MEMBRANE INTERACTIONS BETWEEN SECRETION GRANULES AND PLASMALEMMMA IN THREE EXOCRINE GLANDS

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ABSTRACT

Three types of membrane interactions were studied in three exocrine systems (the acinar cells of the rat parotid, rat lacrimal gland, and guinea pig pancreas) by freeze-fracture and thin-section electron microscopy: exocytosis, induced in vivo by specific pharmacological stimulations; the mutual apposition of secretory granule membranes in the intact cell; membrane appositions induced in vitro by centrifugation of the isolated granules. In all three glandular cells, the distribution of intramembrane particles (IMP) on the fracture faces of the lumenal plasma-lemma appeared random before stimulation. However, after injection of secretagogues, IMP were rapidly cleared from the areas of granule-plasmalemma apposition in the parotid cells and, especially, in lacrimocytes. In the latter, the cleared areas appeared as large bulges toward the lumen, whereas in the parotid they were less pronounced. Exocytotic openings were usually large and the fracture faces of their rims were covered with IMP. In contrast, in stimulated pancreatic acinar cells, the IMP distribution remained apparently random after stimulation. Exocytoses were established through the formation of narrow necks, and no images which might correspond to early stages of membrane fusion were revealed. Within the cytoplasm of parotid and lacrimal cells (but not in the pancreas), both at rest and after stimulation, secretion granules were often closely apposed by means of flat, circular areas, also devoid of IMP. In thin sections, the images corresponding to IMP-free areas were close granule-granule and granule-plasmalemma appositions, sometimes with focal merging of the membrane outer layers to yield pentalaminar structures. Isolated secretion granules were forced together in vitro by centrifugation. Under these conditions, increasing the centrifugal force from 1,600 to 50,000 g for 10 min resulted in a progressive, statistically significant increase of the frequency of IMP-free flat appositions between parotid granules. In contrast, no such areas were seen between freeze-fractured pancreatic granules, although some focal pentalaminar appositions appeared in sections after centrifugation at 50 and 100,000 g for 10 min. On the basis of the observation that, in secretory cells, IMP clearing always develops in deformed membrane areas (bulges, depressions, flat areas), it is suggested that it might result from the...
forced mechanical apposition of the interacting membranes. This might be a preliminary process not sufficient to initiate fusion. In the pancreas, IMP clearing could occur over surface areas too small to be detected. In stimulated parotid and lacrimal glands, IMP-free blisters and vesicles were often observed, whereas in the pancreas they were exceptional. These structures were either attached at the sites of continuity between granule and plasma membranes, or free in the acinar lumen, with a preferential location within exocytotic pockets or in their proximity. Experiments designed to investigate the nature of these blisters and vesicles revealed that they probably arise artifactually during glutaraldehyde fixation. In fact, (a) they were large and numerous in poorly fixed samples but were never observed in thin sections of specimens fixed in one step with glutaraldehyde and OsO₄; and (b) no increase in concentration of phospholipids was observed in the parotid saliva and pancreatic juice after stimulation of protein discharge, as was to be expected if release of membrane material were occurring after exocytosis.

KEY WORDS pancreas exocrine parotid gland lacrimal gland exocytosis membrane fusion freeze-fracture

Among the properties of some types of biological membranes is the ability to fuse with other membranes, thus establishing continuities between different membrane-bounded compartments. This ability is of particular importance for granule and plasma membranes of glandular cells because it is instrumental to the discharge of secretion products by exocytosis.

On the basis of morphological studies carried out by various techniques it was proposed that the fusion process does not occur in one single step, but rather evolves through intermediate stages. These putative intermediate stages have been the object of increasing interest and will be referred to in this paper, together with the establishment of continuity between membranes, under the general term of membrane interactions. Already in the sixties, results obtained by thin-section electron microscopy (reviewed in reference 33) indicated that, before fusion, the interacting membranes come first into close contact, and their adjacent layers merge forming pentalaminar structures. The successive elimination of the internal layers of these structures yields hybrid, trilaminar membranes, and the rapid collapse of the latter results in the opening of the site of fusion and in the establishment of the continuity between the two membrane partners at the opening rims.

Further information on the structural events occurring in exocytosis was obtained more recently by freeze-fracture. Apart from the observation of peculiar arrays of intramembrane particles (IMP), which mark the sites of granule-plasmalemma interaction in some protozoa (34, 37, 38) and in nerve terminals (5, 6, 11, 18, 19, 36, 44), the earliest event detected in a number of mammalian systems was a localized clearing of IMP in the two apposed membranes (2, 7, 21, 31, 32, 42, 43). These observations, coupled with the fact that at least some externally exposed proteins (such as lectin and Fc receptors) disappear from the plasmalemma of mast cells and pancreatic B cells at the sites of granule interaction (21, 30), were interpreted as indications that fusion begins between two apposed bimolecular lipid leaflets (21, 30–32, 43). The subsequent stages of the process are unclear. On the basis of images in mast cells, it was proposed that at least part of the interacting lipid bilayers would bleb out of the cell surface and form IMP-free blisters, which would then be released as discrete, IMP-free vesicles (21).

