A FREEZE-FRACTURE STUDY OF EARLY MEMBRANE EVENTS DURING MAST CELL SECRETION

SUSAN JO BURWEN and BIRGIT H. SATIR

From the Department of Physiology-Anatomy, University of California, Berkeley, California 94720.
Dr. Burwen's present address is the Cancer Research Laboratory, University of California, Berkeley, California 94720.

ABSTRACT

The early membrane events taking place during mast cell secretion were followed in transmission and freeze-fracture electron microscopy. In order to slow down exocytosis and capture intermediate stages of membrane fusion, special conditions of incubation and stimulation were used. These were as follows: (a) the use of incubation media with altered ionic composition, and (b) stimulation with a low dosage of polymyxin B sulfate (4 μg/ml) at low temperature (18°C) for very short incubation times (30-60 s), with or without the presence of formaldehyde (0.8%). Under these conditions, unetchable circular impressions are found on the E face of the plasma membrane, 80-100 nm in diameter, with particles associated with their perimeters. In granule-to-granule fusion, the zone involved is demarcated by one or two rows of particles on the E face. In addition, raised circular areas of varying diameters (43-87 nm) surrounded by similar particles, also found on the E face, may represent potential sites before completion of fusion. Neither the circular impressions on the plasma membrane nor the sites on the granule membrane are permanent, but their appearance coincides with initiation of membrane fusion.

Secretion of the products histamine and heparin from the rat peritoneal mast cell has been shown by transmission electron microscopy to occur by the well-known pathway of exocytosis (1, 10, 13, 18). The normal pattern of release involves an initial fusion between the cell membrane and the membranes of the most peripherally located granules. As secretion proceeds, more interior granules become involved through fusion of adjacent granule membranes. This sequential recruitment of more internally located granules, resulting in the formation of cavities, is referred to as "compound exocytosis" by Douglas (10). Continuity between cell exterior and granule interior is established while the cell membrane and cytoplasm remain intact (18). Exocytosis in the mast cell, as in many other secretory systems, requires the presence of Ca ++ and is energy dependent.

The observations presented here trace the events in membrane fusion during exocytosis by means of freeze-fracture electron microscopy (FEM). Earlier studies of the mast cell in FEM showed that fracture faces of the plasma membrane, in areas conforming to contours of underly- ing granules, appear as smooth zones devoid of particles (7). When the cells were stimulated to secrete in a normal ionic environment, the underlying granules formed large smooth bulges on the plasma membrane fracture face (8), and no fusion-associated rosettelike structures, such as those of Tetrahymena (21), were seen. However, the conditions of stimulation used may not have
caught the very initial stages of exocytosis (19). In the work presented here, not only is secretogogue used at low dosage and low temperature, but the incubation time is shorter, and the ionic composition of the incubation medium is altered to retard the exocytosis response. Under these conditions, in addition to the smooth zones, circular impressions with associated intramembranous particles are observed on both plasma and granule membrane fracture faces, whose appearance coincides with the initiation of exocytosis. These structures may be potential fusion sites.

MATERIALS AND METHODS

Collection and Enrichment of Mast Cells

Mast cells are collected from the peritoneal cavities of male rats, 150–180 g, of the Long Evans strain (University of California Berkeley Colony), using the method of Röhlich et al. (18). Phosphate-buffered saline (PBS) (pH 7.1), containing 1 mg/ml bovine serum albumin (18), is pipetted through an incision into the peritoneal cavity of a previously exsanguinated rat. The fluid is recovered with a medicine dropper after 60–90 s of massage, and centrifuged at 200 g at 4°C for 5 min. The resulting pellet consists of approx. 5% mast cells.

The pellet is enriched for mast cells by a method based on a discontinuous Ficoll gradient adapted from Bach et al. (2). Each pellet is resuspended in 1 ml of PBS, layered onto 1 ml of Ficoll (35 g/100 ml Tyrode’s buffer) in a 15-ml conical centrifuge tube, and centrifuged for 15 min at room temperature in an International clinical centrifuge (International Equipment Co., Needham Heights, Mass.) at 650 g. Mast cells penetrate into the Ficoll layer while the remaining cell types, which accumulate at the saline–Ficoll interface, are removed. The Ficoll is then diluted to 10 ml with PBS, centrifuged and washed twice with PBS (200 g, 4°C, 5 min). A better than 14-fold enrichment (>70% mast cells) is achieved.

Experimental Procedure

Mast cells derived from three to eight rats are pooled after purification in Ficoll. The enriched pellet is resuspended in PBS, or in unbuffered 0.3 M sucrose at neutral pH with CaCl₂ or MgCl₂ (0.9 mM) added, and incubated at 18°C for 30–60 s in a final vol of 1 ml. A controlled amount of histamine secretion is obtained with polymyxin B sulfate (4 µg/ml of incubation mixture) with or without addition of 0.8% formaldehyde. Samples are taken after stimulation for examination by transmission electron microscopy (TEM) and FEM.

