisingly, the pores caused by membrane breakdown resemble the exocytotic fusion pores observed in degranulating mast cells. These observations are consistent with the hypothesis that proposes that the earliest fusion pore is a lipidic pore that forms upon the breakdown of a hemifusion bilayer (Nanavati et al., 1992; Monck and Fernandez, 1992). A preliminary report of this work appeared in abstract form (Oberhauser and Fernandez, 1992).

MATERIALS AND METHODS

Secretory Granules

Mast cell secretory granules were prepared from beige mice, a mutant with the Chediak-Higashi defect (bg^j/bg^j strain, Jackson Laboratories, Bar Harbor, ME) after a procedure described elsewhere (Monck et al., 1991), with some minor modifications that were found to increase the number of intact isolated secretory granules. Briefly, cells were obtained by peritoneal lavage with a solution of the following composition (in millimolar): 136 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 22 $NaHCO_3$, 0.4 K_2HPO_4, 2 glucose, 8.8 units/ml heparin, 0.1% bovine serum albumin (300 mOsm/kg, pH 7.3). The cells were resuspended in 1 ml and layered on 2 ml of 22.5% w/v metrizamide and centrifuged at room temperature for 20 min at 400 *g*. The pellet was resuspended in 1 ml of a Ca^{2+}, Mg^{2+}-free sonication buffer of the following composition (in millimolar): 130 NaCl, 10 KCl, 22 $NaHCO_3$, 0.3 K_2HPO_4, 0.1% bovine serum albumin (300 mOsm/kg, pH 7.3). This suspension of purified mast cells was subjected to four sonication pulses at 25% of maximum power (sonicator model 45; Branson Sonic Power Co., Danbury, CT) and plated onto glass-bottomed chambers and stored at 37°C under 5% CO_2 atmosphere until use. An average of about 200 intact secretory granules per mouse were routinely obtained.

Solutions

The secretory granules were bathed in a standard solution containing (in millimolar): 25 NaCl, 125 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 0.2 ATP, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (300 mOsm/kg, pH 7.3). The standard pipette solution for patch-clamping secretory granules contained (in millimolar): 110 histamine dihydrochloride, 10 $CaCl_2$, 7 histamine, 5 citric acid (330 mOsm/kg, pH 3.5). The histamine concentration and pH of these solutions were shown to inhibit swelling of granule matrix (Monck et al., 1991; Fernandez et al., 1991; Curran and Brodwick, 1991). No monovalent cations could be used, since they were found to prevent long-lasting whole-granule recordings, due to swelling of the granule core.

The standard external solution for whole mast cell recordings was (in millimolar): 130 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES (300 mOsm/kg, pH 7.3). The pipette solution contained (in millimolar): 140 potassium glutamate, 7 $MgCl_2$, 10 EGTA, 0.2 ATP, 10 μM guanosine 5′-3-*O*-(thio)triphosphate (GTPγS), 10 HEPES (290 mOsm/kg, pH 7.2), and various amounts of $CaCl_2$ to buffer free Ca^{2+} between 30 nM and 2 μM. Although the majority of the isolated granule experiments were done in the standard extragranular solution, replacement of the granule bathing solution with this low free Ca^{2+} pipette solution did not change the results.

Patch clamp recording

Due to their small size, patch clamping of secretory granules required small Sylgard-coated, fire-polished pipettes (15–30 MΩ). However, since the input impedance of a granule was typically very high (>10 GΩ), the pipette resistance remained negligible. Changes in membrane capacitance and conductance were followed with a digital phase detector (Joshi and Fernandez, 1988). The phase detector was set to a phase angle such that the phase delays introduced by the recording electronics were compensated and only changes in the admittance of the granule could be recorded. So one output of the phase detector (at an angle ϕ-90 from the stimulus) reflected the real part of the changes in the A.C. admittance (Re(ΔY)) and the second output reflected the imaginary part (Im(ΔY)). Since patch-clamped granules can be approximated by a RpCg equivalent circuit (see Fig. 1 *A*), Im(ΔY) measures changes in membrane capacitance only, and Re(ΔY) measures resistive changes. Every admittance determination was obtained by doing the phase detection over a period of eight sinusoidal cycles (54 mV peak-to-peak, frequency 833 Hz). A calibration signal for the capacitance trace was obtained by unbalancing the C-fast potentiometer of the patch-clamp amplifier (EPC-7, List Elect) by 1 pF. We routinely confirmed that the increase in the input capacitance of the patch pipette was due to the granule membrane by comparing its value with that predicted from the measured diameter of the granule (using a conversion factor of 10 fF/μm^2). In most experiments, the D.C. current was obtained by sampling the current monitor output of the patch clamp amplifier simultaneous with the admittance measurements and computing its average value during the eight sinusoidal cycles (one point every 9.6 ms). In order to increase the time resolution, in some experiments only the D.C. current was sampled (1 kHz). The pipette holding potentials were not corrected for junction potentials, which varied between 7 and 21 mV.