Our previous studies on the pancreas and parotid glands (4, 8–10, 23, 24) indicated, however, that, in exocrine systems, localized IMP clearing at granule-plasmalemma interactions and the formation of IMP-free blisters and vesicles are not general phenomena. Therefore, we decided to extend our observations by a parallel study of three exocrine cells: the acinar cells of the guinea pig pancreas, of the rat parotid gland, and of the rat exorbital lacrimal gland (lacrimocytes). These three cell types were studied by thin-section and freeze-fracture electron microscopy, both while in the resting state and shortly after pharmacological stimulation of secretion. Moreover, since it has been reported that, in some secretory systems, IMP-free areas can form between adjacent granules, both in the cytoplasm of intact cells (29, 42) and in isolated organelles brought into forced physical contact by centrifugation (41), we also
compared the in vivo and in vitro interactions between secretory granules in our exocrine systems. A recent paper on cultured corneal fibroblasts showed that IMP-free areas and blisters can arise artifactually during glutaraldehyde fixation (15). Therefore, we investigated whether formation of IMP-free structures at sites of exocytosis is also dependent on fixation conditions. Also, to establish whether membrane fragments are released during exocytosis, saliva and pancreatic juice secreted in response to well-specified pharmacological stimuli were collected by duct cannulation and analyzed biochemically.

MATERIALS AND METHODS

General

Male albino guinea pigs (350-450 g) and female SpragueDawley rats (180-250 g) were starved overnight. Secretagogues were injected either i.p. or i.v. at the following doses per kilogram of body weight: caerulein, 0.5 μg; secretin, 1 clinical unit; bethanechol, 0.5 mg; isoprenaline, 10 mg.

Electron Microscopy

The animals used for the studies of tissue morphology were sacrificed 0.5-2 min after secretagogue injection, their glands were immediately exposed, and fixative was thoroughly infiltrated in the interstitium by a syringe. The glands were then removed, small tissue blocks were trimmed with razor blades, and the fixation was continued by immersion. Several fixation procedures were used. In many experiments, the samples to be studied by thin-section electron microscopy were fixed for 45 min at room temperature in glutaraldehyde-paraformaldehyde mixtures prepared in 0.12 M phosphate buffer, pH 7.4. Concentrations of the two aldehydes ranged between 1 and 1.5%, respectively. In some cases, the fixative was supplemented with 0.075 M sucrose. After rinsing in 0.12 M phosphate buffer, the samples were fixed with 1% glutaraldehyde-0.5% paraformaldehyde and their pancreatic or parotid ducts with soft polyethylene tubes. After rinsing again, block-stained with uranyl acetate, and embedded in Epon. Other samples were fixed in one step with 2% osmium tetroxide and embedded in epon. For freeze-fracture, the samples were fixed with 1% glutaraldehyde-0.5% osmium tetroxide and then infiltrated with glycerol or frozen directly on gold holders.

Biochemical Experiments

Pancreatic juice and parotid saliva were collected from animals anaesthetized with pentobarbital and acetylcholine in their pancreatic or parotid ducts with soft polyethylene tubes. Secretagogues were injected at the doses specified under General above and at the time intervals shown in Table 1.

RESULTS

Exocytosis

In unstimulated acinar cells of the guinea pig
Guinea pig pancreas, one (B–D) and two min (A) after i.p. injection of caerulein (0.5 μg/kg). Several freeze-fracture images of exocytosis are shown. Note that in this gland the continuities between granule and plasma membranes (long arrows) and between discharged granule membranes (short arrows) occur through narrow, sometimes irregular necks. Even after stimulation, the distribution of IMP on fracture faces of the luminal plasmalemma and granule membranes appears random: no IMP-free areas, blisters or discrete vesicles are seen. J, occluding zonula; * and **, luminal plasmalemma. P and E face, respectively; L, acinar lumen. Fixation with 1.5% glutaraldehyde–1% paraformaldehyde. A, × 34,500; B, × 28,000; C, × 48,000; D, × 50,000.

pancreas, the surface of the luminal plasmalemma is regularly flat, except for the presence of microvilli. The IMP present on the P fracture face are randomly distributed and relatively sparse (~800/μm²). This density is much lower than that of the IMP present on the same fracture face of the basolateral plasmalemma and only slightly higher than in zymogen granule membranes (~600/μm²) (not shown in figures, see references 23 and 24).

0.5–2 min after injection of caerulein, a potent stimulant of pancreatic protein discharge (12), the luminal surface of acinar cells appeared greatly modified. Microvilli progressively disappeared, and front views of the plasmalemma now showed pits and small circular openings (Fig. 1A). Lateral views revealed that these openings were continuous through narrow necks with large cytoplasmic vacuoles characterized by low density of IMP on the P face of their limiting membrane (Fig. 1A–D). Some of these vacuoles were connected through narrow openings to other vacuoles located deeper in the cytoplasm (Fig. 1A and B). Sausage-like rows of up to six vacuoles were observed. That these images correspond to exocytoses (sometimes multiple or compound) and not to endocytoses or infoldings of the luminal plasmalemma is indicated, on the one hand, by the correspondence in size between vacuoles and undischarged granules and, on the other, by parallel thin-section studies (not shown in figures). Our freeze-fracture finding that discharged granules are connected to the lumen by means of narrow necks explains why, in this system, the observation of continuities between granule and plasma membranes is exceptional in thin sections, even after stimulation of secretion (24).
Except for the changes in gross geometry, the structure of the luminal surface of pancreatic acinar cells was little modified. In particular, the density and random distribution of P face IMP was unchanged: no IMP clusters on rosettes, IMP-free areas, or blisters were observed at the sites of granule-plasmalemma proximity and continuity, and IMP-free vesicles were seen only in a very few cases in acinar lumena, irrespective of the conditions used for tissue fixation (Fig. 1 A–D).