Transmission Electron Microscopy

For TEM, cells are fixed with 9 ml of ice-cold 2% glutaraldehyde in 0.2 M Millonig’s buffer (pH 7.4) for 1 h at room temperature. The cells are then washed in Millonig’s buffer containing 6% sucrose, and postfixed in 1% OsO₄ in the same buffer for 1 h at 4°C. The samples are resuspended in 2% gelatin and pelleted in a microfuge for 5 min, dehydrated in an alcohol series, en bloc stained with 2% uranyl acetate in 70% alcohol for 10 min, and embedded in Epon 112. Silver sections are cut on a Reichert model Om U2 microtome (American Optical Corp., Scientific Instrument Div., Buffalo, N.Y.) and stained with uranyl acetate and lead citrate. Electron micrographs are taken on Siemens 1A and 101 electron microscopes at 80 kV.

Freeze-Fracture Electron Microscopy

Cells are fixed with 4% glutaraldehyde in Millonig’s buffer for 10 min at room temperature, centrifuged, resuspended in 20% glycerol in Millonig’s buffer at 4°C for 2–24 h, and frozen in liquid Freon 22. Samples are fractured with a Balzers apparatus (Balzers AG, Balzers, Liechtenstein) at −115°–120°C. Replicas are recovered after washing in bleach overnight, mounted on naked grids, and examined on Siemens 1A and 101 electron microscopes.

RESULTS

Transmission Electron Microscopy

A representative view of the enriched mast cell population is shown in Fig. 1. The cells are characterized by their bilobed nuclei, the densely packed electron-opaque secretory granules surrounded by unit membranes, and the fingerlike surface projections which resemble microvilli in TEM but have been recognized as microfolds in scanning electron micrographs (6, 12, 23). This population of cells was incubated in 0.3 M sucrose and 0.9 mM CaCl₂ at 18°C and stimulated with polymyxin B sulfate (4 µg/ml) for 30 s. Under these mild conditions, only a few granules are induced to release, forming cavities as, presumably, more internally located granules become involved in compound exocytosis (arrow). Other mast cells appear unaffected by this short treatment, indicating that the cells are heterogeneous with respect to their sensitivity to stimulation. This low level of stimulation was purposely selected in order to catch the very initial membrane events associated with exocytosis.

The first of these events is an association between plasma and granule membranes shown in Fig. 2. The two membranes come into physical contact by the formation of a depression approx. 16 nm deep in the plasma membrane. The area of contact between the two membranes is about 34 nm long. Similar profiles of contact between plasma and granule membranes have been seen by
Figure 1 Representative group of mast cells from a population incubated in 0.3 M sucrose + 0.9 mM CaCl₂ and stimulated to secrete with polymyxin B sulfate (4 μg/ml) at 18°C for 30 s. Most of the cells appear relatively unaffected by this mild stimulation, while the remaining cells have a few discharging granules and accompanying formation of cavities (arrow). × 7,800.
Anderson et al. (1) and Lagunoff (13). Pentalaminar associations between plasma membrane and granule membrane have not been observed for this system. However, these associations are observed between adjacent granule membranes (Fig. 4) (13) (see below). The next recognizable step in the process of membrane fusion is the classic inverted omega profile of exocytosis (Fig. 3). No intermediate stages between these two profiles (Figs. 2 and 3) have been observed for mast cells in TEM.

Formation of a pentalaminar configuration between two adjacent granules is shown in Fig. 4. In this case, the arrangement of membrane is not associated with a secretory event, insofar as the granule contents are not solubilizing. Associations of this nature between adjacent granule membranes occur with about equal frequency in both control and stimulated populations of mast cells. The inset (Fig. 4) shows a high power view of the pentalaminar association, designated as membrane fusion in other systems (17).

Freeze-Fracture Electron Microscopy

The mast cell can be positively identified in FEM by three key features shown in Figs. 5 and 6: (a) the overall size and shape of the freeze-fracture image (Fig. 5) resembles that of the cell as seen in thin section (Fig. 1), (b) the contours of underlying granules are clearly visible (Fig. 5), and (c) the surface microfolds appear as snakelike cross-fractures on the fracture face of the plasma membrane (Figs. 5 and 6). Fig. 6 shows the cross-fractured folds in greater detail. The fracture plane occasionally skips down from the cell surface to the membrane of closely underlying granules (arrows).