The breakdown of the membrane patch under the pipette in a patch-clamped granule created a pore that connected the lumen of the secretory granule with the pipette interior. Since these pores resembled the exocytotic fusion pore we wanted to study their formation with high time resolution. Thus we applied a technique that can capture changes in pore conductance with microsecond time resolution. The patch pipette D.C. current was continuously monitored upon establishment of a gigaseal, low-pass filtered at 10 kHz (eight-pole Bessel), and sampled at 100 kHz. A threshold detector was set two times above the noise level (about 3 pA peak-to-peak). If a point crossed the threshold the next 924 points and the preceding 100 points were stored (one record of 1024 points). If such an event was detected, the membrane capacitance was measured, using the phase detection method, by superimposing a burst of 16 periods of sinusoidal waves (54 mV peak-to-peak, frequency 833 Hz) onto the holding potential.

The plasma membrane capacitance and conductance of beige mast cells were measured using the whole cell mode of the patch clamp technique and the standard phase detection method. The correct phase detector angle was obtained by using the phase tracking method (Fidler and Fernandez, 1989). Voltage-ramp stimuli were often used to assess the membrane conductance rapidly. The voltage was ramped from −100 to +100 mV within 225 ms every 2 s (sampling rate, 300 μs per point). The command potentials were corrected for junction potentials. All experiments were done at room temperature (22–24°C). Measurements give mean ± S.E.

RESULTS

Patch clamp of secretory granules of beige mouse mast cells

Fig. 1 *A* shows a Nomarski optics photograph of a patch pipette sealed to the membrane of an isolated secretory

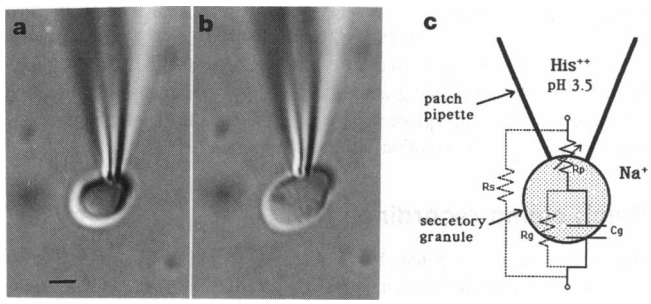

FIGURE 1 Patch clamp of isolated secretory granules of beige mouse mast cells. (*A*) Nomarski optics photograph showing a patch pipette sealed to a secretory granule, just after obtaining the "whole-granule" recording mode. The pipette contains an acidic histamine solution that prevents the swelling of the granule core. Bar = 1 μm. (*B*) The same secretory granule after a large voltage pulse (400 mV, 10 ms) porated the granule membrane, communicating the lumen of the granule with the Na^+- and K^+-containing bathing solution. (*C*) Electrical equivalent circuit diagram of a patch-clamped granule. R_s is the resistive leakage pathway between the granule membrane and the pipette tip; R_p is the access resistance into the granule through the membrane patch isolated by a sealed pipette; R_g is the whole-granule membrane resistance; and C_g the granule capacitance.

granule. The recording is being made in the "whole secretory granule" mode where the pipette solution was in contact with the lumen of the secretory granule. Due to the acidic histamine pipette solution, the secretory granule retains a condensed proteoglycan core and an intact membrane. This pipette solution imitates the ionic environment inside the secretory granule (Alter and Schwartz, 1989), and has been shown to prevent the swelling of the granule matrix without any effect on the formation and development of fusion pores (Monck et al., 1991). Fig. 1 *B* shows the same secretory granule after a large voltage pulse (400 mV, 10 ms) porated the membrane, connecting the lumen of the granule with the Na^+- and K^+-containing bathing solution. Similar to what is observed during exocytosis, exposure to the Na^+- or K^+-containing saline causes a violent expansion of the granule core resulting in the destruction of the granule membrane.

Fig. 1 *C* shows an electrical equivalent circuit diagram of a patch-clamped granule. The mean impedances for each element in this model circuit were estimated from the granule-attached resistance and the whole-granule resistance. The leak resistance, R_s, was 110 ± 38 GΩ (n = 9); the whole-granule membrane resistance, R_g, was 47 ± 12 GΩ (n = 9); upon formation of a pore, the patch resistance, R_p, changed abruptly to a value of about 1 GΩ which then decreased to several megaohms. In comparison, the membrane and leak conductances were considered negligible (*dotted lines*) and therefore a patch-clamped granule can be approximated by a series combination of R_p and C_g.

Beige mouse mast cell secretory granules do not contain spontaneously active ion channels

Like the "whole-cell" mode of the tight-seal patch clamp technique (Hamill et al., 1981), our experimental protocol depended on the measurement of the capacitive current to determine whether the current recordings were from the entire membrane of the secretory granule or from a small membrane patch (Fig. 1 *A*). The expected increase in capacitance, however, is small (100–300 fF). To detect these changes in capacitive current, we monitored the admittance of the patch pipette using a dual channel phase-sensitive detector operating identically to the mode used to monitor exocytotic secretion (Joshi and Fernandez, 1988).

