Arrest of Membrane Fusion Events in Mast Cells by Quick-freezing

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ABSTRACT   We have used quick-freezing and freeze-fracture to study early stages of exocytosis in rat peritoneal mast cells. Mast cells briefly stimulated with 48/80 (a synthetic polycation and well-known histamine-releasing agent) at 22°C displayed single, narrow-necked pores (some as small as 0.05 μm in diameter) joining single granules with the plasma membrane. Pores that had become as large as 0.1 μm in diameter were clearly etchable and thus represented aqueous channels connecting the granule interior with the extracellular space. Granules exhibiting pores usually did not have wide areas of contact with the plasma membrane, and clearings of intramembrane particles, seen in chemically fixed mast cells undergoing exocytosis, were not present on either plasma or granule membranes. Fusion of interior granules later in the secretory process also appeared to involve pores; granules were often joined by one pore or a group of 2-4 pores. Also found were groups of extremely small, etchable pores on granule membranes that may represent the earliest aqueous communication between fusing granules.

Ultrastructural studies have shown that histamine secretion by mast cells, in response to either antigens or synthetic polycations, is mediated by a rapid, compound exocytosis (1, 3, 15, 16, 18, 19, 28). Early in the secretory process, single granules fuse with the plasma membrane, whereas at later stages membranes of adjacent granules in the cell interior fuse in succession to produce deep invaginations in the cell surface (1, 19, 28). These morphological observations correlate well with chemical measurements of release showing that 40-80% of the total cell histamine is secreted within 2 min (3, 28). There is good evidence that secretion is initiated by a rise in intracellular calcium activity (9, 10, 16, 17, 22), and for this reason the mast cell represents an important model of calcium-mediated stimulus-secretion coupling (8, 12).

This rapid exocytotic response of the mast cell has proven extremely useful for studying membrane fusion because numerous fusion events occur within a short period; electron microscopy studies have already demonstrated what appear to be preliminary stages in this process. In thin sections of stimulated mast cells, granule membranes often contact the plasma membrane at sites where the intervening cytoplasm has been expressed (7, 21), forming what Palade and Bruns (25) termed a "pentalaminar figure." The occurrence of these structures in many types of secretory cells during exocytosis has suggested that they are a preliminary stage in membrane fusion (2, 24, 29, 30). In mast cells these contacts can be extensive and are

often seen where the granule bulges against the plasma membrane. In freeze-fracture, these bulges frequently have plasma membrane domes that have been cleared of intramembrane particles (IMP) (7, 21). Furthermore, Lawson et al. (21) have shown in thin sections that a variety of ferritin-conjugated surface ligands will not bind to the plasma membrane where it contacts an underlying secretory granule, suggesting that the binding sites for these ligands have been cleared away (21). These studies and similar observations in other secretory cells (11, 23, 30) have been interpreted as indicating that plasma membrane macromolecules withdraw from the site of incipient fusion and that fusion actually takes place between protein-depleted membranes.

In this study, we have found early stages of exocytosis in mast cells that were "quick-frozen" by the method of Heuser et al. (14). This technique halts biological processes rapidly without the use of fixatives or cryoprotectants. Our results indicate that the initial fusion event between the granule and plasma membrane is highly localized and results in formation of a tiny pore; IMP clearing and extensive contact between fusing membranes, however, are not present.