Fig. 2 illustrates the findings in the rat parotid acinar cells. In this system also, the portions of the luminal plasmalemma not covered by microvilli are flat before stimulation, and their P face IMP are evenly distributed (8). The density of these IMP (~2,100/μm²) is much higher than in the corresponding face of secretion granule membrane (~500/μm²) and only slightly lower than in the basolateral region of the plasmalemma (8).

Stimulation of protein discharge by isoprenaline (3) induced rapid and profound structural changes at the luminal pole of all cells examined. In freeze-fracture front views, the luminal surface appeared quite irregular, with alternation of protruding ridges and profound infoldings continuous with the lumen through large, circular openings (Fig. 2 A). In the remaining portions of the plasmalemma, discrete areas, 0.1–0.3 μm in diameter, either devoid of or very poor in IMP, were sometimes seen (Fig. 2 A). These areas usually appeared as bulges toward the lumen, and occasional fractures revealed granules apposed to these areas in the underlying cytoplasm. Microvilli were greatly decreased in number and apparently replaced by linear folds of the plasmalemma (Fig. 2 A). Freeze-fracture lateral views revealed the continuity of the large openings with cytoplasmic vacuoles (Fig. 2 B and C). The latter were identified as discharged granules by their size and by the density of IMP on the P face of their membranes, which was much lower than that of the surrounding plasmalemma (Fig. 2 C). Other discharged granules appeared connected to the lumen by means of thinner necks (not shown). The sites of continuity of granule and plasma membranes appeared marked neither by arrays nor by clearing of IMP, but only by sharp transitions from one density pattern to the other (Fig. 2 C). Thin sections of parallel samples revealed the close apposition between granule and luminal plasma membranes, with formation at some sites of focal pentalaminar and trilaminar structures (Fig. 2 E and F), as well as numerous exocytoses (not shown in figures, see reference 3). Blisters of various sizes and discrete vesicles were observed by both freeze-fracture and thin sections (Fig. 2 A, B, and D). The images were suggestive of the formation of these structures during granule-plasmalemma interaction. Thus, blisters were preferentially located at the sites of apposition or continuity between the two membranes, whereas free vesicles were often seen either within exocytotic pockets or adjacent to their openings (Fig. 2 A, B, and D). The size and frequency of these IMP-free membrane structures varied. In general, they were large and numerous in poorly fixed samples and relatively small and rare in better fixed samples, especially when the glutaraldehyde-paraformaldehyde mixture was prepared with purified glutaraldehyde. In thin sections of blocks fixed in one step with glutaraldehyde and OsO₄, no free vesicles were seen in acinar lumena (Fig. 2 F). In some discharged granules, the limiting membrane was quite irregular as a result of the presence of pockets and dimples (Fig. 2 B). These structures might correspond to initial stages of membrane recovery, agreeing with the recent tracer studies of Herzog and Farquhar (17).

Also in resting lacrimocytes, the distribution of IMP on the P face of the luminal plasmalemma is uniform, and their density (1216 ± 18/μm²)1 is higher than in secretion granule membranes (846 ± 13/μm²). With respect to the other glandular cells investigated, the luminal surface is more irregular, so that infoldings and recesses of the lumen are frequently encountered, also in unstimulated conditions (not shown, see reference 16).

After injection of betahanechol, a cholinergic agonist, striking changes developed at the luminal plasmalemma. Large, IMP-free bulges, nearly the same size as secretion granules, rapidly appeared (Fig. 3 A and B). In fortunate fractures, the concomitant exposure of both the plasmalemma and the underlying organelles revealed the close apposition of these bulges to secretion granules, whose membrane fracture faces also appeared poor in IMP in the areas of apposition (Fig. 3 B). In most exocytoses, granule membranes, identified by their relatively low density of IMP on the P fracture face, appeared connected to the plasmalemma, whereas free vesicles were often seen either within exocytotic pockets or adjacent to their openings (Fig. 2 A, B, and D). The images were suggestive of the formation of these structures during granule-plasmalemma interaction. Thus, blisters were preferentially located at the sites of apposition or continuity between the two membranes, whereas free vesicles were often seen either within exocytotic pockets or adjacent to their openings (Fig. 2 A, B, and D). The size and frequency of these IMP-free membrane structures varied. In general, they were large and numerous in poorly fixed samples and relatively small and rare in better fixed samples, especially when the glutaraldehyde-paraformaldehyde mixture was prepared with purified glutaraldehyde. In thin sections of blocks fixed in one step with glutaraldehyde and OsO₄, no free vesicles were seen in acinar lumena (Fig. 2 F). In some discharged granules, the limiting membrane was quite irregular as a result of the presence of pockets and dimples (Fig. 2 B). These structures might correspond to initial stages of membrane recovery, agreeing with the recent tracer studies of Herzog and Farquhar (17).

