# FREEZE-FRACTURE OF MEMBRANE FUSIONS DURING EXOCYTOSIS IN PANCREATIC B-CELLS

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ABSTRACT

To examine the freeze-fracture appearance of membrane alterations at sites of exocytosis in mammalian cells, we studied the secretory granule and plasma membrane of rat pancreatic B-cells during glucose-stimulated insulin secretion. Constant features observed were the scarcity of particles in secretory-granule P-fracture faces and the almost total clearance of intramembranous particles in P- and E-fracture faces of the plasma membrane in areas of close apposition of these two membranes preceding fusion; also observed was the temporary persistence of particle-cleared regions after the fusion was completed. Our observations thus support the concept that membranes fuse at sites of closely apposed, particle-free regions and that the physiologically created clear areas found in freeze-fracture replicas of the plasma membrane are the hallmarks of incipient or recent membrane fusion.

KEY WORDS B-cell · exocytosis · freeze-fracture · membrane · islet of Langerhans

Fusion between a secretory granule membrane and the plasma membrane has been thoroughly studied by electron microscopy of thin sections. Observed in such sections, the secretory granule first moves close to the plasma membrane; then the two form a transient pentalaminar membrane, and subsequently a trilaminar membrane is restored during granule-content expulsion through the resultant orifice (4, 6, 10, 14, 20, 25, 26). The examination of freeze-fracture replicas yields more information. In two protozoan genera—*Tetrahymena* (23) and *Paramecium* (21, 22)—a distinctive arrangement of membrane particles, designated the "rosette," characterizes the plasma membrane at the presumptive site of fusion. So far, no counterpart of this protozoan rosette (or necklace) has been convincingly demonstrated in mammalian secretory cells. Rather, as we report here, a clearance of membrane particles apparently occurs at the point of exocytotic membrane fusion in the pancreatic B-cell concomitant with insulin release. In B-cells, insulin is contained in specific secretory granules which can be induced to fuse with the plasma membrane by glucose stimulation (8). In the rat, B-cells comprise about 80% of the total mass of the islets of Langerhans, and, by means of mild collagenase digestion (9), can be freed from exocrine tissue, permitting the attainment of a pure fraction of islets for freeze-fracture studies. The B-cells can be detected in freeze-fracture replicas, due to their central location in the islet (7). Images of exocytosis taking place in this region during glucose stimulation are therefore validly attributable to secreting B-cells (14, 16). The system we have selected here is of advantage in studying exocytosis because: (*a*) it has a well-defined stimulus for inducing insulin secretion, (*b*) it shows a sustained secretory response during stimulation, and (*c*) it has been

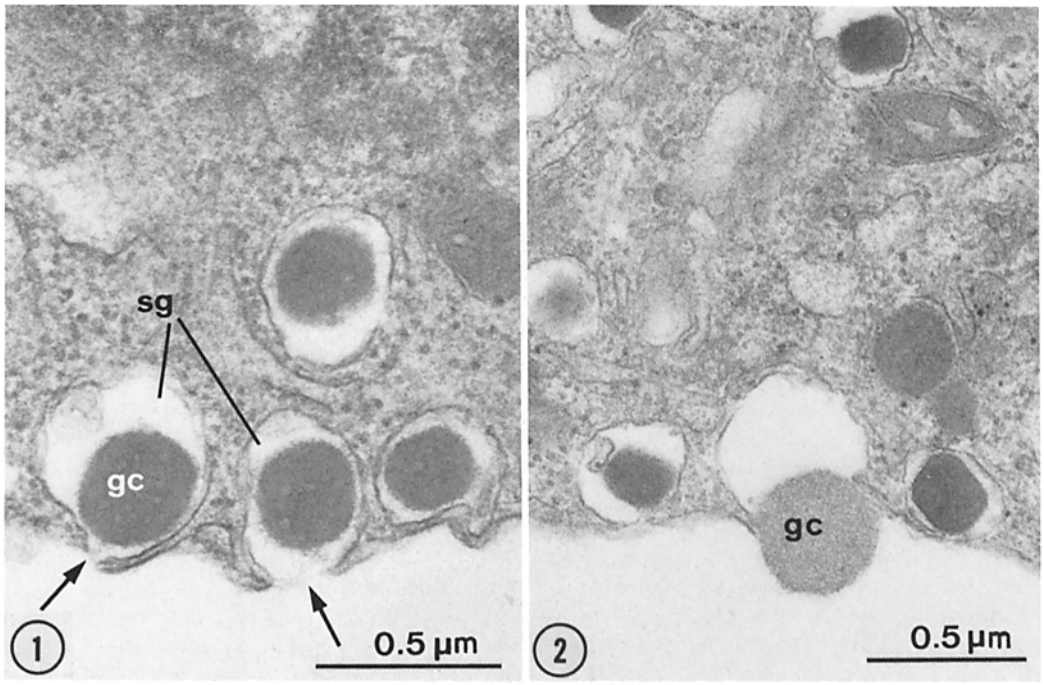
amply documented in freeze-fracture/thin section correlates of cellular membranes (14–17, 19).

