MOLECULAR EVENTS DURING MEMBRANE FUSION

A Study of Exocytosis in Rat Peritoneal Mast Cells

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ABSTRACT

We have used thin section and freeze-fracture electron microscopy to study membrane changes occurring during exocytosis in rat peritoneal mast cells. By labeling degranulating mast cells with ferritin-conjugated lectins and anti-immunoglobulin antibodies, we demonstrate that these ligands do not bind to areas of plasma membrane or granule membrane which have fused with, or are interacting with, granule membrane. Moreover, intramembrane particles are also largely absent from both protoplasmic and external fracture faces of plasma and granule membranes in regions where these membranes appear to be interacting. Both the externally applied ligands and intramembrane particles are sometimes concentrated at the edges of fusion sites. These results indicate that membrane proteins are displaced laterally into adjacent membrane regions before the fusion process and that fusion occurs between protein-depleted lipid bilayers. The finding of protein-depleted blebs in regions of plasma and granule membrane interaction raises the interesting possibility that blebbing may be a process for exposing the granule contents to the extracellular space and for the elimination of excess lipid while conserving membrane proteins.

In most secretory cells, the triggered release of specific materials stored in secretory granules occurs by exocytosis (2, 18, 37, 43). This process involves the fusion of granule membrane with plasma membrane, and results in the exposure of the granule contents to the extracellular space (2, 18, 37, 43). The molecular events in exocytosis, and in membrane fusion in general, are unknown. We have studied the behavior of membrane lipids and proteins during and after the fusion events occurring in the process of degranulation of rat peritoneal mast cells. The extensive membrane interactions occurring all around the circumference and inside degranulating mast cells make these cells particularly attractive for studying membrane fusion.

Mast cells can easily be obtained in homogeneous cell suspension (9). Having high affinity surface receptors for the Fc region of immunoglobulin E (IgE) (so-called Fc receptors) (5), mast cells obtained from appropriately immunized rats have cytophilic IgE antibodies on their surface which serve as receptors for the specific antigen (6). Such sensitized cells can be stimulated to secrete histamine (and other substances) by exposure to the specific antigen (4, 6), or to anti-Ig antibody (6, 25), or to the lectin, concanavalin A (Con A) (29); these ligands bind to and cross-link the cell surface IgE (26, 29, 31, 32), and exocytosis ensues within seconds (10, 34, 43).

Ligand-mediated degranulation of mast cells is energy-dependent (39), requires extracellular Ca^{2+} (20, 33), and does not kill the cell (27). There is increasing evidence to suggest that it is an influx of Ca²⁺ that initiates the exocytosis process: Ca^{2+} is a required (30, 33) and sufficient (43) ion for degranulation, and the artificial introduction of Ca^{2+} into the cell either by microinjection (28) or by the use of the divalent cation carrier molecule A23187 (12, 21) induces energy-dependent degranulation. Moreover, an increase in the permeability of the cells to ⁴⁵Ca²⁺ occurs after the binding of antigen to sensitized rat mast cells (21, 22). After the fusion of the membranes of peripheral granules with the plasma membrane (and subsequently of granule membranes with other granule membranes) (3, 7, 30, 40), the contents of the granules are exposed to the extracellular space and histamine and other amines are liberated from the anionic-binding sites on heparin and protein by exchange with extracellular cations (43). The release of amines is associated with readily detectable alterations in the ultrastructural appearance of the granule, including the enlargement of the space between the granule and its membrane, granule swelling, and reduced electron density of the granule contents (3, 7, 8, 10, 30, 36, 40).

We have studied resting and degranulating rat peritoneal mast cells by thin section and freezefracture electron microscopy, using cationized ferritin, ferritin-conjugated lectins, and anti-Ig antibodies to follow the behavior of various membrane molecules during the process of degranulation. Our findings indicate that membrane proteins are displaced laterally, away from areas of membrane contact, so that fusion occurs between protein-depleted lipid bilayers.