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1 IMP density measurements were carried out as described previously (23) on freeze-fracture pictures printed at 45,000 ×. 50 and 40 flat areas, each 0.05 μm², were analyzed for the luminal plasmalemma and secretion granules of lacrimocytes, respectively.
FIGURE 2  Rat parotid gland, 0.5 (A and D–F) and 1 (B and C) min after i.p. injection of isoprenaline (10 mg/kg). Panels A–C illustrate the freeze-fracture appearance of the luminal surface of acinar cells, which is very irregular as a consequence of the active granule discharge. Luminal recesses (probably exocytotic openings, *) are often large and sometimes continuous or adjacent to small IMP-free vesicles and blisters (arrows). In several cells, the distribution of IMP on the P face of the luminal plasmalemma is irregular: IMP-free areas are encircled in A. Panel C illustrates the abrupt change in IMP density that occurs at the site of continuity between granule and plasma membranes. The membrane of some discharged granules shows protuberances that might correspond to initial stages of membrane recapture (arrowhead in B). Panels D–F are thin sections showing granule-plasmalemma appositions. In samples fixed with aldehydes and then postfixed with OsO₄ (D and E), these appositions are often marked by blisters protruding into the lumen (arrow in D), and clear spaces, apparently devoid of cytoplasmic material, appear between apposed granule membranes (arrowheads in D and E). In contrast, after one-step glutaraldehyde-OsO₄ fixation, similar images were never seen (F; see also Fig. 4 B). L, acinar lumen; J, zonula occludens. Fixation: A, 1.5% glutaraldehyde–1% paraformaldehyde; B–E, 1% glutaraldehyde–0.5% paraformaldehyde; F, one-step fixation with glutaraldehyde-OsO₄. A, ×31,600; B, ×42,600; C, ×27,000; D and F, ×58,000; E, ×41,600.
lemmata through large openings (Fig. 3 C). Usually, IMP were present at the continuities between granule and plasma membranes, although their distribution was sometimes irregular at these sites (Fig. 3 A). Moreover, IMP-free blisters and vesicles of various sizes were commonly seen attached to the luminal plasmalemma or free in acinar lumena, especially within infoldings or in their proximity (Fig. 3 A).

The apposition of secretion granules to the plasmalemma to form bulges was also studied by means of thin sections. In all cases, the two adjacent membranes appeared very close (Fig. 3 D), sometimes with focal merging of the adjacent leaflets to form pentalaminar or even trilaminar structures (Fig. 3 E; see also reference 16). The various images observed might correspond to successive stages of one single process. Granule-plasmalemma bulges were seen in most samples investigated, irrespective of the fixation conditions used. In contrast, blisters and vesicles were visible after glutaraldehyde-paraformaldehyde fixation, particularly in poorly preserved samples, but were never seen in thin sections of glutaraldehyde-OSO₄ one-step-fixed samples (Fig. 3 D and E).

**Granule-Granule Interactions In Vivo**

The interactions between undischarged granules within the cytoplasm of the three glandular cells were also investigated. In each case, essentially the same situation emerged both before and shortly after the injection of secretagogues. Therefore, the observations made in resting and stimulated cells will be considered together.

In the cytoplasm of pancreatic acinar cells, mature zymogen granules always exhibited a regularly spherical or slightly ovoidal shape. These granules were usually not mutually apposed to each other but separated by intervening cytoplasmic matrix. Only in cells containing a heavy complement of zymogen granules were these organelles seen in close contact. However, contact was always restricted to very limited surface areas, and no peculiarities of membrane structure were detected at these sites by thin-section electron microscopy. Analogously, neither distortions of the granule surface curvature, nor clearing of IMP, which might correspond to membrane apposition, were detected by freeze-fracture (not shown in figures).

A different pattern emerged from the studies on the parotid gland. In the acinar cells with a heavy complement of secretory granules, many of these organelles, located both near the cell surface and more deeply in the cytoplasm, appeared apposed to other granules. In thin sections, the appearance of the granule-membrane appositions varied, depending on the fixation conditions used. In sam-
ples treated with glutaraldehyde-paraformaldehyde and then with OsO₄, adjacent membranes did not appear in direct contact but were separated by a clear space, 40-50 nm wide, from which the cytoplasmic matrix was apparently excluded (Fig. 2D and E), whereas, after one-step glutaraldehyde-OsO₄ fixation, the two membranes were closely apposed, with focal merging of adjacent layers to form typical pentalaminar structures over portions of the apposed area (Fig. 4B). On the other hand, in freeze-fracture these appositions appeared as flat, roughly circular areas, bearing little or no IMP on either of their fracture faces (Fig. 4A).

In the rat exorbital lacrimal gland, the freeze-fracture images were very similar to those described for the parotid gland (Figs. 3D and 4C). However, after glutaraldehyde-paraformaldehyde fixation, the thin-section images did not reveal clear spaces, and the two adjacent membranes remained apposed, as with the one-step glutaraldehyde-OsO₄ fixation (Fig. 4D).