## MATERIALS AND METHODS

Islets were isolated from rat pancreases by collagenase digestion (9). The isolated islets were then incubated for 15–60 min at 37°C in a bicarbonate-buffered medium equilibrated with a 95% $O_2$-5% $CO_2$ gas mixture containing 0.5% albumin (wt/vol) and glucose at a stimulatory concentration (3.0 mg/ml). After incubation, the stimulated islets were packed into pellets by centrifugation, and the pellets were fixed in 2% glutaraldehyde buffered with phosphate (0.1 M, pH 7.4, 30 min). After fixation, the pellets were soaked for 30–120 min in a 30% glycerol solution also buffered with phosphate. They were then frozen in Freon 22 (E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.) cooled in liquid nitrogen, and finally fractured and shadowed in a Balzers BAF 301 freeze-fracture device (Balzers A. G., Balzers, Liechtenstein) according to the technique of Moor and Mühlethaler (13). Tissue replicas were cleaned in a sodium-hypochlorite (Clorox R) solution, rinsed in distilled water, and mounted on copper grids. Replicas were examined in a Philips EM 300 electron microscope.

## RESULTS

Islets stimulated for 15, 30, and 60 min were examined and yielded essentially similar results. Electron microscope images of exocytosis, commonly encountered in thin sections, show the continuity between the plasma membrane and the secretory granule membrane, exposing the granule core to the extracellular space (Figs. 1 and 2). Stages preceding the one where the actual union of the membranes can be observed – namely, the merging of the granule membrane (first the outer leaflet, then the inner) with the leaflets of the plasma membrane (in converse order) – are rarely documented (26). Thin sections disclose no significant differences in the morphology of the two unit membranes involved. Freeze-fracturing exposes the inner structure of both the granule membrane and the plasma membrane and provides new information about the fusion process (Fig. 3) (2, 11, 17). The inner leaflet of the B-cell plasma membrane (P face) contains a large number of intramembranous particles (~2,000/$\mu m^2$). Apart from specialized regions of this membrane, such as




FIGURES 1 and 2 Thin sections of glucose-stimulated (3 mg/ml) pancreatic B-cells showing several images of exocytosis. The granule core (*gc*) of secretory granules (*sg*) is variably exposed to the extracellular space through openings (arrows) created by the merging of the limiting membrane of the secretory granule with the cell (plasma) membrane. Fig. 1: × 49,000; Fig. 2: × 43,000.