Fig. 2 shows a typical experiment. The majority (69 of 87) of the gigaseals formed between the glass pipette and the granule membrane occurred spontaneously without application of suction to the pipette interior. The figure shows the time course of the components of the measured admittance (capacitance and A.C. conductance) during the transition from "granule-attached" to "whole-granule" recording.

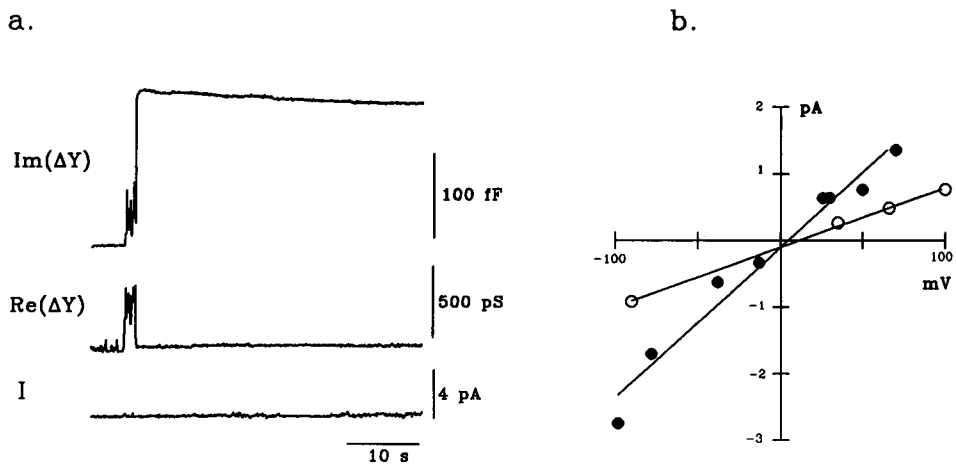

FIGURE 2 Whole secretory granule electrical recordings. (*A*) After about 30 s of obtaining a gigaseal, the capacitance (Im(ΔY)) and A.C. conductance (Re(ΔY)) of the membrane patch increased spontaneously. The measured increase in membrane capacitance (193 fF) was similar to the capacitance estimated from the granule diameter (2.6 μm). Throughout this experiment the holding potential was 36 mV. (*B*) Current versus voltage plots before (*open circles*) and after the transition to whole-granule recording (*filled circles*) of the experiment shown in *A*. The slopes correspond to a conductance of 8.6 pS in the granule-attached configuration and to 23.2 pS in the whole-granule configuration.

These large changes in both components of the admittance were due to an abrupt decrease in the access resistance, R_p, without accompanying changes in either the seal resistance, R_s, or the granule membrane resistance, R_g. In the whole-granule configuration the membrane of the intact secretory granule was studied at different membrane potentials (Fig. 2 *B*). In this example, the granule membrane had a conductance of about 20 pS. The mean slope was 21.6 ± 7.0 pS (n = 5), in standard recording solutions. These recordings did not show any ion channel activity such as voltage-dependent conductances or abrupt conductance changes corresponding to the opening or closure of single ion channels (1-kHz sampling frequency, peak-to-peak noise ~0.6 pA).