Granule-Granule Interactions In Vitro

It was recently reported by another group (41) that flat IMP-free areas can be induced in the membrane of closely apposed chromaffin granules (isolated from the bovine adrenal medulla) by forced physical contact brought about by centrifugation in an ionic medium. Inasmuch as the images thus formed resemble those observed between apposed granules in intact parotid and lacrimal acinar cells, we thought that studies on the effects of forced physical contact of isolated granules could be of some help in the understanding of the in vivo findings. To this end, we compared granules isolated from the parotid and pancreatic glands. Because both these organelles are very fragile, we found it convenient to use for these experiments granule preparations that were only

![Figure 4](image)

**Figure 4** Granule-granule appositions in intact parotid (A and B) and lacrimal (C and D) acinar cells. The images come from animals injected with secretagogues 0.5 (B) or 1 (A, C, and D) min before sacrifice. However, similar images were also observed in nonstimulated cells. The flat, IMP-free areas of frozen-fractured membranes are indicated by arrows. Samples were fixed in 1% glutaraldehyde-0.5% paraformaldehyde (A and C) or in glutaraldehyde-OsO₄ (one step, panels B and D). A, ×45,000; B, ×62,000; C, ×31,500; D, ×45,500.

446  THE JOURNAL OF CELL BIOLOGY - VOLUME 84, 1980
were finally studied by electron microscopy con-
partially purified and that had not been exposed
0.3 M sucrose and 0.15 M KCl buffered at pH 6.8
of IMP-free areas was very low in granules pelleted
pentalaminar structures (Fig. 5 E). The frequency
appeared closely apposed, with the formation of focal
membranes were carried out using a medium containing
5 mM phosphate. Under these conditions,
fracture and in thin sections, by a variety of criteria: size, shape, electron density, and low IMP
complement of their membranes. The results obtained
these experiments are illustrated in Fig. 5. A quantitative analysis of the data on parotid
granules is reported in Fig. 6. Most of the experiments were carried out using a medium containing
0.3 M sucrose and 0.15 M KCl buffered at pH 6.8
with 5 mM phosphate. Under these conditions,
flat, circular IMP-free areas formed at the sites of
apposition of parotid granules (Fig. 5A, B and D).
In thin sections, the participating membranes appeared closely apposed, with the formation of focal
pentalaminar structures (Fig. 5 E). The frequency
of IMP-free areas was very low in granules pelleted
at low speed and increased progressively when higher speeds were used. As can be seen in Fig. 6,
the correlation between frequency of IMP-free areas formed in the membrane of pelleted parotid
granules and the centrifugal force applied was highly significant when analyzed statistically. The
clearing of IMP at the sites of granule apposition was observed with similar frequency in all samples
centrifuged at the same speed, irrespective of the
fixation procedures used. This was also found to be true in unfixed, unglycerinated samples (Figs.
5 D and 6). This process was not dependent on the presence of isotonic concentrations of monovalent
ions in the suspending fluid (as has been reported for chromaffin granules, reference 41) because it
also occurred when granules were centrifuged in plain buffered sucrose (Figs. 5 C and 6). However,
this process seems to be influenced by divalent cations because a higher frequency of IMP-free areas was found in granules centrifuged at low speed in the presence of millimolar concentrations of Ca" (see also reference 40) rather than K" (Fig. 6).

In contrast to the results obtained with the parotid granules, no flat IMP-free areas were seen
between isolated pancreatic zymogen granules, even when centrifuged at an acceleration double
the maximum used for parotid granules (100,000 g, Fig. 5 F). In thin sections, most adjacent zymo-
gen-granule membranes appeared not to be closely apposed. However, in some cases, small
appositions and even typical pentalaminar structures were detected (Fig. 5 G).

Because the guinea pig pancreas granules and rat parotid granules have approximately the same
size and buoyant density (25, 26), they were exposed to a similar force when spun at equal speed.
The results we obtained demonstrate, therefore, a difference between the two granule types in the in vitro formation of forced-contact-induced mem-
brane appositions.

Biochemical Analysis of Pancreatic Juice and Parotid Saliva

It is now generally agreed that during exocytosis only the content of secretion granules is released
to the extracellular space, while the membrane is retained within the cell where it is probably reu-
tilized in further secretory cycles (33). However, as already mentioned in the introduction, the possibility of a limited release of membrane material in the form of IMP-free vesicles corresponding to the portions of granule and plasma membranes that participated directly in the fusion process has been recently raised on the basis of results from stimu-
lated mast cells (21). If this were the case, one would expect small amounts of membrane mate-
rial, such as phospholipids, to appear in parallel with the discharge of macromolecules stored within secretion granules. To test this possibility, we analyzed guinea pig pancreatic juice and rat parotid saliva obtained by cannulation of gland
ducts. In both cases, the animals were first injected with drugs known to stimulate mainly the secre-
tion of water and electrolytes (secretin for the pancreas and bethanechol for the parotid gland) to wash out the ducts and establish the reference values of phospholipid concentration in juice and saliva and then switched to treatments adequate
to maintain a high secretion rate while strongly stimulating discharge by exocytosis (caerulein and
isoprenaline with bethanechol, respectively). The results of three (pancreas) and four (parotid) ex-
periments are summarized in Table I. Two more experiments were carried out for each gland, with different collection schedules, but with qualita-
"tively similar results (not shown). After the begin-
ing of stimulation of water and salt secretion, phospholipids appeared in the collected fluids,
whereas, after some time (20-40 min), only traces were detected. These very low concentrations were not increased when protein discharge by exocyto-
sis was stimulated.