The P-fracture face of a secreting mast cell is shown in Fig. 7. As secretion proceeds, extensive intergranular fusion occurs, and the membranes of six to seven adjacent granules can be observed on one continuous E-fracture face. This type of sequential fusion (compound exocytosis) forms the cavities seen in thin sections of secreting cells (Fig. 1). The zone of fusion between adjacent granule membranes is demarcated by a row of particles of varying sizes (arrows). Face-on views of this area are shown in Fig. 8a-c. The cross-fractured region of granule membrane seen in Fig. 8a (arrow) corresponds to the narrowed area between two fused granules on Fig. 7. Some of the particles associated with the fusion zone are now visible in the rim of the 250–300 nm opening on the E face of the granule membrane. On other granule mem-
FIGURE 4 Formation of a pentalaminar association between membranes of adjacent secretory granules. This particular cell is from a control population, but associations of this nature occur with about equal frequency in both control and stimulated populations. This pentalaminar structure is shown in higher power in the inset. $\times 102,000$; inset $\times 168,000$.

Careful examination of E-fracture faces of the plasma membranes of cells stimulated in an altered ionic environment (polymyxin, 30 s, 18$^\circ$C, in the presence of 0.3 M sucrose + 0.9 mM CaCl$_2$) reveals the presence of circular impressions (Fig. 9). Incubation in sucrose enhances the occurrence of these impressions. The inset in Fig. 9 is a higher magnification of the area within the rectangle and shows one such circular impression in detail (arrow). More of these circular impressions are shown in Fig. 10. They are all found on E faces of plasma membranes from three different preparations. In Fig. 10a the cells were stimulated, as described above, in the presence of sucrose and calcium. In Figs. 10b and c, Mg$^{2+}$ was substituted...
for Ca²⁺, but conditions of stimulation were otherwise identical. Mg²⁺ seemed to facilitate capture of the rosettelike images. In addition, the cells in Fig. 10c were slightly etched. In all cases, the diameters of the circular impressions are relatively uniform, ranging from 80 to 100 nm. Particles are often observed in the perimeters of these structures (arrow). The circular impressions are shown to be non-etchable in contrast to other areas of the same membranes (Fig. 10c), thus indicating that the zone within the impression is true membrane.

The resemblance of these structures to nuclear pores is striking (Figs. 11a–b). The outer diameters of the pores also range from 80 to 100 nm. Due to this resemblance, great care has been taken to ensure that the circular impressions (Figs. 10a–c) are located on the plasma membrane.

DISCUSSION
Membrane events accompanying exocytosis have proved difficult to observe with conventional electron microscopy, either because they take place...
too rapidly or because conventional fixation techniques cannot arrest intermediate stages. However, with the recent application of the freeze-fracture technique, which allows interpretation of site-specific information concerning the interior of membranes (4, 22), fusion rosettes and a sequence of membrane events were obtained accompanying secretion of mucocysts and trichocysts in the ciliated protozoa *Tetrahymena* and *Paramecium* (3, 21). In the case of mast cell secretion observed in FEM, previous incubation conditions used (8) did not succeed in catching
FIGURE 8 a-c Potential fusion sites on granule membranes. (a) The cross-fractured area on this granule membrane (arrow) corresponds to a face-on view of the area of fusion in Fig. 7. The opening is approx. 250–300 nm in diameter, and particles can be seen in the rim which correspond to the rows of particles demarcating the zone of fusion in Fig. 7. (b and c) Smaller raised areas of membrane, surrounded by particles but not cross-fractured, are observed on some granule membranes (arrows). Areas range in size from 43 to 87 nm. Conditions of stimulation: control. A11: × 46,000.

FIGURE 9 Circular impressions on the E face of the plasma membrane of stimulated cells. Inset shows a higher power view of one such circular impression (arrow) within the outlined rectangle. Conditions of stimulation: incubation in 0.3 M sucrose + 0.9 mM CaCl₂, stimulation with polymyxin (4 µg/ml), 18°C, 30 s in the presence of 0.8% formaldehyde. × 19,000; inset: × 58,200.

early membrane events associated with exocytosis. Therefore, in these experiments, the following special incubation procedures were employed: (a) stimulation at 18°C with a low dosage of polymyxin B sulfate (4 µg/ml incubation medium), as developed by Lagunoff and Wan (14); (b) in some cases, slight prefixation with formaldehyde before stimulation, which, in the case of the frog neuro-
FIGURE 10a-c  Circular impressions all on E faces of plasma membranes from mildly stimulated cells. They range in size from 80 to 100 nm and have particles associated with their perimeters (arrow). Three different preparations are shown: (a) Conditions of stimulation: incubation in 0.3 M sucrose + 0.9 mM CaCl₂, stimulation with polymyxin (4 µg/ml), 18°C, 30 s. × 60,200. (b) Conditions of stimulation: incubation in 0.3 M sucrose + 0.9 mM MgCl₂, stimulation with polymyxin (4 µg/ml), 18°C, 30 s. × 58,200. (c) This preparation has been slightly etched. The conditions of stimulation are the same as for Fig. 10b. × 58,200.