MATERIALS AND METHODS

Ferritin-Conjugated Ligands

Sheep antibodies to rat immunoglobulin (S anti-RIg) and their monovalent Fab fragments (Fab anti-RIg), and goat antibodies to rabbit immunoglobulin (G anti-Rab Ig) were prepared and conjugated to ferritin (FT) (Calbiochem, San Diego, Calif.) with glutaraldehyde as previously described (31). Con A (Miles-Yeda) was similarly conjugated to ferritin (17), and all of the conjugates were separated from unferritinized proteins by ultracentrifugation in a sucrose density gradient as previously described (15). Rabbit antiserum to phytohemagglutinin (R anti-PHA) was prepared by immunizing a rabbit with 200 μg of purified PHA (Wellcome Foundation, Ltd., Kent, Great Britain) in complete Freund adjuvant, given in multiple subcutaneous and intramuscular sites on two occasions 1 mo apart, and bleeding 2 wk later. The antiserum was heat-inactivated (56°C for 60 min) before use. The unconjugated S anti-RIg used in some experiments was the IgG fraction prepared by DEAE-cellulose chromatography (31).

Preparation of Mast Cells

Male Wistar rats were immunized with ovalbumin in pertussis vaccine (31). 15-30 days later, a cell suspension, containing 2-5% mast cells was obtained by peritoneal lavage and the mast cells were purified to better than 90% by centrifugation through a discontinuous density gradient of human serum albumin (Kabi, A. B., Sweden), as previously described (31).

Mast Cell Stimulation, Ferritin Labeling, and Processing for Thin-Section Electron Microscopy

Mast cells, incubated in Tyrode's solution (NaCl, 137 mM; KCl, 2.7 mM; NaH₂PO₄, 0.4 mM; MgCl₂, 1 mM; Hepes, 20 mM; CaCl₂, 1.8 mM; glucose, 5.6 mM; adjusted to pH 7.5) were stimulated to degranulate by treatment with ovalbumin (10 μ g/ml), S anti-RIg (500 µg/ml), S anti-RIg-FT (600 µg S anti-RIg/ml), Con A-FT (500 μ g of Con A/ml), or A23187 (6 × 10⁻⁶ M) for various times, from 20 s to 30 min at 37°C. Degranulation was stopped by the addition of cold glutaraldehyde (3% in 0.1 M Na cacodylate buffer, pH 7.3), and the cells were fixed for 15 min on ice, followed by 60 min at room temperature. In most experiments, the cells were stimulated with ovalbumin for 20 s or A23187 for 3 min, fixed as described, washed twice in lysine (25 mg/ml in Na cacodylate buffer), and then labeled at room temperature in one of the following ways: (a) cells were suspended in 400 μ l of Con A-FT for 45 min; (b) cells were suspended in 400 µl of purified PHA (Wellcome Foundation, Ltd.) (200 µg/ml) for 30 min, washed twice, suspended in R anti-PHA (1:50) for 30 min, washed twice, incubated in 800 µl G anti-RIg-FT (450 µg G anti-RIg/ml) for 45 min, washed, and refixed in 3% glutaraldehyde for 60 min (since for PHA labeling, the initial prefixation was done in 0.5% glutaraldehyde); (c) cells were washed in Veronal-buffered saline (pH 7.3) and suspended in cationized-ferritin (Miles-Yeda) (0.23 mg/ml) in the same buffer for 30 min to label negatively charged cell-surface sites (14).

In most cases the cells were then washed, postfixed in 1% osmium tetroxide (OsO₄) in 0.1 M Na cacodylate buffer for 60 min at room temperature, washed in Veronal acetate buffer (pH 7.4), and treated en bloc with 50% uranyl acetate in Veronal acetate buffer for 60 min at room temperature, washed in distilled water, and

resuspended in one drop of 1% agar at 47°C. The agarsuspended cells were centrifuged in the warm, cooled on ice, and the pellet was cut into $1-2 \text{ mm}^3$ pieces; these were dehydrated in ethanol followed by propylene oxide, and embedded in Araldite or Epon 812. Thin sections were stained with uranyl acetate followed by lead citrate (unless otherwise stated) and examined with an AEI-6 EMB electron microscope at 60 kV.