Most of the ion channels that are thought to be present in secretory vesicles and granules should have been detectable under the conditions of the experiment shown in Fig. 2, since the extragranular solution contained K^+, Na^+, Ca^{2+}, and Cl^-. Chloride channels of about 30 pS would give fluctuations of current of about 2 pA at a holding potential of −80 mV (Rahamimoff et al., 1988; Stanley et al., 1988; Lemos et al., 1989), and a single cation channel of 80 pS would give a current of about 4.6 pA at −60 mV and 3.8 pA at +80 mV (Rahamimoff et al., 1989; Hirashima and Kirino, 1992). We also studied the ion conductance of the membranes of beige mouse mast cell secretory granules bathed in a K^+-glutamate saline containing 10 μM GTPγS, 200 μM ATP with 30 nM free Ca^{2+} or 2 μM free Ca^{2+}. These solutions invariably triggered exocytotic degranulation of mast cells minutes after entering the cytosol through the patch pipette. The mean slope conductance of the whole-granule membrane was 14.1 ± 3.0 pS (n = 3) in 30 nM free Ca^{2+} and 13.2 pS (n = 2) in 2 μM free Ca^{2+}, with no detectable fluctuations in conductance typical of ion channels (data not shown). This result suggests that secretory granules from the beige mouse mast cell do not have Ca^{2+}-activated cation channels. These results contrast those of Hirashima and Kirino (1992) who reconstituted a Ca^{2+}-activated cation channel in lipid bilayers that was thought to originate from the membrane of mast cell granules. If there are ion channels in the granule membrane that play a role in the mechanism of exocytosis, they should become apparent in whole-cell recordings as the secretory granules fuse with the cell membrane. Fig. 3 *A* shows a simultaneous measurement of the membrane capacitance and the D.C. current of a patch-clamped beige mouse mast cell. The pipette solution included 2 μM free Ca^{2+} to activate putative Ca^{2+}-activated ion channels in the secretory granules. As Fig. 3 *A* shows, the incorporation of secretory granule membranes to the plasma membrane (detected as discrete increases in the membrane capacitance) is not associated with a measurable increase in the D.C. current at a holding potential of 40 mV. We searched for ion channel openings at different potentials by recording the current in response to a voltage ramp. Four ramps 225 ms in duration were elicited every 2 s, about 30 s after obtaining the whole-cell configuration and throughout a degranulation. Fig. 3 *B* shows that the membrane conductance before (*left*) and after (*right*) the fusion of all the secretory granules with the plasma membrane, is very similar (98 and 91 pS, respectively). Similar results were obtained in four other cells, where an exocytotic increase of the plasma membrane area by a factor of about two (1.8 ± 0.23) did not significantly increase the cell membrane conductance. The mean conductance and reversal potential values before degranulation were 95 ± 11 pS and 35 ± 8 mV, and 93 ± 9 pS and 30 ± 10 mV after degranulation (n = 5). Evidently, exocytotic fusion in beige mouse mast cells occurs without a significant activation of ion channels in the cell membrane, and the membrane of the secretory granules contains no open ion channels. We cannot rule out that under different ionic conditions, the activity of ion channels might become apparent. However, their relevance to exocytosis would not be obvious.

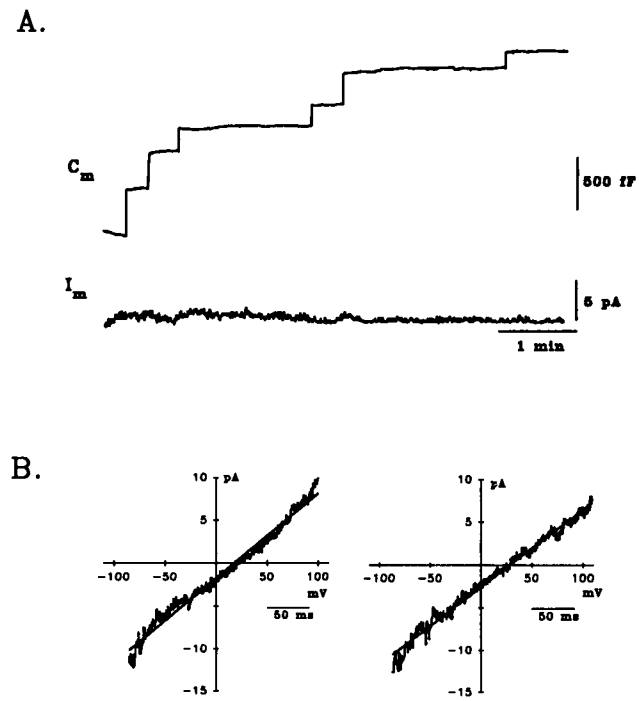

FIGURE 3 Incorporation of secretory granule membranes to the plasma membrane does not increase the whole-cell membrane conductance. (*A*) Cell membrane capacitance and D.C. current changes of a degranulating mast cell. The traces start about 2 min after the whole-cell configuration was established. Each step increase in capacitance represents the exocytosis of a single secretory granule. The pipette solution included 2 μM free Ca^{2+}, and 10 μM GTPγS. The holding potential was +40 mV. (*B*) Whole-cell conductance before (*left*) and after (*right*) degranulation of the same cell. The lines represent a linear fit to the data with a slope of 98 pS (before any fusion event) and 91 pS (after degranulation).

Pores induced by an electromechanical perturbation of the membrane of secretory granules

A mechanical stress or a large voltage pulse applied to the granule membrane triggered the opening of an ion conductance with the characteristics of membrane breakdown. Fig. 4 shows the opening and time course of the pores that form in the granule attached membrane patch upon breaking into

