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EVIDENCE OF de novo MEMBRANE GENERATION IN THE MECHANISM OF MAST CELL SECRETORY GRANULE ACTIVATION

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Summary - Evidence which suggests the occurrence of a rapid new membrane assembly has been observed in the secretory granules of the rat peritoneal mast cell during the early stage of granule activation. The rapid insertion of these newly generated vesicles enables the perigranular membrane of the activated granule to enlarge and expand prior to fusion with the plasma membrane and/or with the neighboring granule membranes. If the newly inserted membrane represents "specialized fusogenic membrane patches", then the presence of de novo membrane generation as an integral step in the mechanism of mast cell granule exocytosis would constitute a fail-safe mechanism in histamine release.

Overwhelming evidence has suggested that granule matrix swelling and the enlargement of the perigranular membrane are early morphological indicators of mast cell granule activation (1-3). Since excess membrane in the form of folds or inclusions has not been observed in the quiescent granule and it is known that membrane cannot stretch beyond 2-3% of its original area (4), it is necessary to consider that new membrane is generated during granule activation. Fusion of this newly generated membrane with the perigranular membrane would enable the granule to expand and the perigranular membrane to lift from the surface of the granule matrix.

By using various ultrastructural techniques, we found that: First, rapid-freezing and freeze-substitution of mast cells reveals the presence of membrane vesicles associated with activated or secreted granules. Second, purified granules after removal of their perigranular membrane can generate membrane vesicles. And third, membrane vesicles and the fusion of these vesicles with the perigranular membrane have been observed using a simultaneous activation, fixation, and membrane enhancement (SAFME) procedure.

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Based on these findings, we proposed that de novo membrane generation can occur as part of the activation process in the mechanism of mast cell granule exocytosis.

MATERIALS AND METHODS

Preparation of mast cells and mast cell granules: Rat peritoneal mast cells were purified according to Sullivan et al. (5) and mast cell granules were purified according to Kruger et al. (6). When perigranular membranes were to be removed, the washed intact granules were sedimented and resuspended in cold deionized water with gentle vortexing. The osmotically lysed granules were then pelleted by centrifugation at 1100xg for 10 min.

Specimen preparation for ultrastructural studies: For routine electron microscopy, samples were fixed in 2.5% glutaraldehyde buffered in 100 mM sodium cacodylate containing 4 mM $MgCl_2$ and osmicated in 1% osmium tetroxide made up in the same buffer. After dehydration the samples were embedded in Epon-812. For rapid-freezing and freeze-substitution, purified mast cells were placed in 7% serum albumin made up in HEPES buffered Hank's balanced salt solution and rapid-frozen and freeze-substituted in acetone containing 4% osmium tetroxide (7). For freeze-fracture, purified granules with their membranes removed, were placed in succeeding concentrations of glycerol to a final concentration of 30%. Following routine sample application procedure, specimens were fractured in a Balzers freeze-etch instrument. The replicas were cleaned in sodium hypochlorite prior to examination.

The SAFME procedure: To achieve simultaneous activation, fixation, and membrane enhancement, mast cells were fixed in 100 mM sodium cacodylate containing 4 mM $MgCl_2$, 0.5 mg/ml saponin, 2 mg/ml tannic acid and 1% glutaraldehyde pH 7 for 30 min at room temperature. After several washings with 100 mM cacodylate buffer the specimens were post-fixed in 1% osmium tetroxide before dehydration and embedding.

RESULTS AND DISCUSSION

Early evidence which suggested that mast cell granules could be the source of the new membrane stemmed from the observation of membranous "blebs" in association with activated granules as well as with purified granules (6,8). Although "blebs" are often considered an artifact of glutaraldehyde fixation, their appearance in activated granules suggests that the granules may have the potential to generate membrane. To avoid glutaraldehyde fixation artifacts, we used rapid-freezing and freeze-substitution techniques to investigate if the granule can indeed serve as the source of new membrane. As shown in Fig. 1, membrane vesicles were found in association with activated and with extruded granules in the absence of glutaraldehyde. This observation supports the concept that the granule can serve as the source of new membrane.