MATERIALS AND METHODS

Mast cells were obtained from Sprague-Dawley rats of either sex, weighing 300-350 g, by procedures similar to those used in previous studies (e.g., see reference 16). Each rat was anesthetized with ether and decapitated, and a midline incision
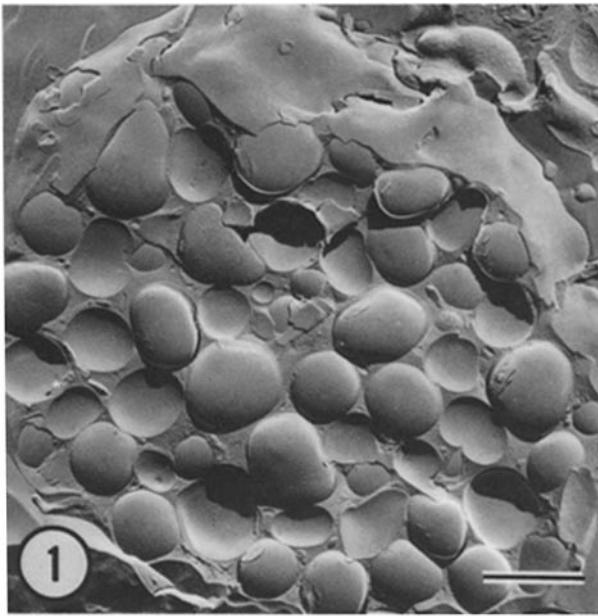
FIGURE 1 Freeze-fracture replica of a "quick-frozen" mast cell. Numerous granules lie beneath the P face of the plasma membrane. This and all subsequent figures have been photographically reversed and platinum deposits appear white. Bar, 1 μm. × 12,500.
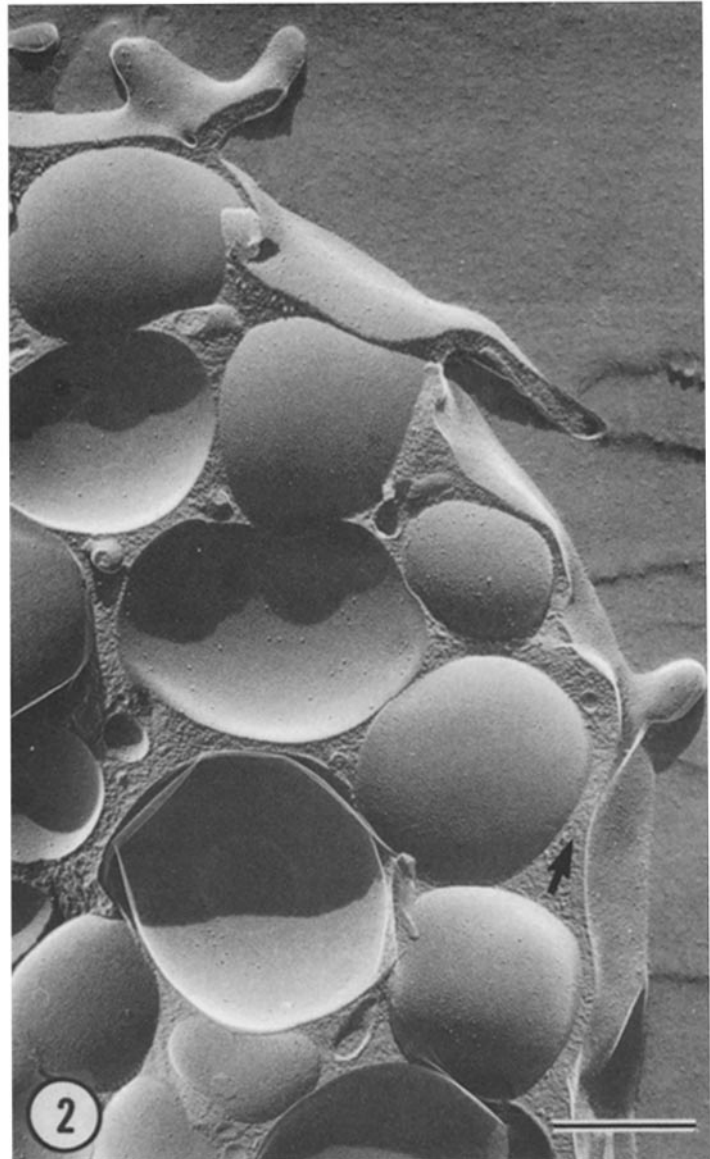
FIGURE 2 Periphery of an unstimulated, quick-frozen mast cell. Granules are well rounded and separated from the plasma membrane by a layer of cytoplasm (arrow). Bar, 0.5 μm. × 37,500.

was made in the abdominal wall. 12 ml of phosphate-buffered saline (PBS) was added to the abdominal cavity, the animal gently rocked for 90 s to circulate the fluid, and the fluid removed. The peritoneal washes from 3–6 rats were combined; the cells were centrifuged at 200 g for 5 min, and the pellet was washed once with PBS before final suspension in 3–6 ml of PBS. All experiments were carried out at 22°C in PBS containing (in millimoles per liter) NaCl, 150; Na_2HPO_4, 4; KH_2PO_4, 2.7; KCl, 2.7; $CaCl_2$, 1; and glucose, 5.6; with the addition of 1 mg/ml bovine serum albumin, 10 units/ml heparin, and adjusted to pH 7.0. Just before freezing, a 0.4-ml aliquot of the final cell suspension (containing ~10% mast cells) was centrifuged at ~100 g for 3 min, the supernate removed, and the loose pellet transferred to a small square of filter paper moistened with PBS. Cells and filter paper were then dipped into PBS containing 4 or 8 μg/ml of the synthetic polycation 48/80 (Sigma Chemical Co., St. Louis, Mo.), a well-known histamine-releasing agent (3, 15, 16, 28). The sample was quick-frozen on the machine designed by Heuser et al. (14). Briefly described, the sample sat on the tip of a plunger, which fell by gravity, pressing the cells and filter paper against a pure copper block cooled to ~4°K with liquid helium. After temporary storage in liquid nitrogen, the samples were fractured in a Balzers 301 unit (Balzers Corp., Nashua, N.H.) either at −130°C without etching or at −115°C with etching for 10 s. Replication was done with platinum-carbon from an electron beam gun at 45° followed by carbon applied from overhead. Replicas were cleaned in sodium hypochlorite and viewed at 80 kV in a JEM 100 B electron microscope.

In other experiments (where noted), mast cells were incubated in PBS at room temperature, stimulated with 2 μg/ml polymyxin B sulfate for 15–30 s, then fixed by adding an equal volume of 4% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.0. Fixation was continued for 1 h at room temperature, after which the cells

were washed three times in the Na cacodylate buffer, resuspended in 22% glycerol/78% Na cacodylate buffer for 1.5 h, then packed by centrifugation at 200 g for 5 min. A drop of packed cells was sandwiched between two gold "hats" that were coated with a concentrated solution of polyvinyl alcohol so that they would stick together after freezing; compare with Pauli et al. (26). These sandwiches were frozen in melting Freon 22 at −150°C and fractured in a Balzers 301 unit by pulling the two sandwich halves apart. Replica production, cleaning, and viewing were done as described above.