muscular junction, was shown to capture intermediate stages of exocytosis (11); and finally, (c) the use of nonionic incubation media with or without substitution of Mg⁺⁺ for Ca⁺⁺. An ionic milieu speeds up granule dissociation osmotically (5, 19, 24). Therefore, by delaying this dissociation in nonionic media, concomitant membrane events appear to be slowed down. The substitution of Mg⁺⁺ for Ca⁺⁺ in the incubation medium also seems to enhance accumulation of sites, probably due to the antagonistic role of Mg⁺⁺ common to many Ca⁺⁺-mediated secretory systems (10).

Plasma Membrane Fusion Sites

The circular impressions seen on fracture face E of the plasma membrane (Figs. 9 and 10) are most often observed in cells stimulated under the conditions mentioned above and appear to be exclusively associated with the E fracture face of the cell membrane. One possible explanation for this oc-
Nuclear pores visible on the fracture faces of the nuclear envelope of a control mast cell. (a) × 27,000. (b) High power view of the area enclosed in the rectangle, showing the structure of the nuclear pores in greater detail. Cf. circular impressions in Fig. 10. × 60,000.

currence is that the stimulus for secretion takes place via interaction with receptor molecules on the surface of the cell which need not be deeply embedded in the lipid bilayer. Another example of an E face-associated array is found in phytohaemagglutinin-stimulated lymphocytes (15). Since
the circular impressions are most often observed in preparations in which exocytosis has just been triggered, they appear to be nonpermanent but inducible sites, in agreement with the findings for the fusion rosettes in Tetrahymena (20).

The sites are 80-100 nm in diameter with particles associated with their perimeters and have a striking resemblance to nuclear pores (Figs. 10 and 11). The fact that these sites resemble nuclear pores strongly suggests that they are associated with membrane fusion, since nuclear pores are the final result of fusion between the inner and outer membranes of the nuclear envelope. Furthermore, formation of nuclear pores is inducible. They are not permanent sites, and their distribution can vary (16), suggesting that in this system, as well, membrane fusion is a regulated event, site and time specific.

It is clear from etched preparations (Fig. 10c) that the circular impressions represent true membrane differentiations because they remain unaltered by the treatment. If, however, cells are stimulated for longer than 30 s, cross-fractured circular areas, having approximately the same dimensions as the circular impressions, appear. These structures resemble craters and are now etchable. They probably represent postmembrane fusion images and not cross-fractured microvilli, since it is known from scanning micrographs (6, 12, 23) that microvilli are exceedingly scarce features of the mast cell surface.

Another possible explanation for the presence of the circular impressions is that they represent sites for pinocytosis. However, our TEM observations indicate that pinocytotic activity is not usually observed under these conditions.

The clustering of particles on the P face of the plasma membrane, observed by Chi et al. (7) in TEM images of control mast cells, is rarely observed in our preparations, and it has not been possible to correlate their occasional appearance with any fusion event.

Granule Fusion Sites

The process of granule-granule fusion leading to compound exocytosis is a controlled, regulated process that generally does not take place until an opening to the external milieu has first occurred, via fusion of one of the peripherally located granules with the plasma membrane. This fusion must somehow induce a change in the granule membrane that allows other granules subsequently to fuse with it. One might speculate that this change is the inducer necessary for compound exocytosis, since before this event spontaneous fusion between granules is inhibited.

It is of interest to note that the lines of particles demarcating the zone of fusion between adjacent granules in the mast cell (Fig. 7) are also observed in in vitro fusion of isolated β-granules from the islets of mouse pancreas by Ca++ (9). It appears, however, that the β-granule preparation consists mostly of twins (two fused vesicles), with very few triplets, in contrast to in vivo systems. Upon addition of Ca++, the intramembrane particles on the β-granules cluster before pore formation.

In mast cells, we observe rings of particles on the E face of the granule membranes and small particle-rimmed blebs (Figs. 7 and 8) which are not cross-fractured. It is not possible to ascribe any polarity to the location of these arrays, as can be done with the arrays of the secretory organelles of Tetrahymena and Paramecium, which are always located at the anterior end of the granules (3, 19).

In the case of the mast cell, one granule is capable of multiple fusions, as shown in Fig. 7, in which a granule is clearly fused with at least three others. It should be kept in mind, however, that compound exocytosis does not take place in protozoa.

In summary, we have presented evidence that points to a role for these arrays in the regulation of the fusion event, similar perhaps to that for the fusion arrays, in Tetrahymena and Paramecium.

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