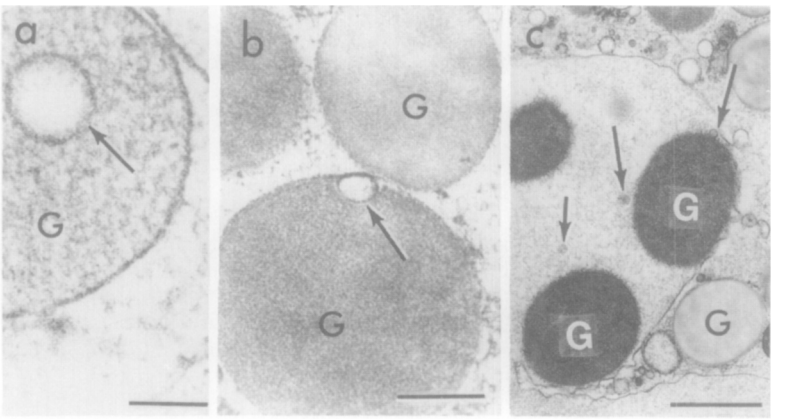

Fig. 1 Vesicles associated with spontaneously activated and released mast cell secretory granules examined using rapid-freezing and freeze-substitution technique. Vesicles (arrows) are found within the granules (G) in an early stage of activation (a and b) and in association with released granules (c). In rapid-freezing, granules in an earlier stage of activation appear less electron-dense than released granules. The bars represent 0.1 micrometer in (a and b) and 1 micrometer in (c).

To further demonstrate that the granule matrix can be the source of new membrane, mast cell granules were purified according to the method of Kruger et al. (6). Perigranular membranes were removed by osmotic shock. Incubation of the demembraned granules in deionized water can result in the formation of bilayer vesicles (referred to as "beads" in ref. 9) at the periphery of the granule matrix or in regions of water infiltration (Fig. 2). When freeze-

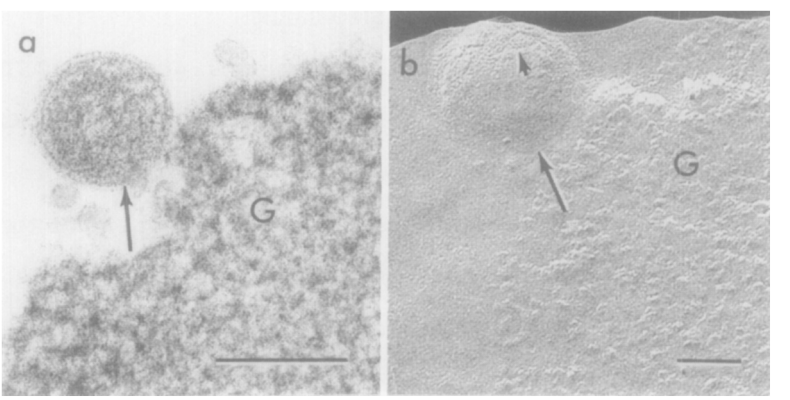

Fig. 2 Purified granules (G), after their membranes have been removed, can generate new membrane in an aqueous environment. (a) Glutaraldehyde-fixed demembraned granule showing the formation of a bilayer bead (arrow) at the periphery of the granule matrix. Frequently electron-dense materials are trapped within the beads. (b) Freeze-fracture of demembraned granule (arrow) reveals a typical bilayer fracture plane (arrowhead) containing no intramembrane particle. The bar represents 0.1 micrometer.

fractured, these newly formed beads showed the typical bilayer fracture plane (Fig. 2b). The absence of intra-membrane protein particles might suggest that the beads are newly formed membrane. During mast cell activation the infiltration of water from the cytoplasm and/or from the nucleus into the granule may result in the rapid formation of bilayer beads in the periphery of the granule matrix situated directly under the perigranular membrane. These newly generated membranes may fuse readily with the perigranular membrane causing it to expand and lift from the granule matrix.

The process of mast cell degranulation has been measured in the millisecond time range (10). The plant glycoside, saponin, was used in low concentration to activate the granules while simultaneously permeabilizing the cells to glutaraldehyde and tannic acid (SAFME procedure). A typical result obtained with the SAFME procedure is shown in Fig. 3. In this figure, membrane vesicles can be seen in the space between the granule matrix and the perigranular membrane. Some appear to be in the process of fusing with the perigranular membrane. These vesicles are not artifacts due to the presence of saponin since their presence has also been observed recently in our laboratory in granules activated by other means and in the absence of saponin. These vesicles may be a product of de novo membrane generation originating from within the granule caused by the activation of the granule by saponin. This interpretation is based on the following reasons. (1) The amorphous and electron-dense quiescent granule can be activated to form membrane vesicles (Fig. 1-3). Thus the granule is capable of serving as the source of the new membrane. (2) There are no observable membrane vesicles congregating outside the granules before or after their activation, suggesting that these membrane vesicles seen in Fig. 3 could not have originated from the cytoplasm. Furthermore, there is no known mechanism by which membrane vesicles can traverse a large membrane and reappear as vesicles on the other side of the membrane. (3) These vesicles shown in Fig. 3 could not have resulted from the process of "granule endocytosis" since the occurrence of such a process would lead to perigranular membrane shrinkage rather than the rapid expansion

