FREEZE-FRACTURE IMAGES OF DISTRIBUTION OF FILIPIN- CHOLESTEROL COMPLEXES IN SECRETORY CELLS OF THE ANTERIOR PITUITARY AND ADRENAL MEDULLA OF MICE*

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In order to clarify the distribution of cholesterol in plasma- and cyto- membranes of secretory cells of the anterior pituitary and adrenal medulla, freeze-fracture images of the filipin-treated tissues were observed.

The distribution of cholesterol in all the secretory cells of these organs shows almost the same pattern. The filipin-sterol complexes are distributed somewhat heterogeneously but in moderately high density on the fractured plasma membranes of all the cells examined. The small shallows on the plasma membrane, corresponding to coated pits and showing aggregates of membrane particles (MP), lack the complexes. The limiting membranes of almost all the secretory granules are very rich in complexes.

The membranes of rough endoplasmic reticulum, nucleus, mitochondria, and Golgi apparatus, which are considered to contain rich enzymatic protein and have active functional properties, are very poor in distribution of cholesterol, though small clusters consisting of a few complexes are scattered in these organelles except for mitochondria. In the trans-side of the Golgi apparatus, where the immature secretory granules arise, the complexes are relatively denser in distribution as compared with those of the cis-side.

Though the cholesterol contents in the membrane have been considered to influence the fluidity, permeability, electric resistance and hydration properties of the artificial and natural membrane (5, 6, 7, 10, 30), many problems remain to be solved as to the distribution of cholesterol and its functional properties in plasma- and cytomembranes.

A polyene antibiotic, filipin, has been known to bind specifically cholesterol and form filipin-sterol complexes (11, 13, 21, 22), which are easily recognized as protuberances or pits in freeze-fracture images (1, 2, 8, 12, 28, 31, 32), and the detection of cholesterol distribution in the plasma- and cytomembranes of various kinds of cells has been the subject of some recent published papers.

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However, nothing has been reported as to the fine structural localization of cholesterol in the plasma- and cytomembranes of secretory cells, except for the papers dealing with pancreatic exocrine and endocrine cells by Orci *et al.* (25) and thyroid follicle cells by Fujita *et al.* (9).

The aim of the present study is to observe the freeze-fracture images of the filipin-treated cells in the anterior pituitary and adrenal medulla, in order to clarify the distribution of cholesterol in the plasma- and cytomembranes of some endocrine cells. Interesting results obtained could be useful in the future for knowing the functional morphology of the secretory cells and detecting the function of cholesterol in the membrane.

MATERIALS AND METHODS

Male and female mice, 2–5 months of age, were used for this study. Thirty animals were perfused with 2% glutaraldehyde solution buffered at pH 7.4 with 0.1 M cacodylate, and the anterior pituitaries and adrenal glands removed were cut into small pieces, and immersed in the same fixative for 1 hr. Then some of the specimens were treated with 0.02–0.05% filipin (a gift of Upjohn Co., Kalamazoo, Mich.) buffered at pH 7.4 with 0.1 M cacodylate for 12–24 hr at room temperature, and the others with 0.02–0.05% filipin in a 1.5% glutaraldehyde solution buffered at pH 7.4 with 0.1 M cacodylate for 12–24 hr at room temperature. After soaking in 30% glycerol solution for 10–12 hr, the tissues were rapidly frozen in liquid nitrogen, cleaved, shadowed with platinum-palladium, and stabilized with carbon in an Eiko FD-2 type freeze-fracture apparatus. The replicas made were examined in a Hitachi H-500 type electron microscope.

Some other materials fixed with glutaraldehyde and treated with filipin were postfixed with 1.0% OsO₄ solution buffered at pH 7.4 with Millonig's phosphate for 1 hr. After dehydration with graded concentrations of alcohol, the tissues were embedded in Epon epoxy resin. The sections, cut on a Porter-Blum MT-2 ultramicrotome, picked up on copper grids, stained with uranyl acetate and Millonig's lead, were examined in a Hitachi H-500 type electron microscope.

OBSERVATIONS

Freeze-replica images

The filipin-sterol complex density of specimens prepared with the filipin solution after the fixation was similar to that with a mixture of filipin and glutaraldehyde solution after the fixation. All kinds of secretory cells of the anterior pituitary and those of the adrenal medulla showed an almost similar pattern to one another in freeze-fracture images of filipin-treated tissue. Freeze-fracture of the membrane of these cells showed unique structural alterations consisting of numerous 25 to 30 nm protuberances or pits for filipin-sterol complexes. No distinct differences were observed in population density of the complexes between P-face and E-face.

The filipin-sterol complexes were distributed somewhat heterogeneously, but in moderately high density on the fractured face of all the plasma membranes of the secretory cells of both organs. Small shallow depressions, suggesting coated pits, where the intramembrane particles form an aggregate, lacked the filipin-sterol complexes (Fig. 1).

FIG. 1. Freeze-fracture image of filipin-sterol complexes in the plasma membrane of the secretory cell of a mouse anterior pituitary. Many complexes are distributed somewhat heterogeneously, and a small depression suggesting the coated pit, where membrane particles aggregate, lacks the complexes. $\times 79,000$

The fractured membranes of the rough endoplasmic reticulum, of nucleus, and of mitochondria were almost free from the complexes, though small culsters consisting of 2–10 complexes were rarely scattered (Fig. 2). Among them, it is quite difficult to find the complexes in the mitochondrial outer and inner membranes. Though membranes of most Golgi sacs also lacked the complexes, some parts of the Golgi membrane of the trans-side, and the membranes of small vesicles and vacuoles in the trans-side of the Golgi apparatus showed aggregates of a fairly large number of complexes (Fig. 3).