All figures have been photographically reversed and platinum deposits appear white.

RESULTS

Well-frozen mast cells usually cross-fractured to expose the membranes of numerous secretory granules lying beneath the P face of the plasma membrane (Fig. 1). In unstimulated cells, granule membranes were smooth and well rounded, with peripheral granules always being separated from the plasma membrane by a layer of cytoplasm (arrow, Fig. 2). The E and P faces of the granule membrane displayed distinctly different patterns of IMP; the E face had numerous IMP both large and small, whereas the P face had only a small number of relatively large particles (Fig. 3). Occasionally, small groups of IMP could be seen on either face (arrows, Fig. 3). Adjacent interior
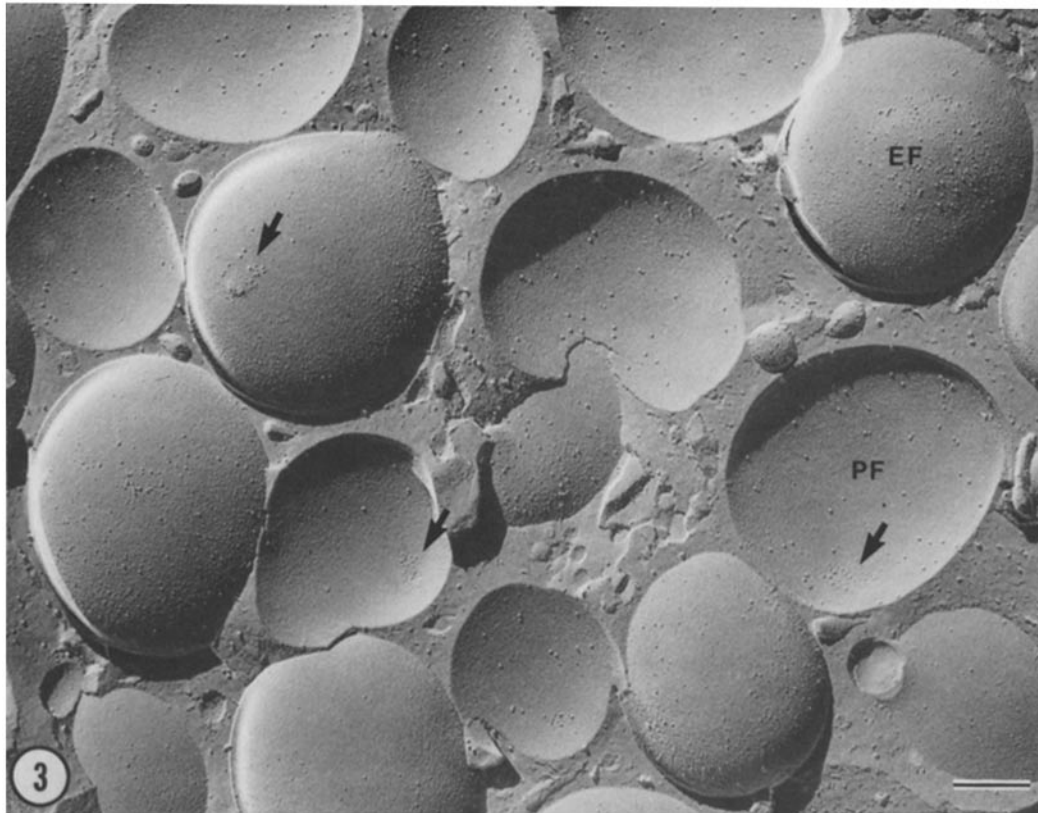
FIGURE 3 Granule membranes in the interior of an unstimulated mast that was quick-frozen. The granule membrane E face (EF) has numerous particles, both large and small, whereas the P face (PF) has only a few large particles. Both faces display occasional groups of particles (arrows). Bar, 0.2 µm. × 62,500.

granules were often packed so closely as to nearly touch, but in no case was there sign of interactions between granule membranes.

To observe early stages in exocytosis, we stimulated mast cells with 48/80 for 10–30 s before freezing. Before exocytosis, unreleased granules were separated from the plasma membrane by cytoplasm (Fig. 4). In some instances (in both stimulated and unstimulated cells), small networks of fibrils reached out from the cytoplasm to impinge on the granule membrane (arrow, Fig. 4). Exocytosis began with formation of a cylindrical membranous neck as small as 0.05 µm in diameter connecting the plasma and granule membranes (Fig. 5). Although it was unclear whether these smallest openings had an etchable center, pores only slightly larger were clearly etchable and thus represented an aqueous channel leading from the extracellular space into the granule interior (Fig. 6). The plasma membrane surrounding these pores always maintained a normal complement of IMP (cf. Figs. 4 and 6) and in no case were any IMP-free areas observed either at or away from these sites.