TANAKA, DE CAMILLI, AND MELDOLESI Membrane Interactions in Exocrine Glands 447
FIGURE 5  Appositions of parotid and pancreatic secretory granules induced in vitro by forced physical contact (centrifugation). Panels A, D, and E refer to partially purified parotid granules suspended in buffered 0.3 M sucrose-0.15 M KCl and centrifuged for 10 min at 50,000 g. The sample shown in panel B was analogous, but centrifugation was carried out at 17,600 g; that shown in C was suspended in buffered 0.3 M sucrose and centrifuged at 50,000 g. Panels F and G show pancreatic zymogen granules centrifuged at 100,000 g for 10 min. All centrifugations were carried out in a Spinco 50.1 rotor. Fixation was in 1% glutaraldehyde-0.5% paraformaldehyde buffered at pH 6.8 with 0.12 M phosphate (panels A–C and E–G), followed by 2% OsO₄ for thin-section specimens (panels E and G). The sample shown in panel D was frozen unfixed. Note that the appositions (circles) between parotid granules were numerous, large, and IMP-free in all samples shown, including the one frozen without prior fixation and glycerol infiltration (panel D), even though, in the latter case, the granules were distorted by the massive formation of ice crystals. In thin sections the adjacent granules appeared closely apposed, sometimes with formation of focal pentalaminar structures (E). In contrast, no IMP-clear areas were detected between adjacent pancreatic zymogen granules, and IMP were distributed in an apparently random fashion (panel F). However, thin sections sometimes revealed small pentalaminar structures between adjacent zymogen granules (panel G). A, × 46,500; B, × 45,000; C, × 34,800; D, × 52,700; E, × 108,800; F and G, × 62,000.
0.20-
0.10-
0.05-

10 20 30 40 50
centrifugal force (g max x 10^-3)

FIGURE 6 Effect of the centrifugal force on the frequency of IMP-free areas in pellets of isolated parotid secretory granules. Partially purified secretory granules were suspended in 0.3 M sucrose, buffered at pH 6.8 with 5 mM phosphate, either without (□) or supplemented with KCl (0.15 M, ○ and □) or CaCl₂ (5 mM, *, or 10 mM, △), and then immediately centrifuged for 10 min at 4°C in a Spinco SW 50.1 rotor under the conditions specified in abscissa, except for the samples treated with CaCl₂, which were incubated at 4°C for 10 min before centrifugation. Most pellets were fixed and infiltrated with glycerol (see figure legend 5) before freeze-fracture, but some were frozen unfixed (□). Each of the values (average ± SE) given comes from the analysis of 10 pictures taken at random and at constant magnification on one freeze-fractured pellet type and printed at 45,000 x. The frequency of IMP-free areas was calculated by dividing the number of these areas by the number of the secretory granule profiles present in the analyzed pictures. Depending on the speed of the final centrifugation, the number of recognizable granule profiles in one picture varied from 20 to 50. The solid line is the calculated regression line of the fixed granules suspended in buffered 0.3 M sucrose-0.15 M KCl (P < 0.001). The correlation coefficient was 0.97.

With the exorbital lacrimal gland similar analyses were impossible because we did not succeed in the cannulation of the duct and because, in this gland, fluid and protein secretion cannot be separately stimulated. However, by studying tears collected at the eye corner before and after bethanochol injection (two experiments), we found that the concentration of phospholipids is low and does not correlate with that of protein (not shown in tables).

DISCUSSION

In previous freeze-fracture studies on exocytosis, essentially two types of structures were revealed at granule-plasmalemma appositions. On the one hand, peculiar arrays of IMP were observed on the presynaptic membrane of nerve terminals (5, 6, 18, 19, 36, 44) and on both the granule and plasma membranes of some protozoa (34, 37, 38); on the other hand, localized clearings of IMP were reported in the two interacting membranes in several mammalian systems: B cells of the endocrine pancreas, polymorphonuclear leukocytes, neurohypophyseal terminals, goblet cells, and mast cells (2, 7, 21, 31, 32, 42, 43). In the latter system, the formation of IMP-free blisters and vesicles, interpreted as end results of membrane fusion, was also reported (21). These findings have led to divergent interpretations of the membrane events occurring during the fusion process. Some, in fact, have postulated that integral membrane proteins (likely the major components of IMP) play a major role (37, 38), whereas many others have concluded that fusion probably occurs between protein-depleted lipid bilayers (2, 7, 21, 31, 32, 42, 43).

