DYNAMIC CHANGES OF THE LUMINAL PLASMALEMMMA IN STIMULATED PAROTID ACINAR CELLS
A Freeze-Fracture Study

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ABSTRACT

In the acinar cells of the rat parotid gland the two membranes participating in exocytosis, i.e., the luminal plasmalemma and the secretory granule membrane, are clearly distinguishable in freeze-fracture because of their different densities in particles. In order to obtain point-specific information about the fusion-fission of these two membranes that occurs during the secretory cycle, glands were studied at various times (5 min to 6 h) after stimulation with isoproterenol. We observed that, in the course of the release of secretion products and shortly afterwards, the enlarged luminal plasmalemma exhibits a mosaic organization consisting of an alternation of membrane patches of high (original plasmalemma) and low (fused granule membrane) particle density. The transition between these two patterns is usually sharp. Later, concomitant with the reformation of acinar canaliculi, the low particle density membrane is found at the cell surface but only bounding vacuolar infoldings, and then it finally disappears.

These results suggest that (a) fusion of these membranes does not result in a random intermixing of the molecular components of the participating membranes, which retain their structural identity; and (b) the enlarged luminal plasmalemma reverts to its original size by a progressive, specific removal of the regions of low particle density from the cell surface.

In secretory cells the exportable proteins are known to be vectorially transported from the site of synthesis to the extracellular space through a pathway which includes a number of distinct membrane-bounded organelles (8, 9, 23, 24): in sequence, the rough endoplasmic reticulum, the Golgi complex, and the secretion granules. The content of the latter is then discharged by exocytosis, i.e., by fusion of the granule limiting membrane with the plasmalemma followed by an opening at the point of fusion (36). Since the transport of the secretion products between adjacent cell compartments occurs in bulk, by means of membrane-bounded vesicles, a concomitant transfer of membranes must be postulated.

The fate of the transferred membrane is still debated. Biochemical studies carried out on various secretory systems have clearly shown that the turnover rate of the molecular components of all the membranes involved is much slower than that of the secretory products (28, 29, 46, 47); thus, it is most likely that these molecular components are reutilized in several secretory cycles (28, 29). However, the mechanisms of this reutilization are still unclear, since it is not known whether membrane patches, after fusing with a membrane of a
different molecules, which are then reassembled at a different site inside the cell.

In order to shed some light on this intriguing problem, it is first necessary to clarify the sequence of events occurring at the point of fusion of two distinct membranes. In fact, it is evident that specific retrieval of intact membrane patches would be more conceivable if the fusion is not followed by a rapid and random intermixing of the individual molecular components of the fused membranes.

Recently, significant progress in the field of membrane structure has been achieved by freeze-fracture studies, because by this technique the membranes are split along their internal hydrophobic domain and reveal the presence of internally located particles. These particles can have different densities and distributions in the different membranes (4, 5). Thus, freeze-fracture appears to be a technique especially suited for investigating at least some of the phenomena occurring at the point of membrane fusion, provided the two participating membranes are characterized by clearly distinct particle patterns. The fusion of the membrane of the secretion granules with the luminal portion of the plasmalemma in the rat parotid acinar cells appears to satisfy this requirement because, in contrast to the situation observed in other exocrine glands, such as the pancreas of the guinea pig (13), the two membranes are clearly distinguishable in freeze-fracture. Furthermore, the system appears favorable for at least two additional reasons: (a) the secretory activity of the acinar cells can be easily synchronized by pharmacological stimulation (3, 7, 12, 27); (b) the radius of the curvature of both participating membranes is large and, therefore, the intramembrane surfaces exposed by fracturing are also large, a situation which is of great help in this type of morphological investigation.

MATERIALS AND METHODS
Female Sprague-Dawley rats weighing ~250 g were starved for 24 h. Secretion was initiated by a single intraperitoneal injection of 0.5 ml of a freshly prepared 1% solution of isoproterenol (IPR) (Sigma Chemical Co., St. Louis, Mo.) in 0.15 M NaCl. Animals were sacrificed by a blow on the head at different times after the injection (5 min to 6 h).

Parotid glands were rapidly exposed by excising the superficial tissues and fixed in situ with 3% glutaraldehyde (in 0.1 M sucrose buffered with 0.1 M phosphate, pH 7.3) injected into the interstitium by means of a syringe. The glands were then rapidly removed and immediately trimmed into small fragments which were kept in the same fixative solution for 2 h at room temperature and then infiltrated with glycerol (at concentrations increasing from 10 to 30%) in 120 mM phosphate buffer, pH 7.3, at 25°C. The infiltration in 30% glycerol lasted for ~1.5 h.