Pore formation appeared to result from a highly localized interaction because the granule membrane around the pore usually remained well separated from the plasma membrane (Figs. 5 and 7). As the pore enlarged, the neck maintained a rounded appearance (Figs. 7 and 8) and the plasma membrane surrounding the neck continued to display numerous IMP (Fig. 8). Remarkably, the P face of newly added granule membrane continued to have the low IMP density that it had before fusion, with the result that a sharp change in IMP density could be seen at the neck of granule opening where granule and plasma membranes met (Fig. 9). This difference in IMP

density was maintained even during extensive degranulation, when it became useful in distinguishing between sheets of fused granule membrane and plasma membrane.

Mast cells frozen after further degranulation exhibited numerous pocketlike invaginations formed by sequential fusion of interior granules with the cell surface (asterisks, Fig. 10). The membranes of these pockets appeared to be actively fusing with neighboring unreleased granules, as they were often dotted with small, circular depressions that represented either pores or contacts between the pocket and adjacent granules (arrows, Fig. 11). Distinguishing between these possibilities was difficult because slightly imperfect freezing produced artifactual contacts between adjacent granules in both stimulated and unstimulated cells (Fig. 12; compare this to the absence of contacts between granules in Fig. 3). However, in fractures that jumped from pocket to granule membrane we found clear examples in which the lip of the pore joined the two fusing membranes (arrows, Fig. 13).

In some cases a single granule was observed to fuse at more than one site (arrows, Fig. 13). We found further evidence of multiple fusion sites but on a much smaller scale. On the P face of membrane pockets, there were groups of small rounded depressions, 0.02–0.07 µm in diameter (Fig. 14). These appeared to correspond in size with groups of small, conelike pores seen on the E face of pockets and neighboring granules (Fig. 15). Etching had removed ice from the center of these pores, suggesting that they were aqueous channels joining two granule interiors. We also saw, between pocket membranes, small contacts that may have preceded the formation of pores (Fig. 16).
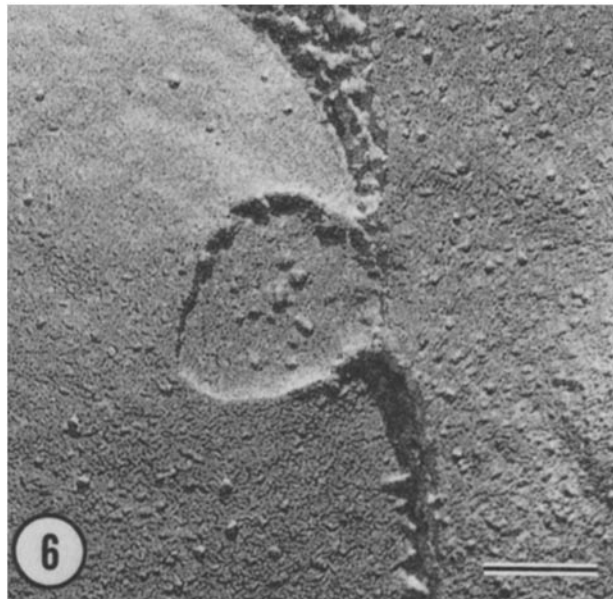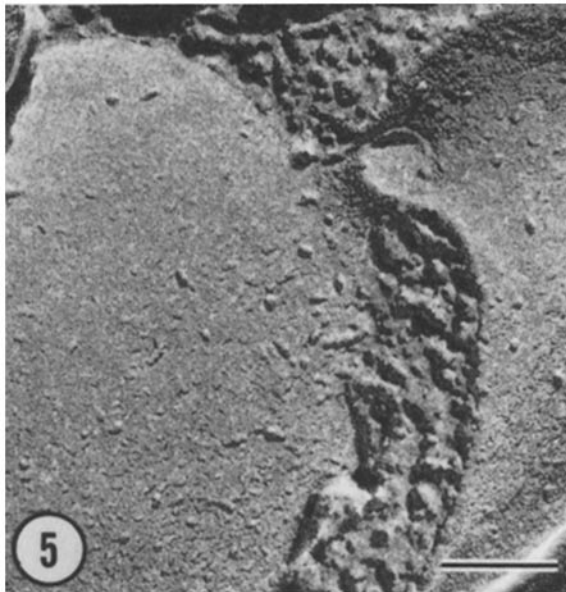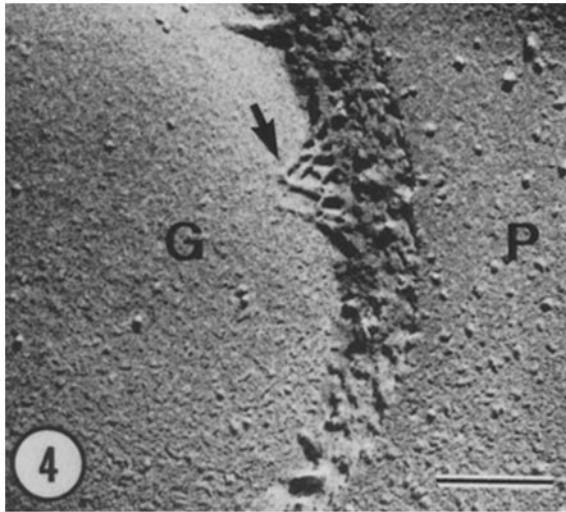
FIGURE 4-7 Pore formation during exocytosis in mast cells stimulated with 48/80, then quick-frozen. In Fig. 4, an unreleased granule (G) lies just under the P face of the plasma membrane (P) but is separated from it by cytoplasm. Occasionally small fibrils reach out of the cytoplasm to touch the granule membrane (arrow). In Fig. 5, granule and plasma membranes fuse to form a narrow pore between them. In Fig. 6, as the pore enlarges, one can see that its center is etched. Note that the surrounding plasma membrane has its normal complement of IMP. In Fig. 7, even after the pore has formed, the remainder of the granule membrane is well separated from the plasma membrane. All specimens were stimulated for 15 s with 8 μg/ml 48/80. Figs. 4-6: Bar, 0.1 μm. X 150,000. Fig. 7: Bar, 0.2 μm. X 62,500.
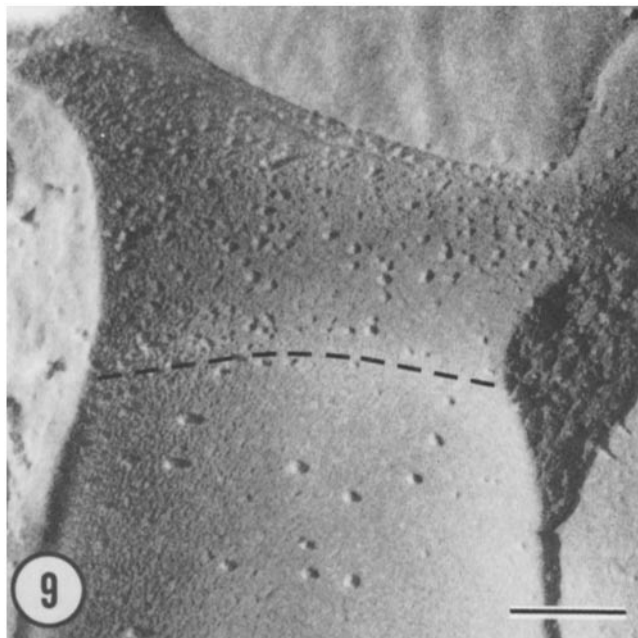
FIGURE 8 and 9 Pore growth after exocytosis. In Fig. 8, as the opening enlarges, its neck remains well rounded and the surrounding plasma membrane still exhibits numerous IMP. In Fig. 9, the granule membrane retains the low density of IMP it had before fusion and the difference in IMP density between it and the plasma membrane results in a boundary at the neck of the opening denoted by the dashed line. Both specimens were stimulated for 30 s with 4 μg/ml 48/80 and then quick-frozen. Bars, 0.1 μm. $\times$ 150,000.