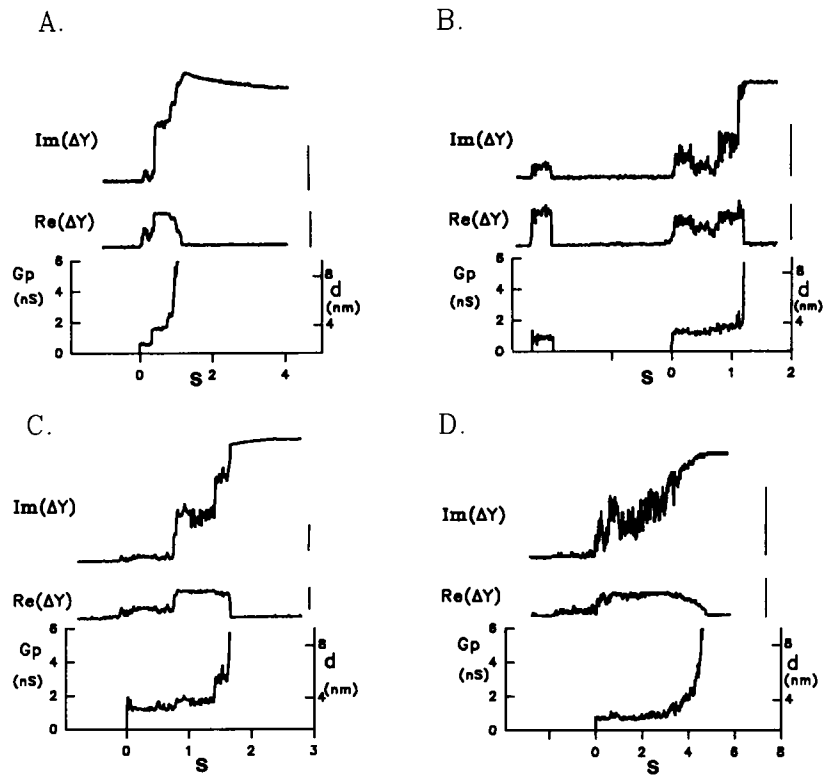

FIGURE 4 Breakdown pores induced in the secretory granule membrane (*A–D*). From top to bottom the figures show a continuous recording of the capacitance (Im(ΔY)), the A.C. conductance (Re(ΔY)) and the computed value for the pore conductance (G_p), during the transition from granule-attached to whole-granule recording. After 94 s (*A*) or 32 s (*B*) of obtaining a gigaseal, G_p increased spontaneously and abruptly and then fluctuated until its value increased to infinity (marked by the sudden decrease of the Re trace to its baseline value). (*C* and *D*) Pores in the granule membrane patch induced by electrical stress. The potential required to induce these pores was 160 mV in *C* and 130 mV in *D*. Right-hand ordinate, pore diameter calculated from Equation 8-1 of Hille (1984), for a resistivity of 100 Ω-cm, and a pore length of 4 nm. The bars represent the calibration for Im(ΔY) and Re(ΔY), 100 fF and 500 pS, respectively.

the "whole-granule mode." Within a few minutes (1.9 ± 0.5 min, n = 31) of obtaining a tight seal, the input admittance of the pipette changed abruptly (Fig. 4, *A* and *B*) without a measurable change in the D.C. current (not shown). This sudden increase in both components of the admittance corresponds to the opening of a pore in the membrane patch connecting the lumen of the secretory granule with the pipette interior. The pore conductance, G_p, is shown in the bottom trace and was calculated as $G_p = (\omega C_g)$ Im(ΔY)/Re(ΔY) (Nanavati et al., 1992). Most of the pores formed spontaneously (31 of 37) and began abruptly with a conductance of about 1.5 nS (mean value of the first 30 ms of the changes in admittance: 1.44 ± 0.22 nS, n = 22) and then expanded, typically within one second, to a conductance of more than 8 nS. In the example shown in *B* a pore opened transiently before expanding irreversibly. The stimulus that triggers the formation of these pores is likely to be the strong mechanical tension that develops in the patch of membrane when a gigaseal is obtained (Sokabe et al., 1991).

In some cases, pores did not form even after about 5 min of obtaining a gigaseal (Fig. 4, *C* and *D*). In these cases, a voltage pulse (variable amplitude, 10 ms long), was applied in order to electroporate the membrane patch. The potential required to induce these pores was 168 ± 13 mV (n = 6) and had an initial conductance of 1.31 ± 0.14 nS (n = 4). The pattern of conductance changes for pores induced by a voltage pulse were similar to those of the pores that formed spontaneously. Interestingly, in some cases several step increases in conductance were observed (Fig. 4*A*). These may correspond to the activity of a single pore or to the formation of several pores in parallel. We cannot distinguish among these possibilities. However, for simplicity, we attribute the entire conductance to a single dilating pore spanning one bilayer (4 nm). These pores have a calculated initial diameter of about 2 nm, corresponding to the diameter of a pore lined by only 10 phospholipid headgroups.

Pores, similar to those formed in the patch of granule membrane trapped by the tip of the pipette, were also observed in the intact membrane of the granule in the whole-granule recording mode. The mean conductance of these pores was 870 ± 193 pS (n = 8), and they opened abruptly after applying a pulse of about 400 mV (430 ± 80 mV, n = 8). The voltages required to electroporate the whole-granule membrane was about three times larger than the voltages needed to porate a patch of granule membrane. One possible explanation for this difference is that in whole-granule recording a bigger voltage drop across the access resistance reduces the voltage applied to the granule membrane. However, since the granule impedance remains very high, we think that this is unlikely. We favor the interpretation that these differences can be explained if the stimulus that opened the pores is membrane tension. Both an isotropic tension or the compressive force of a transmembrane voltage can induce tension in a membrane and their effect is additive (Needham and Hochmuth, 1989). Since the membrane patch is known to be under mechanical tension (Sokabe et al., 1991), a small increase in tension generated by a voltage pulse may be enough to reach the critical amount of membrane tension at which a