The limiting membranes of almost all the secretory granules showed a high density of filipin-sterol complexes distributed homogeneously, though those of a few granules lacked the complexes (Figs. 3, 4).

Thin section images

In the materials treated with filipin after the glutaraldehyde fixation, the plasma membranes except for the coated pits, and the limiting membranes of most secretory granules were usually obscure showing zigzag contour in thin sections, and their unit membrane structure was difficult to recognize in all the secretory cells of both organs (Fig. 5).

The membranes of rough endoplasmic reticulum, mitochondria, Golgi apparatus, coated pits, and coated vesicles were usually well preserved in their fine structure. In addition, a limiting membrane of the coated secretory granule rarely observed near the Golgi region showed also good preservation in its fine structure even after the treatment with filipin (Fig. 6).

FIG. 2. Freeze-fracture image of filipin-sterol complexes in the secretory cell of a mouse anterior pituitary. Not numerous complexes in the limiting membrane of the secretory granules. Membranes of rough endoplasmic reticulum (R), nucleus (N), and mitochondria (M) are almost free from the complexes, though small aggregates are seen. $\times 24,000$

DISCUSSION

Freeze-fracture studies of filipin-treated materials are becoming popular for several kinds of cells, such as erythrocytes (12, 31, 32), sperms (3, 4), tracheal epithelial cells (15), *Tetrahymena* cells (29), smooth muscle cells (16), retinal rod cells (1, 2), fibroblasts (17), mast cells (19), urinary bladder epithelial cells (24), exocrine pancreatic cells (23, 25), kidney podocytes (26), larval urodelan epidermis cells (27), thyroid cells (9), neuromuscular junctions (20), and macrophages (18). However,

FIG. 4. Freeze-fracture image of a secretory cell of a mouse adrenal medulla. Not numerous secretory granules, whose limiting membranes are well labeled by filipin, and the mitochondrial membranes (M) without complexes. $\times 34,000$

the cholesterol contents of plasma- and cytomembranes of various kinds of cells should be further clarified. The present paper concerns the distribution of filipin-sterol complexes in the anterior pituitary cells and adrenal medullary cells of mice.

All the secretory cells of both tissues showed similar pattern to one another in the freeze-fracture images after treatment with filipin.

The plasma membrane and the limiting membrane of almost all the secretory granules of both cells were highly labeled with filipin-sterol complexes. It is considered that cholesterol is necessary for the movement of the plasma membrane and the transport of the secretory granules.

The fact showing the lack of the filipin-sterol complexes at the coated as well as non-coated pits, where the aggregation of membrane particles occur, has been reported in some cells, such as fibroblasts (17), thyroid cells (9), and macrophages (18). Similar results were obtained in the anterior pituitary as well as adrenal medullary cells. Though the functional significance of coated pits or coated granules of the anterior pituitary cells is not clear, they might be implicated with the mem-

FIG. 3. Freeze-fracture images of secretory cells of a mouse anterior pituitary.

a) Not the Golgi apparatus (G), in which the trans-side (lower) is labeled with filipin, while the cis-side is not labeled. $\times 43,000$

b) Not that limiting membranes of most secretory granules are labeled with filipin to form complexes. $\times 37,000$

FIG. 5. Thin section images of the secretory cell of a mouse anterior pituitary immersed in filipin solution after glutaraldehyde fixation. Membranes of mitochondria and rough endoplasmic reticulum are distinct, while the limiting membranes of the secretory granules are obscure. $\times 34,000$

brane retrieval after the release of the secretory granules. In thin sections, the limiting membrane of the coated granule, which is rarely recognized in the cytoplasm, is well preserved in its fine structure. This granule might correspond to the secretory granules, whose membrane almost lack the filipin-sterol complexes, in freeze-fracture images. Nakajima and Bridgman (20) also found an absence of filipin-sterol complexes in the membranes of active zones and acetylcholine receptor aggregates in the neuromuscular junction. It has been shown that cholesterol is present only in the protein particle-free patches of the basal disk and the plasma membrane of retinal rod outer segment (2). These facts suggest that the active region of the plasma membrane showing aggregates of protein particles is low in cholesterol contents.

The membranes of rough endoplasmic reticulum, nucleus, mitochondria and Golgi apparatus are very poor in filipin-sterol complexes, though small scattered clusters are rarely recognized. It is quite difficult to find the complexes in the mitochondrial outer and inner membranes. The complexes are sometimes recognized in the trans-side of the Golgi apparatus, as reported in the pancreatic acinar cell by Orci *et al.* (25). These facts coincide well with the biochemical data that the cholesterol contents in the liver and kidney are the lowest in mitochondria, and somewhat higher in rough microsomes and Golgi apparatus, and the highest in plasma membrane (33). Meldolesi *et al.* (14) also showed biochemically that the membrane of the rough endoplasmic reticulum is markedly lower in cholesterol

FIG. 6. This section image of a part of the secretory cell of a mouse anterior pituitary. Though the limiting membrane of most secretory granules is obscure, that of a coated granule (arrow head) is distinct. G; Golgi apparatus. $\times 66,000$

contents as compared with the zymogen granule membrane and plasma membrane in pancreatic acinar cells. It is reasonable to consider that the membranes of mitochondria and rough endoplasmic reticulum, which contain rich enzymatic proteins and have active functional properties, are low in cholesterol contents.

The trans-side of Golgi apparatus has an activity to form the immature secretory granule, and the limiting membrane of the secretory granules is usually very rich in cholesterol contents as described above. From these facts, it is easily speculated that free cholesterol enters the membrane to be its component at the trans-side of the Golgi apparatus where the secretory granules are formed.

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