In some experiments, different fixation and postfixation regimens were used, but the procedures described above were found to be optimal for the purposes of studying membrane events in rat peritoneal mast cells (Results). The labeling with Con A-FT was inhibited completely in the presence of 0.1 M α -methylmannoside, and no labeling was seen using the three-step procedure for detecting PHA binding, when PHA was omitted from the protocol.

Freeze-Fracture Electron Microscopy

Cells were treated with S anti-RIg (500 μ g/ml) for 30 s or A23187 (6 \times 10⁻⁶ M) for 3 min and then fixed in 3% buffered glutaraldehyde for 15 min on ice, and then 60 min to several days at room temperature. After fixation, the cells were rinsed twice in 0.1 M cacodylate buffer. After resuspension in 25% glycerol in 0.1 M cacodylate buffer for 2 h, the cells were again pelleted, placed on 2-mm cardboard disks and rapidly frozen in the liquid phase of partially solidified chlorofluoromethane (Freon 22 E. I. du Pont de Nemours & Co., Wilmington, Del.). Freeze-fracturing was performed with a Balzers device (model BA 360 Balzers High Vacuum Corp., Santa Ana, Calif.) with a stage temperature of -115° C. The replicas were cleaned with a bleach solution and then examined in a Philips 300 microscope on uncoated grids. The freeze-fracture electron micrographs are mounted with the shadow direction from bottom to top.

RESULTS

Thin-Section Electron Microscopy

Unlike the other cells in rat peritoneal washings, mast cells are extremely sensitive to different fixation procedures. Mast cells fixed in 3% glutaraldehyde on ice for 15 min and then at room temperature for 60 min, followed by postfixation in cacodylate-buffered OsO₄ on ice, show the best overall cell preservation. However, when the post-fixation in OsO₄ is carried out at room temperature, the membranes are better visualized and this is the procedure we normally adopted for studying membrane interactions. Interestingly, in our hands, mast cells are lysed (showing discontinuities in the plasma membrane and loss of cytoplasm) when the initial glutaraldehyde fixation is carried out at room temperature or when the

OsO₄ postfixation is done in Veronal acetate buffer (at 4°C or room temperature), even though the granulocytes, lymphocytes, and macrophages are well preserved in the same cell suspensions.

In general, the results obtained with the various stimulating ligands are indistinguishable in terms of the morphological events that they induce.

GRANULE CHANGES: Unreleased mast cells, or those treated with Fab anti-RIg-FT appear as shown in Fig. 1. The granules are present as homogeneous, dense-staining cytoplasmic inclusions surrounded by their granule membranes. In all such preparations a number of cells show evidence of a small degree of spontaneous degranulation, which generally results in the release of 5% of the total mast cell histamine (20). This occurs in the absence of extracellular Ca2+ and is not prevented by metabolic inhibitors (20). In preparations treated with ovalbumin, S anti-RIg, S anti-RIg-FT, Con A-FT, or A23187, a majority of the cells show varying degrees of degranulation (Fig. 2). As has previously been described (3, 7, 30, 40, 43), the peripheral granules usually appear to be altered first, with the course of degranulation proceeding inward. Granule changes include the enlargement of the space between the granule and its membrane (usually associated with a ruffling of the granule membrane), swelling, and a reduction in its electron density and homogeneity. While some altered granules are discharged from the cell (Fig. 2), most remain within the cell circumference, lying within a network of cavities (Fig. 2) that have been shown to communicate with the extracellular space (40).

MEMBRANE INTERACTIONS: In degranulating cells, particularly those stimulated for only 20 s before fixation, multiple regions of membrane interaction are seen between plasma membrane and granule membranes and between adjacent granule membranes. These include areas where two membranes are in relatively close apposition, with a reduction or loss of visible intervening cytoplasm (Figs. 3-5, 8) or where the two membranes have fused to form a pentalaminar structure of 125-135 Å (Figs. 7, 9). Here, as suggested by Farquhar and Palade (19, 37) the term fusion is used to describe the merging of two trilaminar images to form a single pentalaminar image. The term 'fission' is reserved for the process whereby two bilayer structures form a single bilayer. In the fusion image, the dense intermediate line represents the merged cytoplasmic leaflets of the two membranes. Whereas the majority of



FIGURE 1 Unstimulated mast cell containing many membrane-bound, histamine-containing granules. Note microvilli and the one altered granule (arrow) which probably has released its histamine. (Bar, 1.5 μ m; × 6,500.)