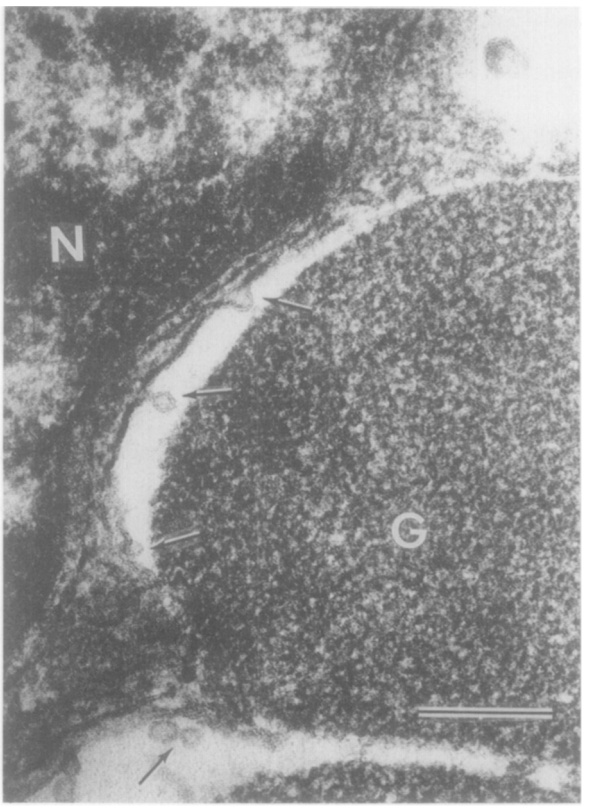

Fig. 3 de novo membrane generation induced by the SAFME procedure. Mast cell granules (G) in early stage of activation are seen near the nucleus (N). Vesicles or beads (arrows) are seen between the perigranular membranes and the granule matrices. Beads in various stages of fusion with the perigranuar membrane can also be seen. The bar represents 0.25 micrometer.

observed following activation. (4) The rapid expansion of the perigranular membrane during granule decondensation, can be accounted for by the fusion of new membrane assembled from membrane precursor elements stored in the granule matrix (9). This entropy-driven process can be very rapid since it does not involve a chemical reaction.

It is not clear if the newly inserted membrane might represent specialized fusogenic membrane patches inserted for the purpose of promoting fusion. If it were so, then the incorporation of the process of de novo membrane generation into the mechanism of secretion would constitute a fail-safe mechanism for secretion. It not only prevents accidental fusion among unactivated quiescent granules, but also assures the success of exocytosis

once the correct activating signal has been received by the granules. Since secretory granules are not known to be able to percolate or move rapidly in the cytoplasmic matrix, it is not clear how an activated granule can come into contact with the plasma membrane in order to form a pore and secrete its contents. The incorporation of the process of de novo membrane generation into the mechanism of secretion, not only enhances the chance of contact between the expanding activated perigranular membrane and the plasma membrane; but it might also assure the success of pore formation.

The evidence presented here is in agreement with the many published observations on mast cell secretion. It is also consistent with our hypothesis that de novo membrane generation occurs as part of the secretory mechanism for the mast cell. It explains how granule swelling and the lifting of the perigranular membrane can occur during exocytosis. The lifting and expansion of the perigranular membrane not only increase the probability of contact between the activated granule and the plasma membrane and/or with the adjacent granules to assure fusion, it can also result in the formation of a large exocytotic cavity to assure rapid washout of the soluble granule mediators. When the expanding perigranular membrane comes into contact with the adjacent granules, the fusion among these granules would result in the formation of a large membrane-lined common cavity before pore formation. This may be the mechanism by which granules activated deep within the cell can be released.

REFERENCES

1. Bloom, G.D. and Haegermark, O. (1965) Exptl. Cell Res. 40, 637-654.
2. Lawson, D., Raff, M.C., Gomperts, B., Fewtrell, C., and Gilula, N.B. (1977) J. Cell Biol. 72, 242-259.
3. Anderson, P., Slorach, S.A., and Uvnas, B. (1973) Acta Physiol. Scand. 88, 359-372.
4 Kwok, R. and Evans, E. (1981) Biophysical J. 35, 637-652.
5. Sullivan, T.J., Parker, K.L., Stenson, W., and Parker, C. (1975) J. Immunol. 114, 1473-1478.
6. Kruger, P.G.,Lagunoff, D.,and Wan, H.(1980) Exptl. Cell Res. 129, 83-93.
7. Heuser, J.E., Reese, T.S., Dennis, M.T., Jan, Y., Jan, L., and Evans, L. (1979) J. Cell Biol. 81, 275-300.
8. Lawson, D. (1980) in Membrane-Membrane Interactions. Gilula, N.B. (ed.) Raven Press, New York. pp 27-44.
9. Chock, S.P. and Chock, E.A. (1985) Fed. Proc. 44, 1324 (534la).
10. Douglas, W.W. (1974) Biochem. Soc. Symp. 39, 1-28.