Samples were frozen by immersion in Freon 22, cooled to -150°C in liquid nitrogen, then freeze-fractured according to the method of Moor and Mühlethaler (31) in a Balzers freeze-etch device (Balzers AG, Balzers, Liechtenstein). The fracturing temperature was -115°C. Platinum-carbon replicas were washed in Na hypochlorite solution to remove organic material, then in distilled water, and finally recovered on 200-mesh copper grids. The replicas were examined in Philips EM 300 and EM 200 electron microscopes. In order to carry out a quantitative analysis of the morphological results, the density of the intramembranous particles in the membranes participating in exocytosis and surface remodeling was determined by counting at different time-points of the experiment (0, 5, 40, and 120 min).

RESULTS
Unstimulated Cells
The ultrastructure of the rat parotid gland, as known from conventional electron microscopy studies (3, 12, 27), can be easily recognized in freeze-fracture replicas. In the unstimulated gland (Figs. 1–4), the acinar cells are characterized by a regularly ordered rough-surfaced endoplasmic reticulum, a large fenestrated Golgi complex, and the abundance of large secretion granules, which appear as rounded cavities (face P)1 or elevations (face E). Mitochondria are scattered, and nuclei are large in size and slightly irregular in outline. Large areas of the plasmalemma are exposed by fracturing: the luminal (secreatory) and lateral regions are separated by tight junctions. The luminal region is characterized by the presence of numerous cylindrical microvilli, while the lateral region has cellular projections that are usually larger and flattened. At the base of the cell, the plasmalemma is in general even, with some pinocytotic vesicles.

The various cellular membranes are characterized by the density of their intramembranous parti-
articles. On the P face, the particle density is very high in the membranes of the rough-surfaced endoplasmic reticulum, nuclear envelope, and Golgi apparatus (Fig. 2), but low in membranes of the secretion granules (Figs. 1, 3, and 4). In the plasma-membrane, regional differences in particle density can be observed on the P face (Fig. 3, insets). The lateral and basal portions are so heavily studded with particles that they display a finely granular appearance (Figs. 3 and 4); on the luminal area, however, the pattern is clearly different: particles, although still abundant, are less numerous than in the other portions of the plasma-membrane, and stand out clearly above the smooth background (Fig. 4). On the E faces, the number of particles is always very low, and no significant differences can be detected among all the above-mentioned membrane types.

Acinar cells are easily differentiated from duct cells: the small size and the occurrence of only a few small scattered granules characterize the cells of the intercalated ducts (Fig. 5), while typical spherical nuclei, abundant mitochondria, absence of granules, and deep unfoldings of the basal plasma-membrane are the hallmarks of the cells lining the columnar ducts (Fig. 6). Moreover, peculiarities also exist in the luminal surfaces of duct cells relative to their acinar counterparts, the particle density on the P face is lower in the intercalated duct cells and even lower in the columnar duct cells (Figs. 5 and 7). In the columnar duct the large diameter of the lumina and the thick network of the tight junctions help in the identification of the cells (Fig. 7).

**Stimulated Cells**

The pharmacological stimulation of the acinar cells by IPR results in profound changes of the acinar lumina, with respect to both their gross architecture and the distribution of the particles associated with their limiting membranes. The changes are illustrated in Figs. 8-12. A quantitative determination of the density of particles on the P faces of the different membranes participating in the processes of protein release and surface remodeling, at the various time-points of the experiment, is reported in Table I. In agreement with previous thin-section studies (3, 12, 27, 42), we observed that a massive discharge of secretion products by exocytosis occurs even a few minutes after IPR injection.

The secretion granule membrane, once incorporated by fusion into the luminal plasma-membrane, acquires the property to fuse sequentially with the membranes of other, undischarged granules. This process produces a dramatic enlargement of the luminal space; 5 min after the injection (Fig. 8), the lumina have lost their regular shape and penetrate deeply into the cells. Microvilli are no longer evident.

The cell surface bordering on the lumen is now characterized by beaded invaginations resulting from multiple granule fusions. Small concave depressions and even smaller conical pits appear at this time on the P face of the luminal surface (Fig. 9 b and c). Similar images have been observed previously in other secretory systems, such as the neuromuscular junction (16, 22), the adrenal medulla (45), and the pancreatic beta cells (34), and interpreted as an early event in the release process, i.e., as points of fusion between the plasma-membrane and the membrane of the secretory granules. Rosettes, annuli, or localized modifications of particle distribution, which have been reported to characterize the points of membrane adhesion in some protozoa (38, 39, 40) as well as endothelial cells (41) and macrophages (44), were never observed around these areas. Analogous observations had been made previously in other glandular cells (34, 45).