FIGURE 2 Degranulated mast cell treated with S anti-RIg-FT for 30 s containing mostly altered granules lying within a network of cavities which communicate with the extracellular space. (Bar, $1.5 \mu m$; $\times 6,500$.)

interacting membranes are seen over altered granules (Figs. 3-5, 7-9) they can also be seen over unaltered granules (Figs. 6, 8), suggesting that such interactions probably precede histamine release. In a preliminary study of a serially sectioned degranulating mast cell, all of the altered granules appear to be in overt contact with the extracellular space at some point, even if this is not apparent in many of the individual sections.

In areas of plasma and granule membrane interaction, multivesiculated membrane structures are sometimes present blebbing from the cell surface (Fig. 10). In most cases their limiting membrane is continuous with the adjacent plasma membrane, while the contained vesicles often appear to arise, at least, in part, from the interacting granule membrane. While we and others (7) have seen similar structures in unstimulated mast cells and in other cell types (35), they are more common in degranulating mast cells and are present in cells stimulated for 15 s or 15 min. In both stimulated and unstimulated mast cells they usually overlie altered granules (Fig. 10), but they also can be detected over unaltered ones. Sometimes they are present over openings in the plasma membrane where altered granules are in overt contact with the extracellular space; such openings are bounded by regions of presumed membrane 'fission,' where plasma and granule membranes are in continuity. The frequency of blebs is greatly influenced by the fixation procedure, being greatest in cells prefixed and postfixed in the cold. These conditions are optimal for the overall preservation of mast cells; however, under these conditions the trilaminar images of the membranes are less distinct when compared to cells that have been postfixed at higher temperatures. No blebs are observed when degranulated cells are fixed in osmium alone, or in a combination of osmium and glutaraldehyde (24), but the mast cells are lysed under these conditions.

Multilamellar membrane whorls (myelin figures) are sometimes seen within cavities containing a number of altered granules in degranulated mast cells (Fig. 11). In some cases, at least, they appear to be formed from the membranes of altered granules. They are most frequently seen under the same conditions of fixation where blebs are maximal.

Displacement of Membrane Ligand-Binding Sites during Degranulation

In degranulating mast cells, a striking and consistent finding is the absence of ligand binding to the plasma membrane in areas where it is interact-



FIGURE 3 Three granules showing (from right to left) increasing degrees of alteration in a degranulating mast cell stimulated with S anti-RIg-FT for 30 min. Note that the FT molecules are densely distributed on the uninvolved plasma membrane region (on right), while they are sparsely distributed on regions where granule and plasma membranes are close together but still separated by intervening cytoplasm (at center). The FT molecules are completely absent from the plasma membrane where it is in close contact and possibly fused with the underlying granule membrane (on left). Unstained section. (Bar, 0.2 μ m; × 47,000.)

FIGURES 4 and 5 Membrane interactions in mast cells stimulated with ovalbumin for 20 s, fixed with glutaraldehyde and then labeled with PHA, R anti-PHA, and G anti-RabIg-FT (Fig. 4) or cationized ferritin (Fig. 5). In both cases, where plasma and granule membranes are interacting, as judged by the lack of intervening cytoplasm, no ligand binding is seen. In Fig. 5, an extruded granule, which is heavily labeled with cationized ferritin, is seen at the top of the picture. (Fig. 4, bar, 0.1 μ m; × 99,000; Fig. 5, bar, 95.2 nm; × 105,000.)



FIGURE 6 Mast cell stimulated with Con A-FT for 15 min. Plasma and granule membranes appear to be interacting at two sites overlying an unaltered granule. In one of these regions the membranes appear to be fused and have formed a simple bleb. In the other region, the oblique plane of section obscures the details of the interaction. In both regions, Con A-FT binding is absent. (Bar, 95.2 nm; \times 105,000.)