We found no evidence of IMP-free membranes either at fusion sites between granules or on the walls of the pockets formed by granule fusion. This absence of IMP clearing at both the plasma membrane and interior granule membranes of quick-frozen mast cells was in marked contrast to previous observations of such areas in membranes of glutaraldehyde-fixed mast cells (7, 21). To be sure that IMP clearing could be seen in our mast cell preparations, we fixed mast cells with glutaraldehyde after 15–30 s of stimulation with polymyxin B sulfate. As a result, we saw distinct IMP-free areas on the P face of the plasma membrane, always on domes where the underlying granule bulged against the cell membrane (Fig. 17). In a few cases, we found within the IMP-free area, a depression that could represent the initial stages of pore formation between the granule and plasma membranes (arrow, Fig. 17). Furthermore, in cells in which granule fusion had formed membrane pockets, we frequently saw IMP-free areas on pocket walls where they closely contacted membranes of neighboring granules (Fig. 18). These results confirm previous studies on glu-

taraldehyde-fixed mast cells and point out that the discrepancy between these results and those on quick-frozen cells is real.

DISCUSSION

By using a new freezing technique to rapidly halt mast cell secretion, we have found that exocytosis begins with formation of a narrow orifice joining the granule and plasma membranes (Fig. 5). Frequently, this orifice formed even while the rest of the granule membrane remained separated from the plasma membrane by as much as 0.1 μm (Figs. 5 and 7). This suggests that the first contact between the plasma and granule membranes is small and focal. Previous thin section studies of glutaraldehyde-fixed mast cells have on occasion demonstrated such localized contacts (1, 4, 20, 28).

