Rings of Membrane Sterols Surround the Openings of Vesicles and Fenestrae, in Capillary Endothelium

NICOLAE SIMIONESCU, FLOREA LUPU, and MAYA SIMIONESCU
Institute of Cellular Biology and Pathology, Bucharest-79691, Romania

ABSTRACT We investigated the distribution of sterols in the cell membrane of microvascular endothelium (mouse pancreas, diaphragm, brain, heart, lung, kidney, thyroid, adrenal, and liver) with the polyene antibiotic filipin, which reportedly has binding specificity for free 3-β-hydroxysterols. In some experiments, concomitantly, cell-surface anionic sites were detected with cationized ferritin. Vessels were perfused in situ with PBS, followed by light fixation and filipin administration for 10 to 60 min. Tissues were further processed for thin-section and freeze-fracture electron microscopy. Short exposure (10 min) to filipin-glutaraldehyde solution resulted in the initial appearance, on many areas, of rings of characteristic filipin-sterol complexes within the rim surrounding stomata of most plasmalemmal vesicles, transendothelial channels, and fenestrae. Such rings were absent from the rims of the large openings of the sinusoid endothelium (liver, adrenal), coated pits and phagocytic vacuoles. After longer exposure (30–60 min), filipin-sterol complexes labeled randomly the rest of plasma membrane (except for coated pits, and partially the interstrand areas of junctions), and also marked most plasmalemmal vesicles. These peristomal rings of sterols were displayed mostly on the P face, and, at their full development, consisted of 6–8 units around a vesicle stoma, and 10–12 units around a fenestra. At their level, the intramembranous particles and the cell surface anionic sites were virtually excluded. Peristomal rings of sterols were also detected on the plasma membrane of pericytes and smooth muscle cells of the microvascular wall, which otherwise were poorly labeled with filipin-sterol complexes as compared to endothelial plasmalemma. It is presumed that the peristomal rings of cholesterol may represent important contributors to the local transient stabilization of plasma membrane and to the phase separation between cell membrane and vesicle membrane at a certain stage of their fusion/fission process.

Cholesterol is recognized as a prominent component of cellular membranes with significant effects on fluidity, permeability, enzyme activity, fusion events, and other membrane properties (10, 18, 31, 32). Together with other lipids, cholesterol may contribute to the creation of lipid domains by lateral phase separation (34), self-segregation, change in the radius of membrane curvature (21), or by being part of a transmembrane lipid asymmetry (5, 8, 20, 36).

To explore some aspects of the cholesterol topology in plasma membrane, we used as system the capillary endothelium, which under its various types displays a complex cell-surface pattern with well defined structural domains such as plasmalemma proper, coated pits, phagocytic vacuoles, plasmalemmal vesicles, transendothelial channels, fenestrae, and large discontinuities. Some of these openings (plasmalemmal vesicles, channels, and fenestrae) are closed by thin, unilayered diaphragms.

The inquiry was based on the use of the polyene antibiotic filipin which, by specifically interacting with cholesterol and other related 3-β-hydroxysterols in cell membranes (3, 9, 27), produces distinctive filipin-sterol complexes (FSC) (11-13, 19, 35, 48, 50) recognizable as ~25-nm protuberances on both freeze-fractured and thin-sectioned membranes. This method has been widely used to investigate the distribution of sterol in a variety of cells (1, 6, 11-13, 14, 19, 23-26, 28-30, 38), including heart capillary endothelia in which a generalized labeling of plasma membrane by FSC was reported (37).

By applying this technique to a large variety of endothelial cells, pericytes, and smooth muscle cells, we can show that

Abbreviations used in this paper: FSC, filipin-sterol complex; MEM, modified Eagle’s medium; DMSO, dimethyl sulfoxide; CF, cationic ferritin; PSRS, peristomal ring of sterols.
the lines of fusion between plasma membrane and plasmalemmal vesicles and its derivatives (channels and fenestrae) are surrounded by characteristic peristomal rings (anuli) of FSC. The latter are virtually absent from the phagocytic vacuoles, coated pits, and the large discontinuities of sinusoid capillaries.

MATERIALS AND METHODS

Reagents: Filipin was a gift from J. E. Grady (Upjohn Co., Kalamazoo, MI); before use, filipin was dissolved in a drop of dimethylsulfoxide (DMSO) (final concentration of DMSO, 1%). Dimethylsulfoxide was purchased from Sigma Chemical Co., St. Louis, MO, and tannic acid (AR code no. 1764) from Mallinckrodt Inc., St. Louis, MO. Cationic ferritin pl 8.4 was obtained from Miles Laboratories, Elkhart, IN; before use, ferritin solutions were dialysed for 24-48 h at 4°C against 0.15 M NaCl and found by electron microscopy to be homogeneous, monodispersed preparations. Dulbecco's phosphate-buffered saline (PBS) and minimal essential medium (MEM) amino acids (× 50 concentrate) were purchased from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY).