FIGURE 7 Mast cell stimulated with ovalbumin for 20 s, fixed with glutaraldehyde, and labeled with PHA, R anti-PHA, and then G anti-Rab Ig-FT. Labeling is dense in all regions of the plasma membrane except where it is fused with the underlying granule membrane to form a pentalaminar structure. Note that the label is bound to the noncytoplasmic side of the granule membrane (arrow) indicating that the inside of the altered granule is open to the extracellular space, although it is not apparent in this plane of section. (Bar, 0.14 μ m; × 69,000.)

ing with underlying granule membrane. This is the case when the ligand is S anti-RIg-FT, Con A-FT, or PHA (visualized with additional layers of R anti-PHA and G anti-Rab Ig-FT) and when the membranes are fused or appear to be interacting at a distance. Where interacting membranes are not fused and some intervening cytoplasm remains, ligand binding is usually present but reduced (Fig. 3), while in places where no intervening cytoplasm is seen, no label is present, even



FIGURE 8 Mast cell stimulated with S anti-RIg for 30 s. The granule membrane surrounding an altered granule is interacting with the overlying plasma membrane (arrow) and has fused with an adjacent granule membrane (bracket) which surrounds an unaltered granule. (Bar, 0.13 μ M; × 75,000.)

FIGURE 9 Mast cell stimulated with ovalbumin for 20 s, fixed with glutaraldehyde and labeled with PHA, R anti-PHA, and G anti-Rab Ig-FT. FT labeling is seen on the noncytoplasmic side of the upper granule membrane but is absent from the pentalaminar region where the membrane has fused with an underlying granule membrane. The reduced amount of label on the granule membrane compared to the usual labeling of plasma membrane (cf. Fig. 7) is probably related to poor penetration of the ligands due to the presence of granule matrix material. (Bar, 0.1 μ m; × 99,000.)



FIGURE 10 Mast cell stimulated with S anti-RIg-FT for 30 s. A multivesiculated bleb is present overlying an altered granule in a well-preserved cell. The outer limiting membrane of the bleb is continuous with the plasma membrane. (Bar, $0.27 \ \mu m$; $\times 36,000$.)

FIGURE 11 Mast cell stimulated with S anti-RIg-FT for 30 s containing a multilammellar membrane whorl (myelin figure) which is adjacent to four altered granules. (Bar, $0.27 \ \mu m$; $\times 36,000$.)

though the membranes are often quite far apart (Fig. 4). Neither simple (Fig. 6) nor multivesiculated blebs are ever labeled by these ligands. A similar labeling pattern is usually seen with cationized ferritin (Fig. 5) except that interacting membranes and blebs are sometimes labeled. In some degranulated cells, Con A-FT and PHA labeling is present on the noncytoplasmic surface of the granule membranes which line the cavities that are open to the extracellular space. Here too, label is always excluded from areas where two granule membranes are interacting (Fig. 9).

Two types of experiments exclude the possibility that the lack of ligand binding to interacting membranes is due to redistribution of membranebinding sites induced by the cross-linking effects of the ligands themselves. Firstly, cells stimulated by A23187, and labeled with Con A-FT after fixation with glutaraldehyde, show a pattern of labeling which is indistinguishable from cells which have been stimulated with cross-linking ligands such as ovalbumin, Con A, or S anti-RIg. Secondly, we have found that Fab anti-Ig-FT, which has been shown not to redistribute Ig on the surface of lymphocytes (16) or mast cells (31) does not bind to areas of interacting membranes.

Occasionally we find an increased density of labeling (by Fab anti Ig-FT on unfixed cells, and by Con A-FT on prefixed cells stimulated with A23187) in the areas immediately adjacent to regions of fusion. This suggests that the ligandbinding sites are displaced laterally in the course of the fusion process.

Freeze-Fracture Electron Microscopy

NORMAL MAST CELLS: Our findings are similar to those reported by Chi et al. (11) on unstimulated mast cells. The cell surfaces are relatively smooth with the notable exception of numerous folds or convolutions that appear in thin sections as microvillar cell processes (Fig. 12). The plasma membranes are characterized by a random distribution of intramembrane particles on both the inner and outer fracture faces (P and E, respectively). The particles range in size from 60 to 125 Å in diameter, and they are distributed asymmetrically on the two fracture faces with the majority of the particles present on the inner membrane half.