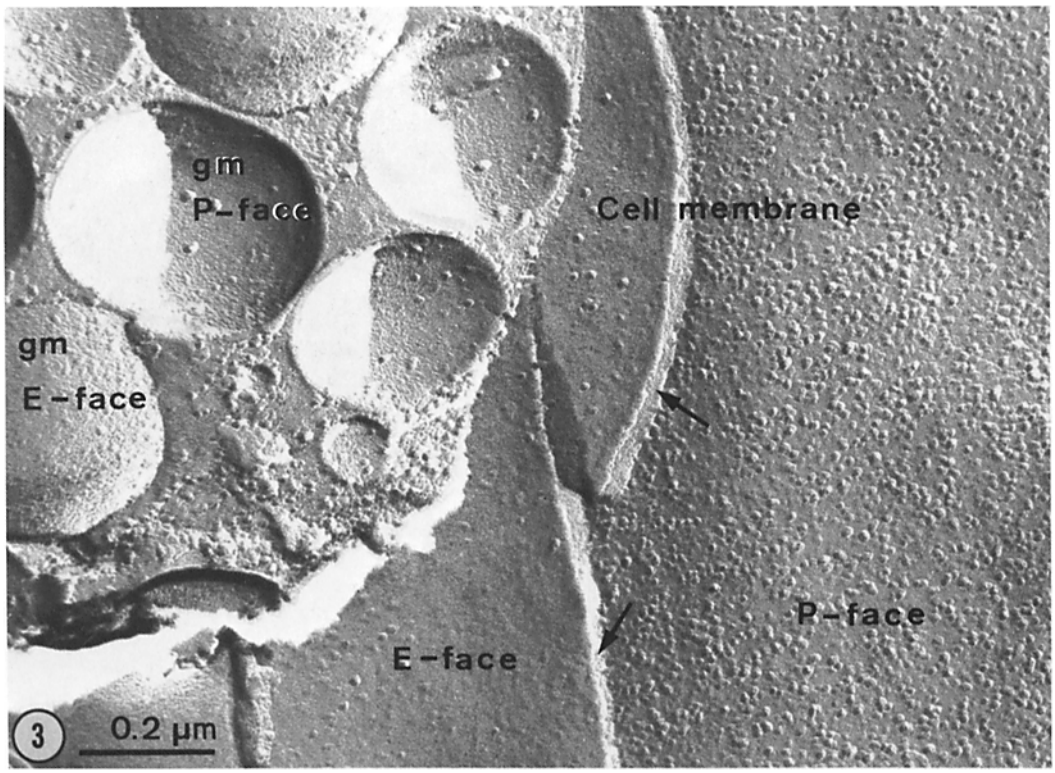
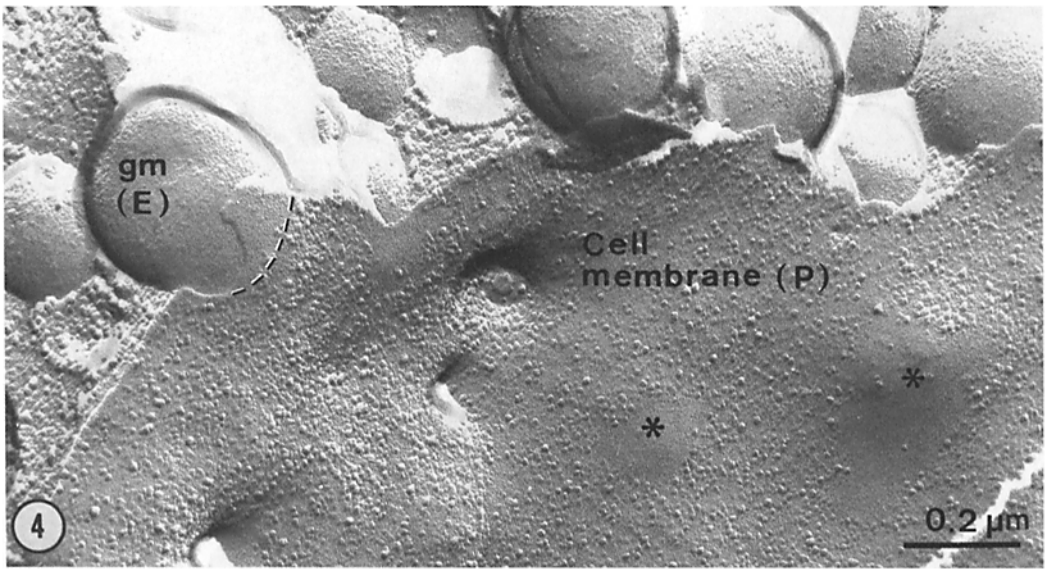



FIGURE 3 Freeze-fracture replica of unstimulated pancreatic B-cells showing the main components involved in exocytosis. On the right, the fracture plane has exposed the plasma membranes of two adjacent B-cells, revealing, respectively, the E face (outer leaflet) of one membrane and the P face (inner leaflet) of the other. The step indicated by the arrows represents the thickness of the intercellular space between the two adjacent cells. On the left, a cross-fracture of the cytoplasm exposes membrane faces of the secretory granules. Concave profiles correspond to P faces of the granule limiting membrane (*gm*); convex profiles represent E faces of the granule membrane (*gm*). Note the striking difference in the intramembrane particle content of the plasma membrane and granule membrane and also the fact that the few particles in the granule membrane are of rather large size. × 91,000.

