FREEZE-FRACTURE OBSERVATIONS OF THE LACTATING RAT MAMMARY GLAND

Membrane Events during Milk Fat Secretion

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ABSTRACT

Membrane events during milk fat secretion were analyzed by freeze-fracture of the rat mammary gland. Two modes of milk fat secretion were observed: extrusion of fat droplets surrounding a portion of the apical plasma membrane of the alveolar epithelial cells and, less frequently, release into the alveolar lumen of fat droplets contained in intracytoplasmic vacuoles. The extrusion process consists of two asynchronous events: clearing of membrane particles (probably including integral membrane proteins) and bulging of the apical plasma membrane. Most fat droplets are extruded with a bilayer membrane envelope (milk fat globule membrane) partially devoid of particles. The segregation of membrane particles may represent the onset of a process of structural degradation of the milk fat globule membrane.

KEY WORDS membrane structure · secretion · lactation · milk fat globule membrane · freeze-fracture

Under the complex hormonal environment and other stimuli of pregnancy and onset of lactation, the resting mammary gland is transformed into an actively secreting organ (8, 40). This process of growth and differentiation leads to the extensive development in the alveolar epithelial cells of rough endoplasmic reticulum, Golgi apparatus, and other endomembranes which are involved with the plasma membrane in the synthesis and secretion of milk (2-4, 9, 15, 16, 20, 28, 35, 37, 42).

The ultrastructural, physiological, and biochemical aspects of lactation have been investigated (4, 9, 16, 23, 35, 42). Early ultrastructural observations demonstrated that, during milk fat secretion, most lipid droplets are extruded into the alveolar lumen with a bilayer membrane envelope derived from the cell plasma membrane (2, 3). This secretion mechanism, as well as biochemical (10, 21, 28, 30) and morphological (10) evidences, led to the deduction that in freshly drawn milk the fat globule is still surrounded by a membrane (milk fat globule membrane, MFGM; for review see references 1, 29). Other studies provided additional aspects of milk fat secretion, namely on the origin, composition, and mechanism of formation and stabilization of the MFGM: (a) Golgi vesicles have been reported to contribute directly to the formation of the MFGM (45); (b) lipid droplets included in intracellular secretory vacuoles can be secreted without participation of the plasmalemma (47); (c) after secretion, the membrane of the milk fat globule may undergo a process of vesiculation and fragmentation and is gradually lost (14, 38, 44, 46); (d) some fat droplets are extruded with a crescent of cytoplasm...
capillaries, and extracellular connective structures uptake of blood-borne substances essential for that surround the alveoli, are involved in the membranes, in close relation with myoepithelial cells, milk biosynthesis. reference 33 for illustrations). The basal membranes present junctional complexes for intercellular contact and sealing of the alveolar lumen (Fig. 1; see reference 33 for illustrations). The basal membranes, in close relation with myoepithelial cells, capillaries, and extracellular connective structures that surround the alveoli, are involved in the uptake of blood-borne substances essential for milk biosynthesis.

Before the onset of fat secretion, a large amount of fat droplets is accumulated in the apex of the mammary epithelial cells. The lumina of the alveoli are already filled with numerous milk protein components. The casein granules are easily identified by their typical profiles consisting of flat, circular clusters of rugosities without enveloping membrane. The fracture-face A of the apical plasma membrane presents an apparently random distribution of numerous membrane particles, and views of microvilli. The particles are also present in the fracture faces of the microvilli.

The observation of the apical plasmalemma during lactation revealed structural alterations related to milk fat secretion (Figs. 2-11, and 13). A mild or pronounced bulging of microvilli-free regions of the membrane was observed. These areas were either smooth, i.e. devoid of particles (Fig. 2) or, less frequently, showed no alteration of particle distribution in relation to the surrounding apical plasmalemma. Fractures which allowed for simultaneous view of the plasma membrane and cytoplasm (Figs. 3, 5, and 7-9) showed that the bulges were occupied by fat droplets, sometimes associated with small portions of intracellular components. The events of fat droplet extrusion could be reconstructed from views of A and B faces of the apical plasma membrane and cross-fracture profiles. These included the gradual enveloping of the fat droplets by the apical plasmalemma. Frequently, the fracture-face A of the fat droplet membrane envelope displayed a smooth, particle-free surface in continuity with apical plasma membrane regions which presented an unaltered pattern of particle distribution (Figs. 3-6). Equivalent aspects of membrane particle clearing on the fracture-face B were also observed (Fig. 8). Less frequently, the fat droplets were enveloped by a membrane presenting a fracture face with particles (Fig. 9). Intermediate aspects consisting of emerging droplets surrounded by a membrane with some particle-free areas and the consecutive extrusion of more than one fat droplet were also observed. Other observations suggested that the secretion of larger fat droplets could also occur by extrusion of its components enveloped by a particle-free membrane as if the fat was being squeezed through a "locus minoris resistance" of the cell membrane (Figs. 10 and 11).