In our study, exocytosis and granule-granule interactions were investigated in three exocrine systems. Two of these systems (the pancreas and the parotid gland) had already been investigated by freeze-fracture (9, 10, 23, 24). However, because, in these previous studies, no attention was given to the phenomena occurring shortly after stimulation of secretion, no direct information on the membrane events during exocytosis was available. The results we obtained revealed some analogies and some differences among the three systems investigated. Thus, in neither of them, could we detect arrays of IMP at the sites of granule-plasmalemma interaction. Taken together with previous data on the other mammalian systems, this negative finding lends support to the view that IMP arrays, in the systems where they are present, probably do not play a direct role in the process of membrane fusion, but rather in the regulation of exocytosis. Thus, in protozoa, IMP arrays have been suggested to function in the attachment of granules to the plasmalemma, a process, which, in these cells, occurs some time before and independent of fusion (34). Another possibility is that the arrays represent clustered ion pores, whose transport activity might be important in triggering the...
**TABLE I**

Concentration of Protein and Phospholipids in Pancreatic Juice and Parotid saliva

| Protein, mg/ml | Pancreas | Parotid |
|----------------|---------|---------|
| Secretin       | 1.75 ± 0.48 | 0.93 ± 0.23 |
| Caerulein      | 16.36 ± 3.34 | 8.60 ± 1.70 |
| Phospholipid, nmol/mg protein | 6.03 ± 0.73 | 2.83 ± 0.63 |

Data given are averages of three (pancreas) and four (parotid) experiments ± SE. Assays were carried out on pooled samples obtained from either three rats (parotid) or two guinea pigs (pancreas). Secretagogues were injected either i.v. (pancreas) or i.p. (parotid) at the beginning of the indicated time periods.

* Minutes.

This second hypothesis was also suggested for the IMP rows of nerve terminals (44). However, recent experimental evidence from the frog neuromuscular junction suggests that, in this system, the IMP arrays might be part of recognition complexes that direct the fusion of synaptic vesicles to regions of the plasma membrane located at the minimum distance from the clusters of cholinoreceptors present in the postsynaptic membrane (5, 6).

In the three exocrine systems investigated in our study, distinct differences were observed in relation to the localized clearings of IMP. Images of this type, which were never observed at the cell surface of unstimulated cells, rapidly appeared, after secretagogue injection, on the apical plasma membrane of most lacrimocytes (in the form of bulges) and of some parotid acinar cells but were never seen in the pancreas. Analogously, circular, flat IMP-free areas were common between adjacent granules in parotid and lacrimal glands, both before and after stimulation, but were not seen in the pancreas. The formation of these structures was also studied in isolated granules brought into forced physical contact by centrifugation. Admittedly, no proof exists that the granule-granule interactions studied in vitro correspond to those observed in intact cells. However, that this might be the case is suggested, on the one hand, by the similarity of the images and, on the other hand, by the correspondence of the in vivo and in vitro findings: in both cases IMP-free areas were present between parotid granules and absent between pancreatic granules.

Before discussing the significance of localized IMP clearings in granule and plasma membranes, two important questions on these structures should be considered. First, it should be asked whether IMP-free areas are real structures present in living cells. In this respect, it is worth mentioning that the appearance of these structures on the plasma membrane was not random, as might be expected for a fixation artifact or an unspecific sign of cell damage, but was restricted to the luminal region, where exocytosis is known to take place. Also, IMP-free areas were observed with similar frequency in samples fixed and processed under different conditions. Moreover, in the in vitro experiments, they were also observed in pellets of unfixed parotid granules. A similar observation had been made previously on isolated chromaffin granules (41). Unfortunately, all the attempts we made in the course of this study to investigate frozen unfixed tissue were unsuccessful. In fact, the granules and luminal surfaces are mostly located deep in acini, so that frozen unfixed samples were always too distorted by the massive formation of ice crystals to be useful in membrane interaction studies. Because of this failure, we feel that caution with regard to IMP-free areas is appropriate, although the data of this paper, as well as that reported in the literature (2, 14, 20, 21, 29–32, 42, 43) seem to support their existence in living cells. The second question concerns the nature of the thin-section image(s) corresponding to the IMP-free areas seen in freeze-fracture. In parotid acinar cells fixed with aldehydes and then treated with OsO₄, a clear space was seen between the parallel surfaces of adjacent granule membranes. Since, however, this type of image was not seen after one-step glutaraldehyde-OsO₄ fixation, it might be the result of an artifact. In all other cases, in vitro as well as in intact cells, the interacting granule and plasma membranes were seen closely adjacent to each other. Focal merging of outer layers, with consequent formation of pentalaminar
structures over portions of the apposition area, were found in some, but not in all, such membrane interactions. Thus, it can be concluded that IMP-free areas correspond not only to pentalaminar structures, but in general to close granule-granule and granule-plasmalemma appositions.