areas where tight and gap junctions exist, these intramembranous particles are usually dispersed at random. They are between 8 and 10 nm in size, with rare extremes of 13 nm. Morphologically, the outer leaflet of the plasma membrane (E face) resembles the inner leaflet, except that it has fewer particles (~300/$\mu m^2$), the dimensions (8–10 nm) of which are comparable to those of inner leaflet particles. The freeze-fractured secretory granule membrane generally splits to unmask either a convex or a concave spherical face (see Fig. 3). The convex fracture face represents the inner leaflet of the granule membrane (the E face, relating to the interior of the vesicle) while the concave fracture face represents the outer leaflet (the P face of the membrane, relating to the cytoplasmic matrix). In the P-fracture face, membrane-associated particles are scant (~200/$\mu m^2$), numbering even less in the E face (~50/$\mu m^2$). Particles in both fracture faces are about 13 nm in diam. Once the different faces of the granule and plasma membranes have been precisely identified, two circumstances favor proper interpretation of membrane interaction during exocytosis: first, the observation of stimulated cells in order to increase the chance of witnessing granule release; and second, the examination of replicas with fracture planes showing both the cytoplasm and the plasma membrane of the same cell. This latter point is of considerable importance in correlating a tentative temporal sequence of morphological phenomena in the fracture faces of the plasma and granule membranes with the spatial relationships between these organelles. Cells thus cleaved are presented in Figs. 4–8,




FIGURES 4–13 Micrographs from isolated islets stimulated in vitro by 3 mg/ml glucose up to 1 h which illustrate details of different morphological stages of exocytosis.

FIGURE 4 Cytoplasmic and cell (plasma) membrane fracture faces showing a secretory granule [*gm*(*E*)] in close relationship with a particle-free area in the cell membrane (P face) (dotted line); two bare patches (*), one of them overlying a cell membrane bulge, may represent future sites of exocytotic fusion. × 74,000.

10, and 11. In the sequence of exocytosis, three major structural stages can be defined: in the first stage, the fracture face of the secretory granule membrane is observed close to the fracture face of the plasma membrane (see Figs. 4, 5, and 8). Now, the E face of the granule membrane and the P face of the plasma membrane can be fully appreciated, and, at the site of most intimate contact between the two membranes, the P face of the plasma membrane reveals an area (usually ovoid) lacking any intramembranous particles. Without clear-cut transition, this area blends with the normally particulate fracture face. Zones without particles in the plasma membrane P face lie also over circular or elliptical bulges which are interpreted as the result of secretory granules' adhering to the inner aspect of the plasma membrane (Fig. 4).

Occasionally, an opportune fracture plane exposed secretory granule membranes (E faces) through a "window" produced by the local removal of the cell membrane (P face) (Fig. 6). The amount of cytoplasm separating the two fracture faces was exceptionally small, indicating intimacy between the granule membrane and the plasma membrane (cf. Figs. 4, 5, and 8). In this case, the granule membrane appeared to have no particular differentiation. The second morphologically visible stage of exocytosis within a given spatial sequence is depicted in Figs. 8, 9, and 11. In general, these images were obtained only from P faces of the plasma membrane and consist of circular depressions, their centers filled with protruding spheroids. Such depressions are continuous with the membrane face, which is devoid of membrane-associated particles. The central bulge is smooth, and we interpret it as the actual granule core confronting the extracellular space.