In the cytoplasm of the lactating cells most fat droplets showed smooth fracture faces. By cross fracture they often displayed a lamellar or paralamel rami.
The freeze-fracture survey of a lactating alveolus of rat mammary gland shows the apex of several cells, a segment of apical plasma membrane (aA), microvilli on longitudinal and cross views (mv), and lateral plasma membranes (lA, lB) where the tight junction band (tj) can be seen. In the alveolar lumen (L), fat flobules (fg) show a smooth fracture face. A cross fracture through a large fat droplet (fd) during extrusion is also observed. × 10,000.
taining lipid droplets surrounded by numerous, small (fat?) globules with a smooth fracture face were occasionally observed (Fig. 12). The following observation suggested that the secretion of these fat droplets followed a mechanism different from that of fat droplets which have no membrane: intracytoplasmic vacuoles were observed in close contact with the apical plasma membrane, and the region of the alveolar lumen directly opposite to these vacuoles presented emerging particle-free globules suggesting that fat was being released through a small opening in the plasma-lemma (Fig. 13).

In the alveolar lumen, milk fat appeared as globules, heterogeneous in size and structure (Figs. 1, 14, and 15). Fracture usually followed a plane close to the surface; most fracture faces were smooth (Fig. 1). Less frequently, a membrane with particles was seen around one or more globules (Fig. 14). In this case, the pattern of distribution of particles on the membranes enveloping fat globules ranged from uniform to segregated (Fig. 14) or reticulated. Cross fracture of the fat in the lumen revealed aspects similar to those observed in the cytoplasm (Figs. 14 and 15). Fat globules surrounded by cytoplasmic crescents were also observed. Other observations suggested the incorporation of small lipid droplets in larger globules, as well as the blebbing off of segments of the membrane envelope of the fat globules (Fig. 15).

DISCUSSION
Analysis of freeze-fracture results indicates two possible modes of milk fat secretion: (a) extrusion of the fat globule surrounded by a portion of the apical plasma membrane, the prevalent mechanism; and (b) release into the alveolar lumen of free fat globules, i.e. without enveloping membrane, a process observed much less frequently and restricted to the secretion of small fat droplets contained within intracellular vacuoles.

The fracture faces of the apical plasmalemma during the extrusion of fat droplets illustrate two membrane events: clearing of membrane particles and bulging of the plasma membrane. In some instances, as the droplets are pushed out of the cell there is a progressive clearing of particles in the areas of membrane bulging so that, at the moment of separation from the cell, the globule is surrounded by a bilayer membrane without particles. In others, the extrusion process is not preceded nor accompanied by clearing of membrane particles, and so, the extruded fat globules are enveloped by a membrane with particles. Intermediate aspects, i.e. fat droplets extruded with a variable degree of membrane particle clearing, are also found. In consequence, our results demonstrate an asynchronism between bulging of the plasma membrane and redistribution of integral membrane components, as revealed by the progressive clearing of particles. The extrusion of fat droplets with a bilayer membrane envelope supplied by the apical plasma-lemma of mammary epithelial cells has been described in thin-section studies in several species (2-4, 11-13, 16, 22, 35, 36, 39, 41-43). Our freeze-fracture results

1 The smooth surface of most fat globules observed in the alveolar lumen probably corresponds to the fracture face of the fat globule membrane in view of the clearing of membrane particles during extrusion, and the concept of membrane splitting in freeze-etching (31). In some instances it might also correspond to the fracture through an outer lipid lamella of the fat globule. In this case, the cross-fracture profile of the fat globule membrane should often be evident (see Figs. 5 and 14).

\[\text{FIGURE 2} \text{ Initial events of fat droplet extrusion. The fracture-face A (a)}_e \text{ of the apical plasma membrane shows bulging of particle-free areas (arrows). Microvilli are observed on cross fracture. } \times 40,000.\]

\[\text{FIGURE 3} \text{ Fracture-face A (a)}_e \text{ of an area of bulging of the apical plasma membrane, allowing a simultaneous view, on cross fracture, of a fat droplet (fd) underneath. The smooth area of the apical plasmalemma (*) is clearly demarcated from the area with particles. L, alveolar lumen. } \times 30,000.\]

\[\text{FIGURE 4} \text{ High magnification of the transition from the area with particles to the smooth area of the apical plasmalemma of Fig. 3 shows the continuity of these membrane segments. } \times 120,000.\]

\[\text{FIGURE 5} \text{ Fracture-face A (a)}_e \text{ of an area of the apical plasmalemma with particle clearing (*). The outer lipid lamella of a fat droplet is exposed, and the cross-fracture profile of the surrounding apical membrane is evident (arrowheads). L, alveolar lumen. } \times 40,000.\]
show that this process is often accompanied by segregation of membrane integral components. It is also possible that, during extrusion, peripheral membrane components at the inner surface are segregated. This could facilitate the extensive bulging of the apical cell membrane during the extrusion process and account for the absence of actin and spectrin in MFGM preparations.