pore forms (O'Neill and Tung, 1991). Furthermore, in most cases if the mechanical tension of the patch is large enough, no voltage pulse would be required. In contrast, the granule membrane that is not subjected to the mechanical tension imposed by the pipette tip will require a larger voltage pulse to reach an equivalent membrane tension. Thus, as in the case for all biological membranes, membrane tension resulting from a mechanical or electrical stimulus can cause membrane breakdown, and the formation of pores in the granule membrane patch (Needham and Hochmuth, 1989). What is surprising, however, is the striking similarity between the breakdown pores and the exocytotic fusion pores of beige mast cells.

Breakdown pores are similar to exocytotic fusion pores

The electromechanical breakdown of biological membranes has been shown to be a very fast event (<30 ms (O'Neill and Tung, 1991)). Since the admittance measurements are slow (9.6 ms per sample), a different method was used to measure the initial pore conductance at a fast sampling rate (10 μs) (Breckenridge and Almers, 1987a; see Methods). A current transient appears when a pore establishes an electrical connection between the pipette solution and the lumen of the secretory vesicle. This transient is the result of the movement of charges necessary to make the inside of the granule equipotential with the pipette interior. In order to capture this current transient, the patch pipette D.C. current was continuously monitored upon establishment of a gigaseal. A threshold detector was set two times above the noise level. If a point crossed the threshold, the current trace was saved and the membrane capacitance was measured.

Fig. 5 shows a current transient through a breakdown pore. The pore conductance is calculated from the discharge current I, and the potential difference driving I ($E_h - E_g$). Where E_g is the transgranule potential (outside minus inside) and E_h is the pipette holding potential. $E_h - E_g$ is calculated from the time integral of I, divided by the capacitance of the granule membrane, C_g. The conductance of the pore, G_p, was then calculated as $I/(E_h - E_g)$. The initial conductance of the pore, G_o, is obtained from the amplitude (I_o) of the transient divided by the initial value of ($E_h - E_g$). In this example G_o was 935 pS (E_h = 60 mV) and the difference in capacitance before and after the current transient was 290 fF. The calculated pore conductance shows that electromechanical pores begin abruptly (<20 μs) with a conductance of about 1 nS (1.12 ± 0.5 nS, n = 3). This value is slightly smaller than the initial conductance measured with the admittance method (1.37 ± 0.18 nS, n = 28; calculated from the first 30 ms of the changes in admittance). Once the pore had opened, its conductance increased at a relatively slower, but highly variable rate (200–1800 pS/ms). These conductance patterns are strikingly similar to exocytotic fusion pores (Spruce et al. 1990).

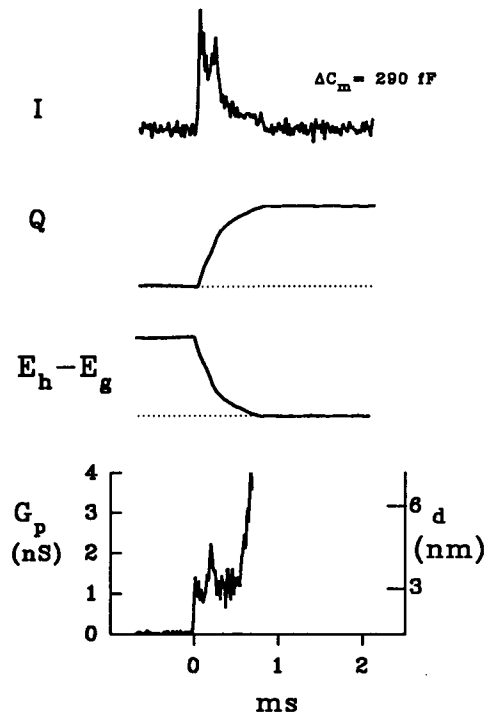

FIGURE 5 Measurement of the initial conductance of a breakdown pore in a membrane patch of a secretory granule. The figure shows a current transient (*upper trace*) that marks the formation a pore connecting the lumen of the secretory granule and the pipette solution. The pore conductance was calculated as detailed in the text.

Fig. 6 shows the time course of the conductance of three different breakdown pores: a pore that expands rapidly; a pore that expands more slowly; and a pore that opens and then closes (Fig. 6, *a*, *b*, *c*, respectively). Similar recordings were obtained from exocytotic fusion pores measured in beige mouse mast cells undergoing exocytosis (Fig. 6, *d–f*). Like exocytotic fusion pores, breakdown pores can expand either rapidly, slowly in a fluctuating manner, or they can form transiently.