However, it has been more common to find in fixed mast cells that granules contact the plasma membrane over a broad region, often producing bulges in the plasma membrane that are IMP-free. Because the IMP-free bulges occur at the right time and place, they probably originate from some early stage
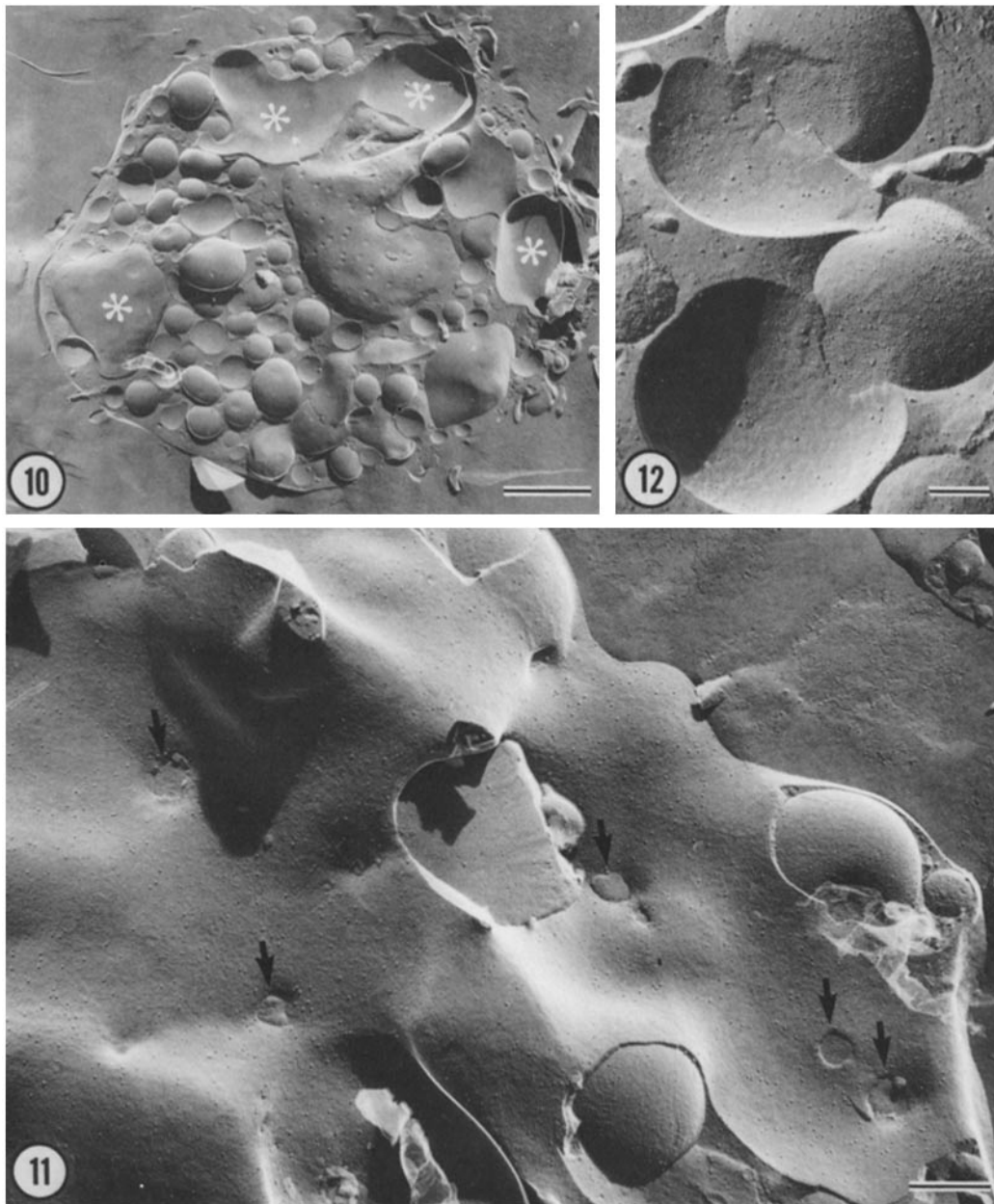
FIGURE 10   Degranulation in a mast cell stimulated for 30 s with 4 µg/ml 48/80, then quick-frozen. Asterisks denote large pockets of granule membrane resulting from fusion of interior granules. Bar, 2 µm. × 6,000.

FIGURE 11   An extensive sheet of fused granule membranes in the interior of a degranulating mast cell. The P face of this membrane has large IMP similar to those seen on the P face of unreleased granules. Circular depressions in the fracture plane (arrows) represent sites at which neighboring granules have either contacted or fused with this sheet. Specimen was stimulated 30 s with 4 µg/ml 48/80, then quick-frozen. Bar, 0.5 µm. × 22,000.

FIGURE 12   Artifactual contacts between granules that result from imperfect freezing. Specimen was stimulated and frozen as in Fig. 11. Bar, 0.2 µm. × 40,000.

in membrane fusion as Chi et al. (7) and Lawson et al. (21) originally suggested. Nevertheless, the fact that we find neither IMP-clearing nor extensive granule contact at the initial fusion site in quick-frozen cells leads us to think that these events occur on a much smaller scale but, during fixation, spread to involve unphysiologically large areas of membrane.