Less frequently, fat droplets seemed also to be released from intracytoplasmic vacuoles into the alveolar lumen. Our findings agree with previous thin-section observations. This exocytotic mechanism presumably involves the fusion of the secretory vacuoles, containing lipid droplets and other secretory products, with the plasma membrane, and results in the release of fat globules without an enveloping membrane.

Morphological and biochemical studies of secretion in different cells, including mammary epithelial cells during lactation, provide evidence for a continuous turnover between endomembranes and the plasma membrane segments related to the secretory processes. These events could explain the presence of Golgi and endoplasmic reticulum enzymes in MFGM preparations and their phospholipid composition intermediate between that of the Golgi and the plasma membranes, even if contamination from cytoplasmic components extruded with some fat droplets could be avoided or if the Golgi vesicles did not contribute directly to the formation of the MFGM. In consequence, the enzymic or chemical analysis alone cannot provide definitive evidence for the origin of the membrane of the fat globule, but must be seen in perspective with cell membrane dynamics.

After fat secretion, electron microscope observations suggest that, in some globules, the membrane is gradually lost by a process of fragmentation and vesiculation. It is possible that during and after structural degradation of the membrane envelope of the fat globules, lipid and protein components of the initial fat globule membrane may be adsorbed to the surface of the fat globules. Thus, the unit membrane profiles observed on thin sections of MFGM preparations, obtained from cream by freezing, sonication, and other procedures, may represent not only initial MFGM but also reflect bilayered structures reconstituted before and during the isolation procedures. The structural rearrangement of the MFGM during or after secretion has been postulated to account for some biochemical and ultrastructural results. However, it has been recently assumed that the overall composition of the MFGM relative to the plasma membrane of the alveolar epithelial cells is not affected. Unfortunately, analysis of the apical plasmalemma of the lactating mammary cell is not accessible.

Our freeze-fracture observations suggest that the events which occur within the apical membrane during milk fat secretion may account for some compositional, physicochemical, and ultrastructural differences between MFGM and plasma membrane preparations. The mechanism of segregation of membrane particles is, at present, unclear. It is possible that the

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**FIGURE 6** Final events of fat droplet extrusion. Membrane particles are absent on fracture-face A (aA) of the apical plasma membrane which envelops the fat droplet. L, alveolar lumen. x 27,000.

**FIGURE 7** Stage equivalent to Fig. 6. The fracture process exposed the lamellar appearance of the fat droplet (fd). Membrane particles are observed on the area of the plasma membrane which surrounds but is not in direct contact with the fat droplet (arrowheads). aA, fracture-face A of the apical plasma membrane. x 40,000.

**FIGURE 8** Fat droplet extrusion as observed from the inside of a cell. A smooth area of membrane bulging (*) under the fat droplet (fd) is demarcated from the surrounding fracture-face B (aA) of the apical membrane. x 40,000.

AMADEU PÊREOTO DE MENEZES AND PEDRO PINTO DA SILVA  
Milk Fat Secretion  
773
reorganization of the membrane integral components results from the interaction between the fat droplet core and the apical plasma membrane, during extrusion. It might also involve the concomitant displacement of peripheral membrane proteins at the inner surface.3

In summary, the process of segregation of integral components from areas of plasmalemma which envelop the fat droplets may differentially restrict the membrane components which form the milk fat globule membrane. This could correspond to an initial event of membrane structural degradation, which progresses after extrusion. Although most of the components found in purified preparations of MFGM were at one time associated with the apical plasmalemma, the fat globule membrane cannot be considered as structurally identical to the apical membrane of the mammary epithelial cells.

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Figure 9 Emergence of a fat droplet (fd) surrounded by a membrane with particles (α₁). α₂, fracture-face A of the apical plasma membrane; L, alveolar lumen. × 30,000.
Figures 10 and 11. Extrusion of fat droplets (fd) suggesting the squeezing through a "locus minoris resistentiae" of the apical plasma membrane. Fig. 10, the fat material is surrounded by the apical plasma membrane, without particles (a). L, alveolar lumen. × 35,000. Fig. 11, dumbbell profile as a result of a constriction at the plasma membrane level. × 17,000.

Figure 12. Intracytoplasmic vacuole containing a fat droplet (fd). × 17,000.

Figure 13 Intracellular vacuole with a membrane with particles (vA) in close contact with the apical plasmalemma. From the opposite outer side of the cell membrane a globule with a smooth fracture face is emerging. L, alveolar lumen. × 35,000.
Figures 14 and 15. Aspects of globules in the alveolar lumen. Fig. 14, fracture-face A of a fat globule membrane with a smooth particle-free area. The arrowheads point to the cross-fracture profile of the fat globule membrane. × 38,000. Fig. 15, vesiculation process of the fat globule membrane. The segment of blebbing (arrowhead) presents a fracture face with an irregular distribution of particles. × 38,000.
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