In order to discuss the significance of IMP-free areas, the results of the present work will be considered together with the data from the literature on other systems. The general picture which emerges is quite heterogeneous. At one extreme are the systems (pancreatic B cells, polymorphonuclear leukocytes, neurohypophysial terminals) in which a good correlation was found between IMP clearing and exocytosis. In these systems, the exocytotic openings were surrounded by a plasmalemma rim devoid of IMP (2, 31, 32, 43). Similar findings were obtained in fusing myoblasts (20). In mast cells (7, 21), goblet cells (42), and lacrimocytes (this study), on the other hand, localized IMP clearing certainly occurs in the plasmalemma at the sites of granule apposition. However, there is no definite demonstration that exocytosis always occurs in the cleared areas because the fracture faces of granule-plasmalemma continuities are not smooth at the openings but studded with IMP. Finally, a dissociation exists in the parotid gland, where the scarcity of IMP-free areas appearing in the lumenal plasmalemma after stimulation does not correlate with the high frequency of exocytoses, and in the exocrine pancreas, where IMP-free areas were never seen (this study). In addition, in another system in which IMP-free areas are usually not seen in exocytosis, namely, the frog neuromuscular junction (5, 11, 18), formation of these areas in the presynaptic membrane at the sites of vesicle apposition was induced by changes in the ionic environment: by incubation in isotonic Mg** solution (a condition which stimulates the vesicle-plasmalemma apposition but suppresses quantal transmitter release) (10) and by stimulation with black widow spider venom during prolonged incubation in Ca**-/free media containing EGTA (5). In the latter case, the fusion of vesicles, which is greatly stimulated, did not occur in the center of the IMP-cleared areas but always at the edges adjacent to the IMP arrays (5). Finally, it should be emphasized that the IMP-free areas at the cell surface are very similar to those occurring within the cytoplasm between undischarged granules, observed in this as well as in previous studies on other systems (29, 42), although these granule-granule interactions do not evolve towards fusion. Thus, even if localized IMP clearings have been observed in relation to exocytosis in a variety of mammalian systems, the two processes do not appear strictly related because (a) localized IMP clearing in interacting membranes is not always followed by fusion, and (b) not all fusions are preceded by detectable IMP clearing in interacting membranes.

In contrast, in all systems so far investigated, a correlation seems to exist between formation of IMP-free areas and changes in geometry of cell structures at the sites of intimate membrane contact: bulges of the plasmalemma (2, 7, 19, 20, 21, 32, 42, 43, this study) and flat circular areas in the membrane of spherical secretion granules, both in intact cells (29, 42, this study) and in vitro, after centrifugation (40, 41, this study). The presence of these deformations might be interpreted as an indication that the membranes were forced together. On the other hand, we know from the in vitro studies that forced physical contact is sufficient to induce IMP clearing in adrenal (41) and parotid (this study) granule membranes. On the basis of these observations, the following interpretation of the membrane events in secretory cells can be tentatively envisaged: IMP clearing, rather than a true intermediate stage of membranefusion, might be the result of a tight mechanical apposition of membranes, a preliminary process not sufficient to initiate fusion. Whether the interacting membranes would proceed beyond the apposition stage to become continuous with each other would, however, depend on additional factors as yet un-
identified. It might be further speculated that, in the case of granule-granule interactions, the driving force for clearing might be the excessive intracellular granule load, with consequent close packing of the organelles (in cells with few granules no clearing is seen); in the case of granule-plasma membrane interaction, it might be a specific process triggered by cell stimulation.

It still remains to be explained why IMP-free areas were never seen in pancreatic acinar cells and were less frequent than expected at the luminal surface of stimulated parotid acinar cells. One possible explanation is that the clearing always occurred but on membrane surfaces too small to be detected over the background of the surrounding fracture faces. This explanation seems particularly attractive for guinea pig pancreatic acinar cells, whose luminal plasmalemma is characterized by a low IMP density and where exocytotic openings are usually very small. Also, the results of the in vitro experiments demonstrate that pentalaminar structures, although small, are also induced in pancreatic zymogen granule membranes by forced physical contact, even though in parallel samples no heterogeneity of the IMP distribution was detected by freeze fracture. The reason why the size of the membrane appositions and exocytotic openings is so different in the various secretory systems investigated is unknown.

Previous studies have suggested that IMP-free blisters and discrete vesicles might play a role in the final events of exocytosis (redistribution of granule and plasma membrane molecules that participated in the fusion process) (21). However, the present data indicate that, in the exocrine systems investigated, these structures result from fixation artifacts. Thus, their size and frequency were greatly reduced in properly fixed specimens, especially of parotid tissue, and they were not encountered after one-step glutaraldehyde-OsO₄ fixation. In full agreement with these morphological results, we found that in the fluids secreted by the pancreas and parotid (glands in which the occurrence of blisters and vesicles in fixed stimulated tissue is very low and relatively high, respectively) phospholipids were present only in traces, independent of the protein concentration. The small amounts of phospholipids that were recovered in the samples collected just after the beginning of the pharmacological stimulations might, in fact, be accounted for by washout of material accumulated in the main ducts during the surgical cannulation and/or shed from acinar and duct cells before the beginning of the experiments. Because, in pancreas and parotid granules, phospholipids are located only in the limiting membrane, and because their concentration is known (25, 27), it can be calculated that, if exocytosis involves the release of membrane material, this would go undetected under our experimental conditions only if amounting to less than 5% of the membranes of the participating granules. On the other hand, a generalized trapping of discharged vesicles in the duct tree seems unlikely in view of the high secretion volumes that were maintained throughout our experiments. Thus, our findings seem to exclude the possibility of a significant release of membrane material during exocytosis and to support the view that the membrane molecules that participate in fusion are redistributed within secretory cells. This redistribution might be impaired during aldehyde fixation, thus explaining the frequent appearance of blisters and vesicles in the samples of stimulated glandular tissue prepared for electron microscopy by conventional techniques.

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