This is possible for two reasons. First, recent studies show that glutaraldehyde fixation actually leaves the membrane bilayer in a relatively labile state. Hasty and Hay (13) found that IMP-free blisters often form in fibroblast plasma mem-

branes after glutaraldehyde fixation. Chandler (5) showed that large IMP-free vesicles pinch off from sea urchin eggs at sites of cortical granule exocytosis, if aldehyde fixation is used. In mast cells, IMP-free blisters filled with vesicles have been seen contacting sites of fusion between the plasma and granule membranes and have been recognized as possible artifacts because they occur only after glutaraldehyde fixation (20, 21). Furthermore, we have demonstrated that, in sea urchin eggs, glycerol will cause artifactual fusion of plasma and cortical granule membranes even if applied after glutaraldehyde fixa-
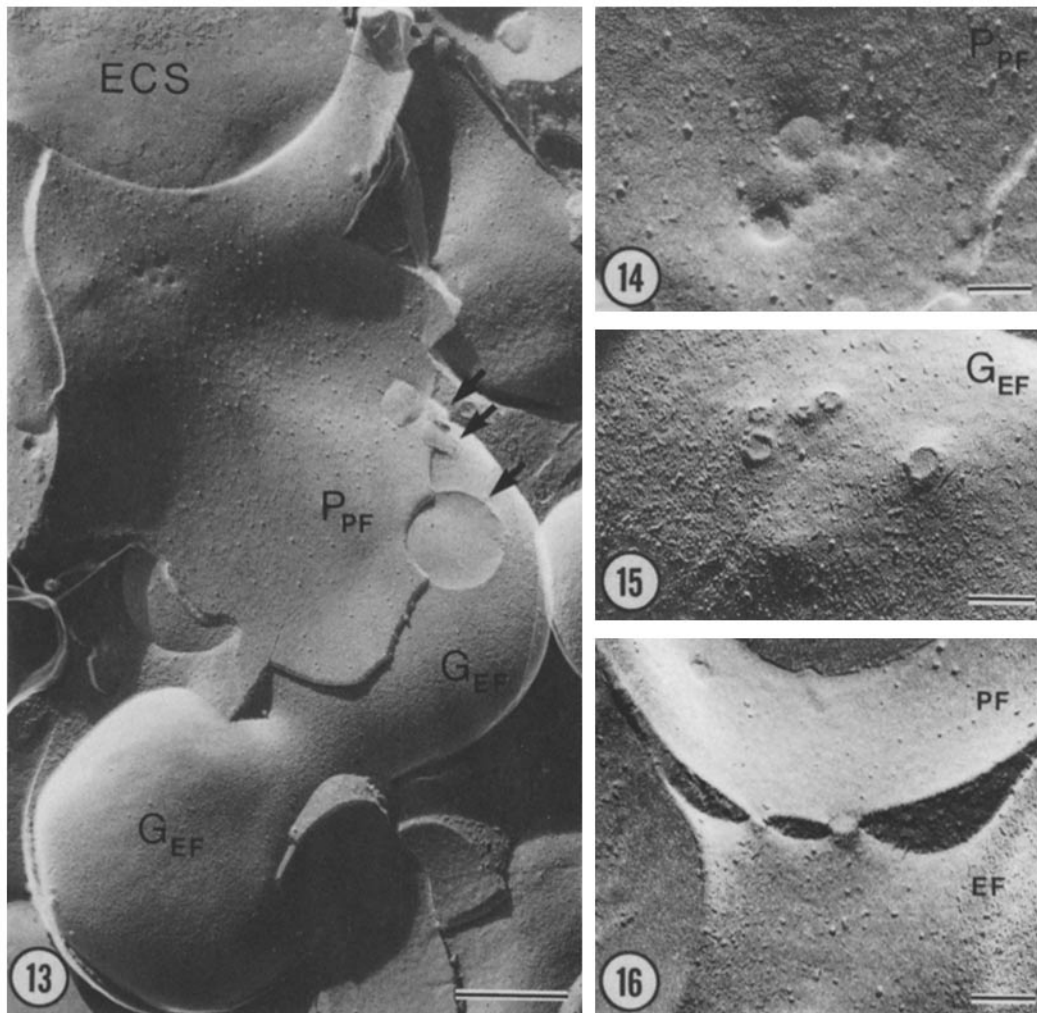
FIGURES 13–16   Contact and fusion between granule membranes in the interior of the mast cell. In Fig. 13, a pocket (P) that was formed by fusion of granule membranes extends from the extracellular space (*ECS*) into the interior of the cell. It continues to grow by fusion with neighboring granules (G) sometimes fusing with a granule at more than one point (arrows). The subscripts *PF* and *EF* denote P and E fracture faces, respectively, in Figs. 13–16. Fig. 14 shows a group of small pores on the P face of a membrane pocket. Fig. 15 shows a group of small, etchable pores on the E face of a neighboring granule. Fig. 16 shows small disklike contacts between two pockets of granule membrane. All specimens were stimulated for 17 s with 4 μg/ml 48/80, then quick-frozen. Fig. 13: Bar, 0.5 μm. × 30,000. Figs. 14–16: Bar, 0.1 μm. × 85,000.

tion (6). This fusion involved formation of an IMP-free bilayer continuous with both "fixed" membranes. Taken together, these studies point out that biological membranes remain labile during and after fixation and that fusing membranes remain particularly vulnerable to postfixation changes.