There are two types of particle clusters or aggregates that are occasionally observed in the plasma membranes of both unstimulated and stimulated mast cells. One of these is characterized by a loose arrangement of large (125 Å in diameter) particles (Fig. 13) which may contain as many as 150 particles. Several of these aggregates may be present at the same time, and there is no apparent association of these regions with specific cell surface or cytoplasmic components. The second type of particle cluster is usually comprised of an aggregation of heterogeneous particles (70-125 Å in diameter) that can fuse or coalesce with each other (Fig. 14). This type of cluster is generally associated with a depression or invagination of the plasma membrane and is also found at the base of some microvilli.

The granule membranes in unstimulated cells have intramembrane particles in approximately similar densities, on both the outer (P face) and inner (E face) halves. The particles on the outer membrane half are randomly arranged, while those on the inner half are frequently present in discrete clusters of two to four particles. These discrete clusters are usually located on slight depressions in the fracture face.

STIMULATED MAST CELLS: The cell surface morphology of the mast cell is altered significantly after stimulation by S anti-RIg or A23187. In most degranulating cells, the surface membrane is frequently interrupted by an extensive network of microvillar folds (Fig. 15). The cross fractures through the folds expose the membranes of many cytoplasmic granules that are closely associated with the microvillar processes.

Granule-related bulges are frequently present in

the plasma membrane as a result of stimulation (Figs. 15, 16). The intramembrane particles are dramatically reorganized in the bulging regions of the membrane. Some bulges are completely devoid of particles, while in others the particles are reorganized into a variety of nonrandom arrangements. In all cases, the rearrangements effectively result in increasing the density of particles at the base of the bulges, and producing a bulge region of membrane that is primarily comprised of a particle-depleted smooth zone.

The earliest detectable plasma membrane changes associated with degranulation are present prior to the appearance of a bulge. These changes are characterized by the appearance of a focal zone of rearrangement in regions where granule and plasma membranes appear to be interacting (Fig. 17). These regions are defined by the close apposition of the two membranes where the fracture plane moves from one membrane to the next without detectable intervening cytoplasm. Most frequently, these putative interacting zones are detected as smooth regions in the plasma membrane. However, in a few instances, a slight protruberance with a central particle appears to be related to the interacting zone. These interacting zones can also be detected on the granule membrane fracture faces.

Blebs of the plasma membrane can be observed during degranulation induced by either S anti-RIg or A23187. The blebs have essentially the same morphology as some of the bulges. Both fracture faces of the blebs are depleted of almost all particles, and a collar of particles is present at the base of the blebs (Fig. 18). When a bleb is released from a cell, the collar of particles is retained by the plasma membrane.

The granule membranes inside the cell are sig-

FIGURE 12 Freeze-fracture appearance of the plasma membrane (PM) of an undegranulated mast cell. The microvillar folds (M) and a granule membrane (G) are indicated. The distinct membrane particle aggregates that are present on some of the cells within the population are enclosed by circles. $\times 17,100$.

FIGURE 13 A higher magnification view of one of the particle aggregates in Fig. 12. The aggregates are comprised of a loose arrangement of large 12.5-nm particles. The particles are generally larger than the other particles in the plasma membrane. \times 72,500.

FIGURE 14 A type of particle aggregate that is present in the plasma membrane of both undegranulated and degranulated mast cells. This type of aggregate is usually associated with an invagination or depression in the plasma membrane. The particles in the aggregate vary in size, and they may fuse or coalesce with each other. \times 96,000.