The mean expansion time for breakdown pores, defined as the time between the first increase in conductance and their expansion to an immeasurably large conductance (i.e., the duration of the transient increase in the Re trace as shown in Fig. 4), was about 800 ms (834 ± 224 ms, n = 26). Exocytotic fusion pores had mean expansion times of about 400 ms (420 ± 80 ms, n = 545; see also Monck et al., 1991). However, the mean expansion time is misleading because of the large variation in expansion times and their non-Gaussian distribution. After plotting the data as frequency distributions, we found that the fitted time constants were similar for both types of pores (250 ms for electromechanical pores and 190 ms for exocytotic fusion pores). The mean pore conductance calculated during the expansion time for breakdown pores and exocytotic fusion pores were also similar, being 2.83 ± 0.87 nS (n = 26) and 3.76 ± 0.38 nS (n = 72), respectively. This striking agreement may be pure coincidence resulting, for example,

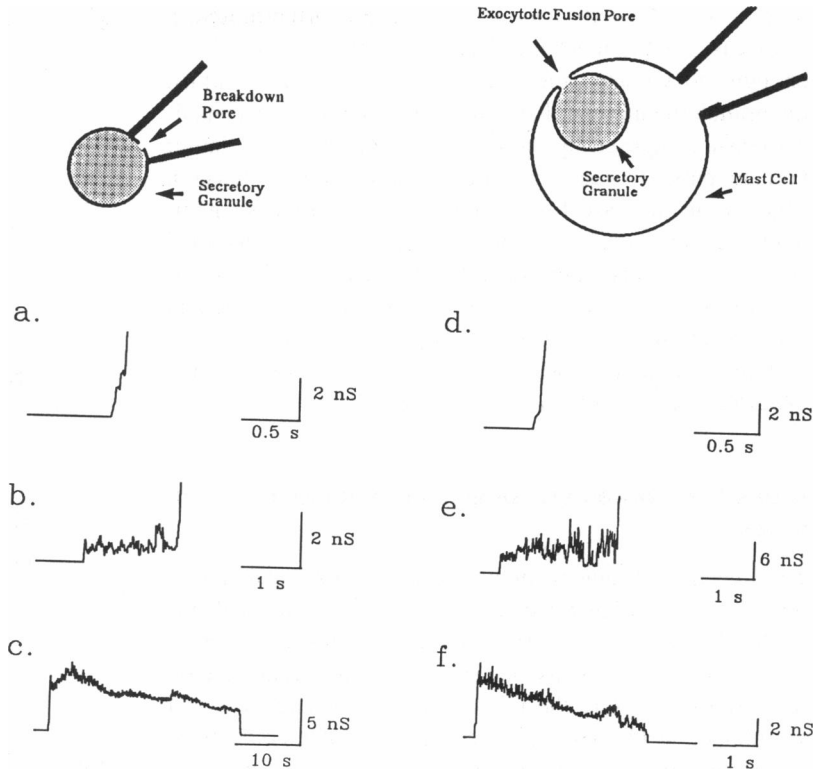

FIGURE 6 Breakdown pores in the secretory granule membrane patch and exocytotic fusion pores share common patterns of ion conductances. Examples of the time course of the ion conductance of breakdown pores (*A–C*) and exocytotic fusion pores (*D–F*).

from a fortuitous choice of membrane tension that may not be equivalent to that generated during fusion. Alternatively, breakdown pores accurately reproduce the physical events that take place during the opening of an exocytotic fusion pore.

DISCUSSION

We have used the patch clamp technique to study the electrical properties of isolated secretory granules from beige mouse mast cells. Our data shows that extragranular solu-