The distribution of particles at the luminal surface is no longer homogeneous. Membrane portions similar to the resting luminal membrane, i.e., with high particle density, are continuous with regions of much lower particle density which most likely arise from the insertion of granule membranes (Figs. 8 and 9). The latter regions, however, have a particle density which is slightly higher than that of the membranes of undischarged granules (Figs. 8, 9, and Table I). This enrichment after fusion seems to be a very rapid event, since it is clearly evident in practically all the membranes of fused granules we have seen.

From 5 to 30 min after IPR injection, protein discharge continues. Lumina increase progressively in size; their invaginations extend deeper and deeper into the adjacent cells. During this process, the membrane heterogeneity that we have already described is retained; in particular, the particle density in the areas which correspond

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Since, as already mentioned, the E leaflets of the two membranes which become continuous during exocytosis (the granule membrane and the plasma-membrane) are very similar in particle density, our description from this point on will concern only the P leaflet.
FIGURE 1 General view of several unstimulated parotid acinar cells. The field includes: the face view of an intercellular secretory canaliculus (ic) bounded by tight junctions (J) and containing cross-sectioned cylindrical microvilli; the lateral plasmalemma (LPM) of two adjacent cells exhibiting flat, irregular projections; the cross section of an acinar lumen (AL); numerous large, spherical secretion granules (SG); cross-sectioned cisternae of the rough-surfaced endoplasmic reticulum (RER). In this and the following figures, the direction of shadowing is indicated by an encircled arrow. × 13,000.
to the original luminal plasmalemma remains as high as in the resting gland (Table I).

At 40 min after IPR injection, most acinar cells have released the majority of their granules. From this time on, the lumina progressively revert to their normal size, as indicated by the observations made between 40 and 180 min. During this period, membrane regions with different particle density remain evident (Fig. 10 and Table I); however, their distribution changes with time. Thus, during the active secretory process the lumen is irregularly invaginated and bounded by a patchy membrane, whereas from 80 to 120 min after injection canaliculi are reformed and the low particle density membrane is present at the cell surface only at the level of large vacuolar infoldings (Figs. 11, 12, and Table I). At the neck by which these cavernae are connected with the canaliculi, a sharp drop in particle density is clearly evident (Fig. 11). Some small blebs invaginated towards the adjacent cytoplasm are scattered on the surface of these cavernae. Concomitantly, numerous vesicles appear in the apical portion of the cytoplasm, which is now completely devoid of granules (Fig. 12).

At even later times, the cavernae progressively decrease in size and finally disappear. In parallel, discrete microvilli are reformed. Eventually (6 h) the secretory lumina appear indistinguishable from those described in the unstimulated glands.

DISCUSSION

In the acinar cells of the rat parotid gland, the secretory portion of the plasmalemma displays peculiar structural characteristics relative to the lateral and basal regions. An analogous observation had been made previously in the exocrine pancreas of the guinea pig, where, in addition, a striking similarity was found to exist between the two membranes (granule membrane and secretory plasmalemma) which become continuous after exocytosis (13). In the parotid gland, on the contrary, these two membranes are clearly distinguishable in freeze-fracture. This situation proved to be particularly favorable to yield point-specific information about the dynamic events occurring at the luminal surface, especially in relation to: (a) protein discharge, and (b) membrane retrieval from the enlarged secretion lumina.

As far as the first phenomenon is concerned, our results clearly indicate that fusion of the membranes does not result in a random intermixing of the molecular components of the participating membranes. In fact, if a generalized intermixing were to take place, one would expect the membrane of the fused granule to be progressively enriched in particles migrating from the adjacent regions of the luminal membrane. This process would result, at the early time-points of the experiment, in the appearance of a gradient of particle density localized across the point of fusion and, later on, in a dilution of the particles in the luminal membrane, with a final distribution being (a) homogeneous and (b) intermediate in particle density between that of the resting luminal plasmalemma and that of the secretion granule membrane.