FIGURE 15 Freeze-fracture appearance of mast cell during degranulation. The plasma membrane fracture face is frequently interrupted by an extensive series of folds that are recognized as microvilli in thin sections. Granule membranes are apparent (G) as well as bulges that are related to granule exocytosis. Note that particle rearrangements are present in some of the bulge regions (arrows). $\times 15,120$.

nificantly altered during the degranulation process (Fig. 19). There is a slight increase in particle densities on both granule membrane fracture faces, and small particle aggregates are frequently present on the outer membrane halves (P-fracture faces). In addition, smooth zones are readily detectable in regions of granule membrane interaction. These smooth zones have not been detected between granules in unstimulated cells, and they are strikingly similar to the regions of granule membrane-plasma membrane interaction in stimulated cells.

DISCUSSION

Our studies confirm and extend the ultrastructural observations of others on the degranulation of rat peritoneal mast cells (3, 7, 10, 11, 30, 40, 42). Using a variety of ferritin-coupled ligands with thin-section electron microscopy, together with freeze-fracture electron microscopy, we have di-



FIGURE 16 Plasma membrane fracture face of degranulated mast cell (from Fig. 15). The fracture face is interrupted by microvillar folds. The exposed cytoplasm (C) and a granule membrane (G) are indicated. Note the bulges (1-3) that are frequently observed during the exocytosis process. The intramembrane particles are randomly distributed throughout the plasma membrane except in the regions of release (bulges). These regions are primarily smooth zones as a result of the particle rearrangements. The small bulges in 2 (arrow) may reflect the vesiculation that is sometimes associated with the exocytosis process. \times 51,500.

rected our attention specifically to the membrane events that occur during the exocytosis process. Since the introduction of Ca^{2+} into the cells by means of A23187 gives results which are indistinguishable from those seen when mast cells are stimulated by ligands which can cross-link IgE receptors (antigen, S anti-RIg, and Con A), it is likely that, in each case, it is an influx of Ca^{2+} that initiates the sequence of events that leads to membrane fusion and exocytosis. How Ca^{2+} acts inside the cell in this respect is unknown. Since Mg^{2+} cannot substitute for Ca^{2+} (20, 21, 28), and since ATP is required after Ca^{2+} influx (21), it is probable that the mechanism is more complex than simple charge neutralization or lipid segregation, which may explain the role of Ca^{2+} in the fusion of negatively charged liposomes (38).

The clearest and most important aspect of these



FIGURE 17 Fusion process in a degranulating cell. The regions of granule membrane (G)-plasma membrane fusion are characterized by smooth zones (X) in the pasma membrane fracture face. In one region (arrow), a small protuberance with a central particle may be associated with a presumptive fusion site. \times 62,500.

studies is the demonstration that membrane proteins are displaced from the 'interaction zones' when granule membranes come together and fuse with the plasma membrane or with each other to form pentalaminar structures. We cannot exclude the possibility that some protein is retained in these areas of interacting membranes, but the absence of IgE (and presumably the Fc receptors to which they are bound), Con A, PHA-binding glycoproteins, and intramembrane particles suggests that most, if not all, of the membrane proteins are displaced and that the pentalaminar fusion struc-



FIGURE 18 Freeze-fracture appearance of a bleb (B) that frequently accompanies degranulation. The inner membrane half of the bleb is practically devoid of intramembrane particles. The particles from the bleb region of membrane are retained in a zone at the base of the bleb (arrow). A transected granule (G) is also present. \times 61,800.

ture is composed of two protein-free lipid bilayers. It is difficult to interpret the inconsistent labeling of interacting membranes with cationized ferritin in view of the uncertainty as to how much of it is binding to protein and how much to lipid. Since cholera toxin binds to the areas of fusion in degranulating mast cells, it is likely that at least some glycolipids (GMI) (13) are not displaced during fusion (D. Lawson and H. Herschman, unpublished observations). The finding that IgE receptors, lectin-binding sites, and intramembrane particles are often increased in density at the edges of fusion sites and blebs suggests that the membrane proteins are retained by a process of lateral displacement into adjacent membrane, rather than

that they are being lost during the exocytosis process.