Figs. 12 and 13 illustrate the third morphologically identifiable stage considered to relate to B-cell exocytosis. Again, observed on the P face of the plasma membrane, this view of the exocytotic event is apparently not associated with any specific structure in the cytoplasm. Third-stage images consist of circular or slit-shaped lacunae in the membrane face. No intramembranous particles are present in the depressed surfaces, which are surrounded by a normally particulate fracture face. Since these concavities, when contiguous with cross-fractured cytoplasm, are not found in association with either secretory granules or the granule cores which we described earlier, we interpret them as late-stage manifestations of the exo-

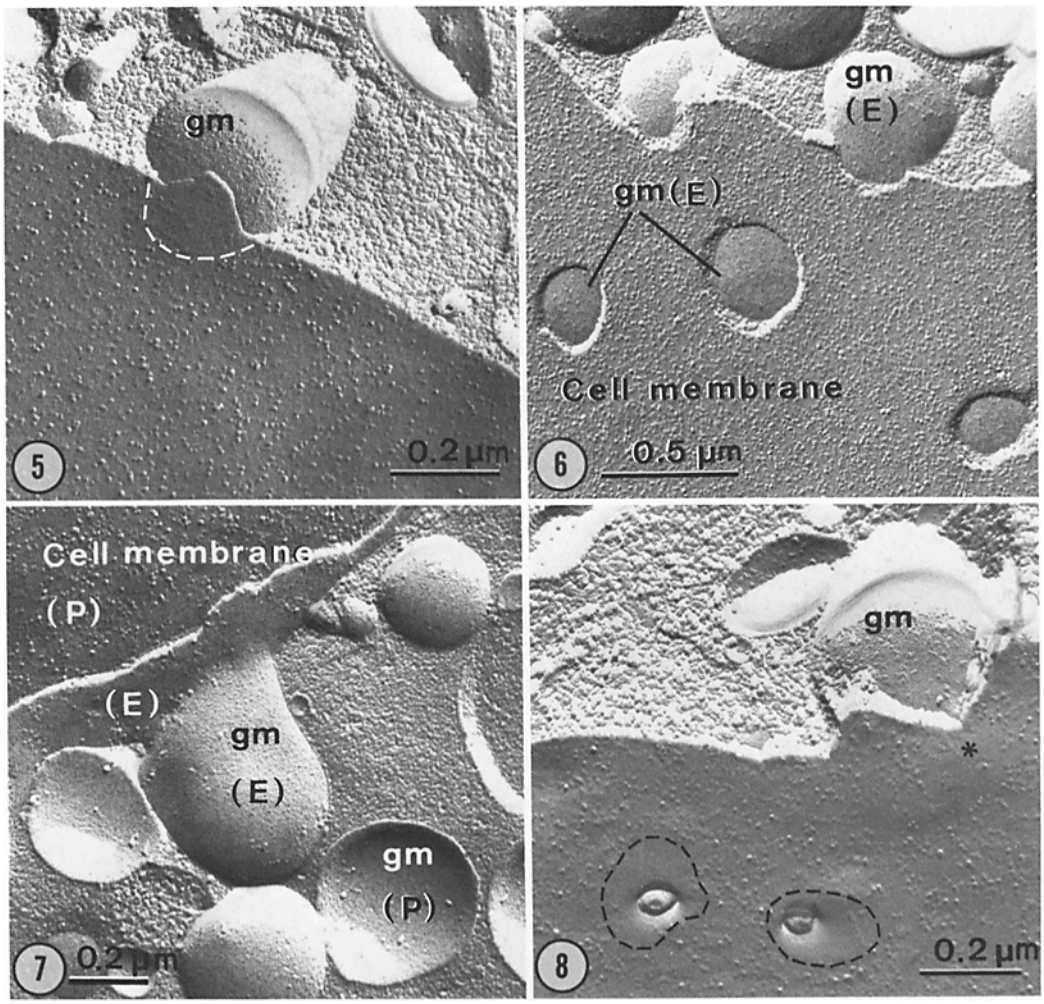



FIGURE 5 Here the proximity of the granule membrane (*gm*) to the cell membrane is demonstrated (the particle-free patch in the latter is encircled by a dotted line). × 71,000.

FIGURE 6 This image discloses several E faces of secretory granule membrane [*gm*(*E*)] both in cross-fractured cytoplasm (upper right corner) and via the windows created by the fracture plane through the islet cell membrane (lower part). None of the exposed E faces of the granules shows signs of morphological differentiation. × 36,000.

FIGURE 7 Cytoplasmic fracture showing the continuity between a granule limiting membrane [*gm* (*E*)] and the E face of the cell membrane. The low content of intramembrane particles in the E face of the cell membrane makes it difficult to visualize a particle-free area at the exocytotic site such as seen on membrane P faces. × 52,000.