A second consideration is that glutaraldehyde fixation requires a number of seconds, a period much longer than a single exocytosis event (8). Thus, numerous granules must attempt to fuse under conditions in which glutaraldehyde has stopped some processes but not others. It is highly likely that macromolecule (IMP) segregation and granule-plasma membrane contact normally precede fusion, but our results would suggest that these changes are limited to a tiny region of the plasma membrane where a small exocytotic pore will eventually form. Indeed, if segregation of membrane macromolecules does occur before fusion, its domain in quick-frozen cells is usually so small as to escape detection. We presume that as aldehyde arrests membrane fusion, there is time for these localized changes to spread over abnormally large areas of the plasma

membrane, giving rise to what are described as IMP-free bulges.

On the other hand, quick-freezing is better able to sample one moment in this membrane fusion process because the interval is short between the time when cooling first starts to affect exocytosis and the time when freezing finally halts the process completely (probably 2 ms or less [14]). Hence there is less chance that intermediates become distorted because a later stage is preferentially blocked.

Quick-freezing has also allowed us to detect formation of multiple pores between adjacent granules (Figs. 14 and 15). We think that these represent an early step in fusion of these membranes because they are seen only in cells where membranes of previously fused granules contacted unreleased granules. Formation of multiple openings into a single granule has been described in sea urchin eggs (6) and zoospores (27). Indeed, in sea urchin eggs these openings appeared to be preceded by numerous small contacts between the granule and plasma membrane (6). Interpretation of multiple contacts and
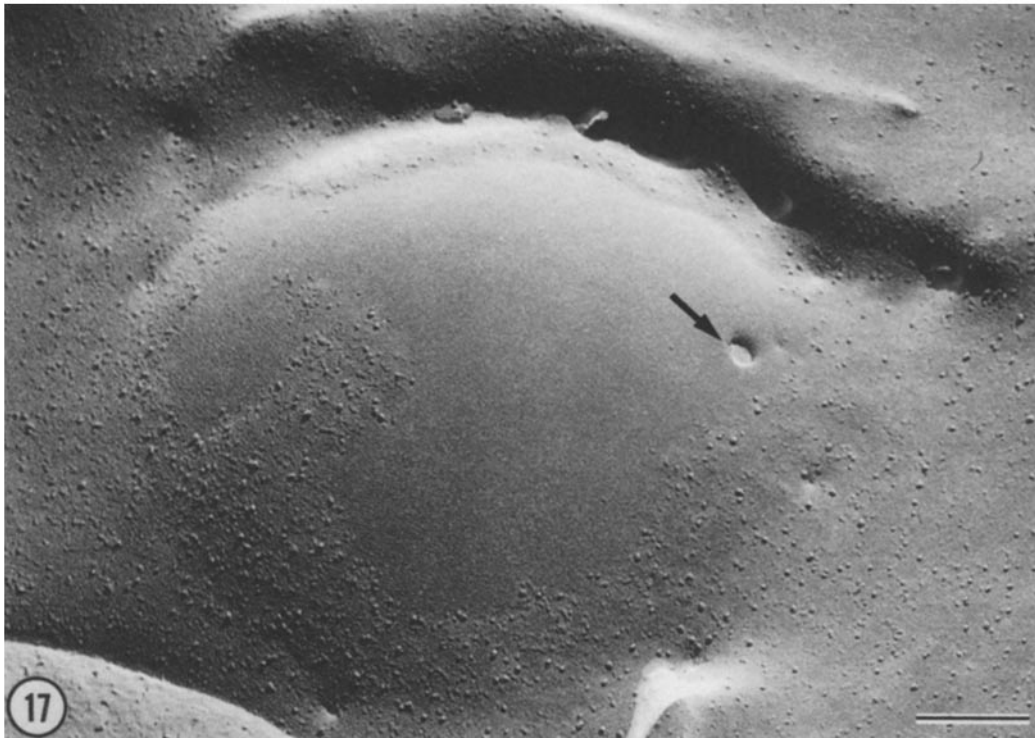
FIGURE 17   An IMP-cleared area on the P face of the plasma membrane of a glutaraldehyde-fixed mast cell. Within this area is a small depression that may be the site of initial contact or fusion between the plasma membrane and the underlying granule membrane (arrow). The specimen was fixed after 30 s of stimulation with 2 μg/ml polymyxin B sulfate. Bar, 0.2 μm. × 75,000.

FIGURE 18   IMP-cleared areas on a sheet of fused granule membrane in the mast cell interior. These areas (asterisks) occur where the sheet has extensive contact with a neighboring unreleased granule. The specimen was stimulated and fixed with glutaraldehyde as described in Fig. 17. Bar, 0.2 μm. × 75,000.

pores in eggs, however, was problematic because in that cell the glycerol used for cryoprotection caused artifactual membrane fusion (6). In the present situation, however, multiple pores are found in mast cells that have been exposed to neither fixatives or cryoprotectants; thus it is more likely that such multiplicity of fusion actually does occur.

Quick-freezing, then, has proven valuable not only in reevaluating the membrane fusion intermediates seen in glutaraldehyde-fixed cells but also in visualizing events not previously seen in fixed cells.

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