Thus, our observations confirm the prediction of others, that membrane fusion occurs, in some instances at least, in regions denuded of 'intramembranous proteins' (1). However, the close apposition of two membranes to form a pentalaminar image does not always involve protein and particle exclusion. For example, tight junctions (zonulae occludens) between epithelial cells, which appear as pentalaminar structures by thinsection electron microscopy (19), are composed of a meshwork of ridges and grooves, and freezefracture observations show that the ridges are usually studded with intramembrane particles (23). It



FIGURE 19 In a degranulated cell, the granule membranes are altered as a result of granule-granule membrane fusion. Smooth zones (X) are present on both granule membranes halves as a result of granule membrane interactions, and particle aggregates (arrowhead) are present on some of the granule membrane fracture faces as a result of the degranulation process. (FG), fused granule; (RG), released granule. \times 51,500.

is probable that the greater thickness of the 'fused' tight junction membranes (140–150 Å) compared with the fused membranes in degranulating mast cells (125–135 Å) is related to the presence of protein in the former. Perhaps, transient fusion excludes proteins while stable fusion interactions includes them.

Our studies provide few clues as to the mechanism of protein displacement or the nature of the recognition system that enables granule membranes to fuse specifically with the plasma membrane and then with each other, but not with other membranes. However, the finding of decreased or absent ligand binding in areas where two membranes are interacting but not fused, strongly suggests that protein displacement precedes fusion; in fact, membrane protein displacement appears to occur hand-in-hand with the displacement of visible cytoplasm. It is possible that displacement of cytoplasm and membrane proteins allows the spontaneous fusion of the two lipid bilayers. We could find no evidence for preformed arrays of intramembrane particles similar to the particle rosettes present in plasma membrane fracture faces of Tetrahymena pyriformis, which serve as specialized fusion sites for mucocyst discharge in these ciliated protozoa (41). We have observed loose aggregates of large particles in plasma membrane fracture faces of mast cells, but since they are present in both resting and stimulated cells, they are probably unrelated to the degranulation process. Furthermore, these aggregates cannot be related directly to the clustering of surface receptors induced by the binding of the multivalent stimulating ligands, as they are also observed in cells activated by A23187. The loose particle aggregates also have no apparent topological relationship to blebs or other cellular components, and so their significance remains obscure.

In mast cell degranulation, and in exocytosis in general, the events following membrane fusion, and which lead to the opening of the granule contents to the extracellular space, are unknown. Our finding of simple and vesiculated blebs in regions of granule and plasma membrane interaction raises the possibility that these structures may play a role in degranulation. Consistent with this view is the finding that blebs are more frequent in stimulated cells than in unstimulated ones and usually are found associated with altered granules – even in unstimulated cells. Since blebs involving plasma and granule membranes can be seen occasionally overlying unaltered granules, it is conceivable that the pinching off of such blebs by fission provides the mechanism, in some cases, for opening the granule contents to the extracellular space; at the same time it would allow the cell to dispose of excess lipid (resulting from the fusion of large amounts of granule membrane with plasma membrane) while conserving membrane proteins. It is possible that a similar process involving adjacent granule membranes leads to the formation of the cytoplasmic multilamellar membrane whorls (myelin figures) that are sometimes seen within cavities containing altered granules. On the other hand, blebs and myelin figures are seen in a variety of cell types and have long been considered artifacts, and we cannot exclude the possibility that they are induced by the fixation process or are the result of inadequate fixation. While their frequency is clearly influenced by the method of fixation we have not found a correlation between their presence and poor fixation. It could be that specific fixation conditions are required for their induction (if they are artifacts) or for their preservation (if they are physiological).

It is difficult to determine from static pictures the sequence of events in a rapidly evolving process such as degranulation in mast cells. Nonetheless, we feel that our observations are consistent with the following tentative hypothesis: a triggered influx of Ca2+ leads to a multifocal lateral displacement of cytoplasm and proteins in plasma and underlying granule membranes (possibly mediated by contractile elements, such as microfilaments) which allows the two membranes to fuse; the fused lipid bilayers, being depleted of protein and thus, perhaps, unstable, bulge from the cell to form a simple bleb, which subsequently vesiculates and pinches off, opening the granule contents to the extracellular space, so that histamine is released by cation exchange (43). It seems likely that at least some of these postulated events occur in other examples of exocytosis and that membrane protein displacement may prove to be a common feature of transient membrane fusion.

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