FIGURE 8 Islet cell membrane (P face) showing a particle-free area (*) overlying the E face of a secretory granule (*gm*) and particle-depleted zones (dotted circles) surrounding two out-flowing granule cores. × 67,000.

cytotic process, i.e., succeeding membrane fusion and granule-core dissolution. If this is true, they represent remnants of the granule membrane now incorporated into the plasma membrane.

## DISCUSSION

At presumptive sites of fusion with the membrane of secretory granules during stimulation of insulin

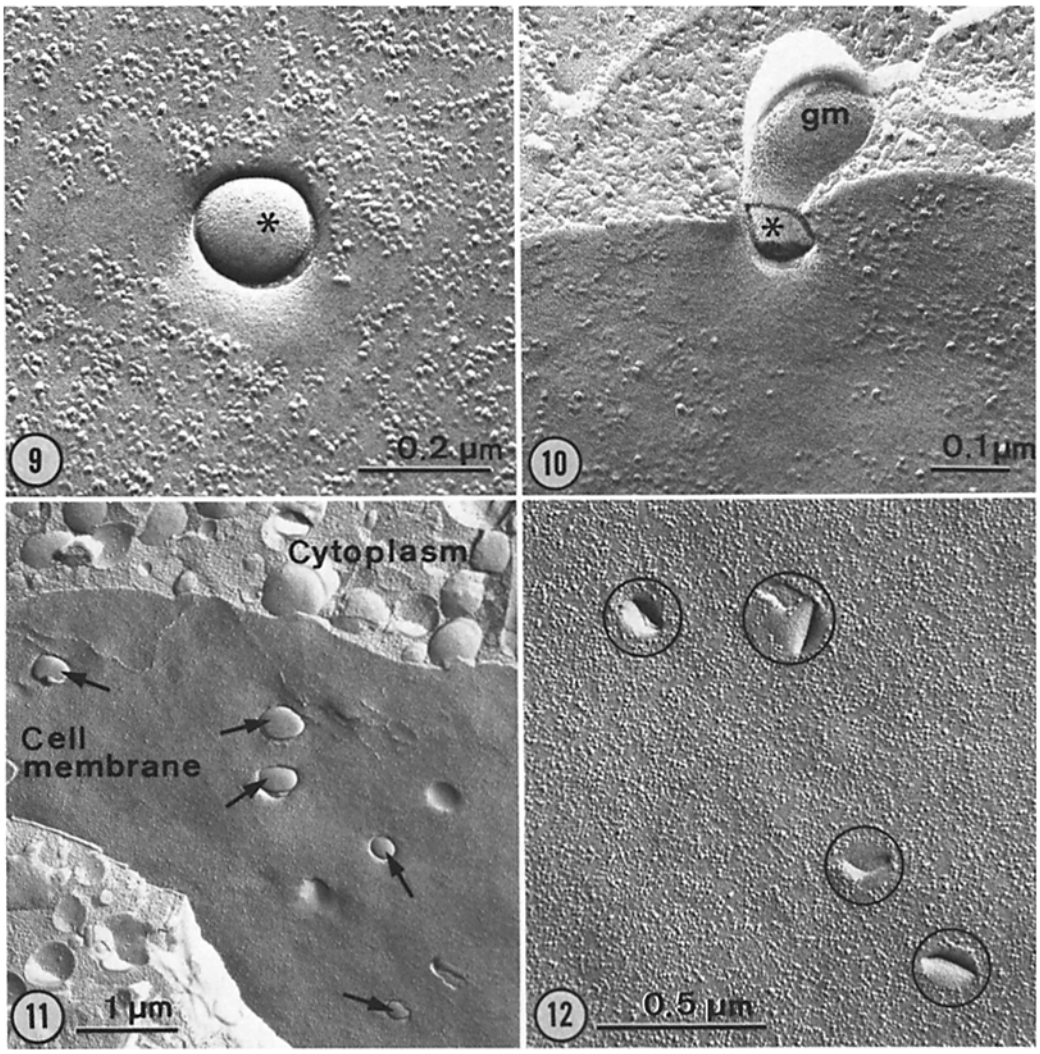



FIGURE 9 The granule core (*) is exposed *en face*, bulging above the fracture face of the membrane. This image is the landmark of a completed exocytotic event. × 86,000.