On the contrary, the apical plasmalemma acquires, on stimulation, a mosaic organization (sharp alternation of low particle density patches with high particle density regions indistinguishable from the resting plasmalemma) and remains heterogeneous until the secretory lumina regain their original size. We are therefore forced to conclude that the molecular movements in the plane of the membrane are severely restricted at the point of fusion. As a consequence, the identity of the individual membrane patches is preserved, at least partially. These conclusions might appear to contradict the widely accepted idea that membranes are fluid and, therefore, expected to be capable of extensive intermixing on fusion (18, 19, 43). However, it has become recently evident that, in fluid membranes, at least some of the molecular components are not entirely free for lateral movement. On the contrary, the mobility of these components appears to be controlled, probably by their association with underlying cytoplasmic structures, such as microfilaments and/or fibrous proteins (2, 14, 17, 18, 19, 33), as suggested by experiments carried out in many types of cells: erythrocytes (where the fibrous peripheral membrane protein spectrin has been implicated [20]), lymphocytes (14, 17), and polymorphonuclear leukocytes (33). It seems likely that similar mechanisms would operate in exocrine cells as well, at least at the secretory portion of the plasmalemma, which appears strictly anchored to the cytoplasm by means of a well-developed system of microfilaments. Thus, it is tempting to suggest that the mechanisms by which the mutual intermixing of granule membrane and secretory plasmalemma is prevented might depend on the association of the intrinsic components of these two membranes with different peripheral proteins, probably located towards the cytoplasm.
FIGURE 2. Tangentially exposed P and E membrane faces of numerous adjacent rough-surfaced endoplasmic reticulum cisternae (RER-P and RER-E, respectively) are shown. The E face of a fenestrated Golgi plate is labeled GC-E. × 36,500. See next page for legend 3.
The slight increase in intramembranous particle density observed in the fused granule membranes in comparison with the unfused granule membranes, even a few minutes after IPR injection, is hard to interpret. It is not even possible to establish whether this phenomenon represents a prerequisite or a consequence of granule discharge. A number of possible explanations are open. The extra particles might originate from the granule membrane itself, as a consequence of either a conformational change or a “relaxation” of the stretched membrane (21) related to granule discharge.

The alternative possibility, i.e., that the extra particles might derive from the adjacent luminal plasmalemma, appears to be in contrast with the constancy of particle density that we found in the latter membrane throughout the experiment. Finally, the slight particle enrichment in fused granule membrane might be due to a possible partial extraction of membrane phospholipids occurring during tissue processing. However, even if this were the case, the conclusions of our work would remain basically valid because the two membranes involved in fusion still remain clearly identifiable.

Previous investigations, carried out with thin sections, are rather conflicting as to the cytological mechanisms of the remodeling of enlarged secretory surfaces. In a number of systems, a population of small vesicles has been found to appear in the apical cytoplasm concomitant with the removal of the redundant plasma membrane originating with granule fusion (1, 3, 11, 15, 25, 26, 27, 32, 35, 37). These vesicles have been often interpreted as membrane patches that have been retrieved directly from the lumen and are in transit to other cell organelles, such as the Golgi complex or multivesicular bodies (1, 3, 25, 27, 35, 37). In other studies, however, larger structures continuous with the cell surface have also been seen and interpreted as corresponding to an intermediate stage in membrane recapture (26, 32). The results that we have obtained provide further information on the process, but are by no means conclusive. By the use of freeze-fracture, we have established that the extensive remodeling of the luminal plasmalemma which occurs in the parotid acinar cells after the exhaustion of protein discharge is a non-
random phenomenon; rather, it consists of the slow and progressive removal of the regions of low particle density from the cell surface. During the active exocytosis period, these regions appear in patches at the luminal surface, alternating with the typical plasmalemma in a mosaic organization: as the experiment continues, these regions are mostly found to bound large invaginations, which are continuous with the acinar lumen. There are at least two possible explanations for this last finding: (a) the invaginations might correspond to the regions where originally many granules have sequentially fused, forming large cavernae which are then retrieved more slowly than smaller invaginations (derived from either a single granule or a few granules); (b) alternatively, the large cavernae might be the result of a segregation of smaller low particle density membrane patches.

The following stages in the membrane dynamics at the secretory surface are the reduction in size and, eventually, the disappearance of the large invaginations, so that no regions of low particle density are visible at the luminal surface any longer.

Concomitant with these processes, we have observed the appearance of small vesicles in the apical cytoplasm. Thus, we are led to suggest that these vesicles might represent the means by which the limiting membrane of the invaginations is internalized. This sequence of events, however, is only tentative because: (a) images of vesicle formation are relatively rare at the surface of the large invaginations, and (b) due to their small radius of curvature, the particle density of the membranes of the small vesicles could not be determined exactly. Hence, the cytological origin of the vesicles could not be established with certainty.