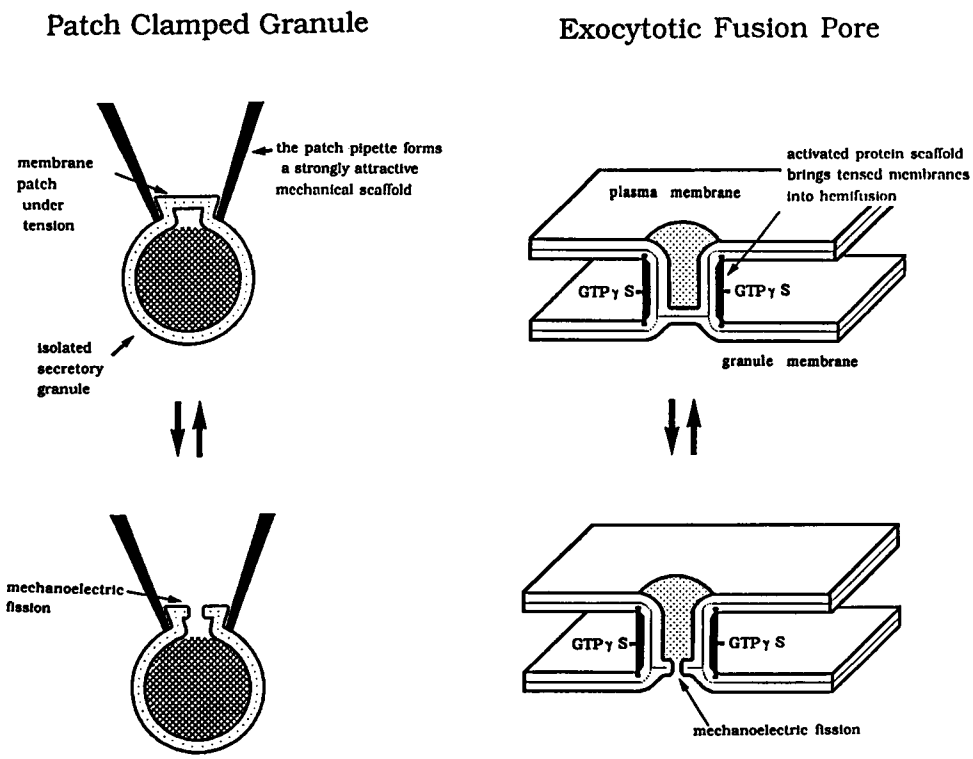

FIGURE 7 Model that explains the similarities between breakdown pores in the granule membrane and exocytotic fusion pores. See text for details.

tions that degranulate patch-clamped beige mouse mast cells are ineffective in activating ion channels in the granule membrane, making it unlikely that open ion channels in the granule membrane play a role in the mechanism of exocytosis in beige mouse mast cells. Moreover, if open ion channels in the vesicle membrane are involved in the molecular mechanism underlying exocytosis, then manipulations that change the electrochemical or osmotic gradients between cytosol and lumen of vesicle should affect the probability of fusion. This is clearly not the case in beige mouse mast cells, since fusion is seen to precede swelling of the secretory granules (Breckenridge and Almers, 1987a), and the osmolality of the cytosol (150–640 mOsm/kg) does not significantly affect the kinetics of exocytosis (Brodwick et al., 1992). Together, these findings rule out the chemiosmotic hypothesis of secretion (Pollard et al, 1979; Stanley and Ehrenstein, 1985; Uvnas and Aborg, 1989; Lemos et al., 1989). However, our experiments do not exclude the possibility that ion channels in the granule membrane of beige mouse mast cells could open transiently just before exocytotic fusion or in response to yet untried saline mixtures.

The membrane tension generated by the deformation of the granule membrane by the patch pipette or by the large voltage pulses created pores in the granule membrane that most likely resulted from membrane breakdown (Needham and Hochmuth, 1989). Recent experiments using rapid-freezing techniques have demonstrated that electrical membrane breakdown creates well-defined, discrete physical pores of 20–120 nm in diameter and that these pores form and expand rapidly but can also remain stable for several seconds before closing (Chang and Reese, 1990). It is widely known that pores resulting from membrane breakdown can be induced in all membranes (Benz and Zimmerman, 1981; Chernomordik et al., 1987; O'Neill and Tung, 1991). Our most important finding is that the breakdown of the granule membrane caused by an electric or a mechanical stress triggers the formation of a pore or pores that closely resemble the pattern of conductance changes of exocytotic fusion pores observed in degranulating beige mast cells. They have similar expansion times and conductance levels during their expansion times. Also the initial conductance of breakdown pores (about 1 nS) is in the range of the initial conductances measured for exocytotic fusion pores (range: 50 pS to 1.5 nS; mean: 329 $\pm$ 8 pS, n = 620 (Spruce et al., 1990)).

Fig. 7 shows a diagram that explains our interpretation of the similarities in conductance patterns between breakdown pores in the granule membrane and exocytotic fusion pores. In the case of a patch-clamped granule (*left*) the spontaneous invagination of the membrane caused by the attractive interaction with the glass will create an isotropic tension along the surface of the membrane patch. When the area is increased to a critical value, membrane breakdown occurs creating a pore. Similarly, during exocytotic secretion (*right*) an activated "scaffold" of proteins tenses the opposing membranes (Monck and Fernandez, 1992). The strong attractive force generated by the active scaffold brings the membranes to coalesce and form a "hemifusion" intermediate. Membrane fusion is completed by the formation of an aqueous pore after rupture of the shared bilayer. Thus, our results provide strong support to the view that the ion-conducting part of the exocytotic fusion pore is a small water-filled pore through a single bilayer and is gated open by isotropic tension. This interpretation is also supported by recent work showing that the exocytotic fusion pore can be modeled as a lipidic structure spanning a single shared bilayer (Nanavati et al., 1992; Monck and Fernandez, 1992). For example, Nanavati et al. (1992) showed that the range of conductances predicted for such pores is very similar to those measured for exocytotic fusion pores. Furthermore, they predicted that such lipidic pores could open abruptly, fluctuate, and expand irreversibly or close. This hypothesis is also consistent with the idea that full membrane fusion proceeds through a hemifused bilayer state (Helm et al., 1989; Burger and Verkleij, 1990; Song et al., 1991).

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