FIGURE 10 A view of cross-fractured cytoplasm including a secretory granule with its limiting membrane (*gm*) and part of the core (*), which protrudes above the P face of the islet cell membrane and covers the site of granule membrane/cell membrane fusion. In both Figs. 9 and 10, the area surrounding the sites of exocytosis is distinctly particle free. × 105,000.

FIGURE 11 An islet cell membrane (P face) demonstrating many completions of exocytosis (arrows designate the bursting granule cores) in a stimulated B-cell. × 14,000.

FIGURE 12 An islet cell membrane (P face) with four slit-shaped depressions (delimited by black circles) devoid of intramembranous particles. Such replicas are believed to represent late stages in exocytosis, after the dissolution of the granule core within the extracellular space (cf. Fig. 10). × 44,000.

release, the plasma membranes of islet cells undergo characteristic morphological changes. Essentially, these alterations consist of the focal clearance of intramembranous particles in well-delimited areas of the plasma membrane (P faces), bare spots which are visible before, during, and after the actual fusion process. Evidently, this succession of structural phenomena accompanying

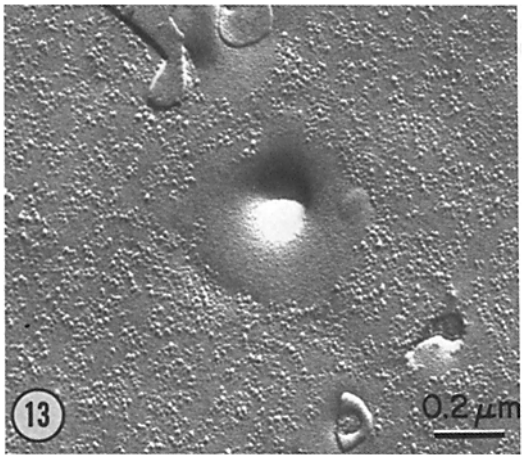



FIGURE 13 A large particle-free area surrounds a depression in the membrane face and is interpreted as the final stage of exocytosis after the granule core (cf. Fig. 9) has dissolved in the extracellular space. See also Fig. 12. × 45,000.

exocytosis is substantively different from the well-studied model of membrane fusion during mucocyst discharge in *Tetrahymena* (23). In this ciliate, the plasma membrane houses specific aggregates of particles—rosettes—at sites of fusion with the secretory granule membrane or mucocyst membrane, which itself possesses an "annulus" in register with the rosette at the time of fusion. In the pancreatic islet-cell system, however, no structural differentiations of the respective secretory granule membrane and plasma membrane appear at presumable sites of fusion. If we assume that the bare areas in a freeze-fractured membrane represents its lipid domains (1, 27, 28), then the exocytotic fusion process in islet cells would only (or mostly) involve nonproteinaceous parts of the membrane, a type of fusion originally proposed by Lucy (12).[1] These data thus question the application of the rosette model of membrane fusion (21–23) to mammalian cell systems (5).

At the moment, interpretations of the mechanism of particle clearance can still be only tentative. What is evident is that the elimination of particles is a prerequisite for the fusion of the two membranes, very diverse in particle content—the islet-cell plasma membrane is rich in particles, while the secretory granule membrane contains but few. Once fusion has occurred, it is likely that the smooth areas serve to accommodate the newly added secretory granule membrane and mediate the equilibration of particle density. The clearing is wholly reversible, for after secretory stimulation ceases (unlike the situation reported for the parotid acinar cell [2]), the islet-cell plasma membrane exhibits a homogeneously distributed particle population.[2]

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[1] The rosette or necklace configurations which have been noted at sites of membrane invaginations in smooth muscle (18) or capillary endothelium (24) do not necessarily indicate the event of membrane fusion, but may represent sites of relatively fixed attachment.

[2] After this paper was submitted for publication, two reports appeared on the freeze-fracture aspects of exocytosis during mast cell degranulation (3, 11). In this system, particle clearing on the plasma membrane and granule membrane fracture faces was also found as the main sign of exocytotic interaction. In addition, one of the studies (11) reported the absence of ligand-binding sites in regions of the plasma membrane interacting with or closely apposed to granule membrane. A similar observation although not specifically related to the present context was made independently in the pancreatic B-cell (19).

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