In conclusion, our results suggest a possible explanation for the apparent conflict in the results obtained in a number of morphological and biochemical studies on various cell systems. In fact, it

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<th>Membranes</th>
<th>min after IPR injection</th>
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<tr>
<td></td>
<td>0</td>
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<tr>
<td>Secretion granules</td>
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<tr>
<td>Fused secretion granules</td>
<td></td>
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<tr>
<td>Low density patches of the luminal plasmalemma</td>
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<td>Cavernae of the luminal plasmalemma</td>
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<tr>
<td>Typical luminal plasmalemma</td>
<td>2,132 ± 206</td>
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* Values given are averages ±SE of particle densities (number/square micrometer). Micrographs of each experimental time-point were printed at constant final magnification (×80,000). 10-20 P face areas of each type of membrane were chosen at random. However, in order to minimize errors due to the different curvatures of the various structures under study, only relatively planar portions (0.08-0.4 μm² in surface) were considered. Figures relate to total surface areas ranging from 1.5 to 7.2 μm². The number of the particles was determined by direct counting.

FIGURE 5 Intercalated duct. The apical cytoplasm of the duct cells contains a well developed Golgi complex (GC), mitochondria and heterogeneous vesicles but is devoid of the large spherical secretion granules which are characteristic of the acinar cells. Note that the P face of the luminal plasma membrane (*) has a low particle density. J = tight junction. DL = duct lumen. × 40,500.

FIGURE 6 Low magnification view of a columnar duct. The cells are characterized by the deep infoldings of the basal plasmalemma and by the abundance of mitochondria (M). The round nuclei (N) are located centrally. DL = duct lumen. × 9,000.

FIGURE 7 Columnar duct. The picture illustrates the structure of the P face of the lateral (LPM) and luminal (*) plasmalemma. Note that the latter is extremely poor in particles. m = cross-sectioned microvilli; J = tight junctions. × 30,500.
The figure shows the dramatic modifications of the luminal morphology 5 min after IPR injection. * = P face of the original luminal plasmalemma. Many fused granule membranes are shown; in some of these the continuity with the typical luminal plasmalemma is visible in the plane of fracture (**); in others (**), it is suggested by the pleomorphic shape of the organelle. SG-P and SG-E = unfused secretion granule membranes, P and E faces. × 51,000.

Figure 8
FIGURE 9 5 min after IPR injection. The P face of three fused granule membranes (**) in continuity with the typical luminal plasmalemma (*) is shown. Note the sharp transition in particle density occurring at regions of continuity of the two membrane types (arrows). On the P face the particle density is slightly higher in fused (**) than in unfused granules (SG-P). AL = acinar lumen; J = tight junction; SG-E = secretion granule membrane, E face; *** = granule membrane which has probably fused with the luminal membrane. 9 a, x 40,000; 9 b, x 43,500; 9 c, x 30,000.
FIGURE 10 80 min after IPR injection. The figure shows that the distribution of particles on the P face of the luminal plasmalemma is greatly heterogeneous. I = fracture through the neck joining the acinar lumen with three cavernae underlying the fracture plane. J = tight junction. × 68,000.

FIGURE 11 The figures show the appearance of the P face of the luminal membrane in several acinar cells 2 h after IPR injection. The acinar canaliculi, similar in size and in particle density of the limiting membrane to those present in the unstimulated gland (*), are continuous with large cavernae bounded by a low particle density membrane (**). The transition between the two types of membrane is sharp and occurs near the neck of the infoldings. AL = acinar lumen, J = tight junction. 11 a, × 24,000; 11 b, × 37,000; 11 c, × 26,500; 11 d, × 34,000.
FIGURE 12 Low magnification view of parotid acinar cells 2 h after IPR injection. The apical portion of the cytoplasm is occupied by one large caverna bounded by a low particle density membrane (**) as well as by a population of small vesicles. GC = Golgi complex; RER = rough-surfaced endoplasmic reticulum; N = nucleus; C = reforming canalculus. × 22,500.
is generally accepted that the dimensional control of the cell surface, in relation to discharge of secretion products, is effected through the coupling of membrane fusions (= exocytosis) and fissions (= endocytosis). Furthermore, the molecular components of the participating membranes turnover much more slowly than secretion products (28, 29, 46, 47), suggesting that they are reutilized in several cycles; yet, these repeated interactions do not affect the identity of the granule membrane and the plasmalemma, which retain their morphological and biochemical peculiarities (10, 28, 29, 30). According to our data, this phenomenon is likely to depend on the immiscibility, at least partially, of these two fluid membranes (10, 28, 29, 30). According to our data, this phenomenon is likely to depend on the immiscibility, at least partially, of these two fluid membranes.

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