Animals: Experiments were carried out on 26 male 120-130-g Wistar rats, and 32 male 25-30-g RAP mice: animals were kept in standard housing and were studied in similar conditions for ten days before experiments.

Experimental Procedures: After general anesthesia and laparotomy, the general protocol consisted of the following steps: (a) blood removal, (b) fixation by perfusion, (c) filipin administration, (d) removal of excess filipin, (e) collection of specimens, and (f) processing for freeze-fracture and thin-section electron microscopy.

Blood Removal: The abdominal aorta and vena cava caudalis were catheterized with polyethylene tubing (Clay Adams, Division of Becton, Dickinson & Co., Parsippany, NY). The aortic catheter was connected to a Harvard infusion pump and the vasculature was washed free of blood by retrograde perfusion at a flow of 4 ml/min for 5-6 min with PBS, pH 7.2, or with 0.15 M NaCl and found by electron microscopy to be homogeneous, monodispersed preparations. Dulbecco's phosphate-buffered saline (PBS) and minimal essential medium (MEM) amino acids (× 50 concentrate) were purchased from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY).

Administration of Filipin: Perfusion of same buffered fixative as above, containing 50 μM filipin (in 1% DMSO): the solution was maintained by infusing pump and the vasculature was washed free of blood by retrograde perfusion at a flow of 4 ml/min for 5-6 min with PBS, pH 7.2, or with 0.15 M NaCl and found by electron microscopy to be homogeneous, monodispersed preparations. Dulbecco's phosphate-buffered saline (PBS) and minimal essential medium (MEM) amino acids (× 50 concentrate) were purchased from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY).

Removal of Excess Filipin: This was accomplished by brief washing with buffer.

Collection of Tissues: To secure exploration of a broad structural pattern of endothelial cells, the following types of capillaries were investigated in collected specimens: for continuous endothelium, heart, lung, brain, and diaphragm: for fenestrated diaaphragmed endothelium, pancreas, renal peritubular capillaries, and thyroid; for fenestrated nondiaphragmed endothelia, kidney glomerular capillaries; for sinusoid-fenestrated endothelium, adrenal cortex; for sinusoid endothelia, liver. Within these tissues, in addition to endothelium, we also examined the distribution FSC on other cells of the vessel walls especially pericytes and smooth muscle.

Processing for Electron Microscopy: For freeze-fracture, specimens were immersed in 25% glycerol in 0.1 M HCl-Na cacodylate buffer, pH 7.2, for 2 h at 22°C, mounted on gold disks and quickly frozen in the liquid phase of partially solidified Freon 22 cooled with liquid nitrogen, and fractured at −115°C in a Balzers-301 apparatus. Replicas were cleaned with sodium hypochlorite and chloric acid and recovered on coated 200-mesh cooper grids.

For thin sectioning, fragments from same specimens prepared for freeze-fracture were postfixed for 90 min at 4°C in 2% Oso4 in 0.1 M HCl-Na cacodylate buffer, pH 7.2, then treated in block with either 0.5% magnesium uranyl acetate in 0.15 M NaCl for 30 min at 50°C or with arsenate or HCl-Na cacodylate buffer, pH 7.2, for 2 h at 22°C, mounted on gold disks and quickly frozen in the liquid phase of partially solidified Freon 22 cooled with liquid nitrogen, and fractured at −115°C in a Balzers-301 apparatus. Replicas were cleaned with sodium hypochlorite and chloric acid and recovered on coated 200-mesh cooper grids.

To investigate the topological relationships between cationic ferritin-detectable anionic sites and the FCS, we conducted a set of experiments in which the administration of filipin was preceded by perfusion with cationic ferritin (CF pl 8.4, for 3 min at 37°C, followed by washing with PBS. In a few experiments, the perfusion with filipin-glutaraldehyde was followed by quenching with 0.1 M glycine (45) and subsequent CF administration. In the last two types of experiments, the specimens were prepared for thin-section electron microscopy only.

RESULTS

Endothelial cells exposed for 10 to 30 min to filipin-glutaraldehyde solution revealed on their luminal plasma membranes typical FSC (Fig. 1), visible on the P faces as distinct ~20-30-nm-diameter hemispherical protrusions and depressions appearing preferentially around the openings of plasmalemmal vesicles, transendothelial channels, and fenestrae. Occasionally, corresponding pits could be observed on the ectoplasmic faces. In some regions, one to three FSC could be seen in the rims of these openings (Figs. 4 and 6), whereas in others they form a complete peristomial ring of sterols (PSRS) consisting of 6 to 8 units around a vesicle (or channel) stoma, and 10 to 12 units around a fenestral opening (Figs. 4 and 5). In the endothelium of sinusoid capillaries of liver and areatal cortex, PSRS could be also detected around openings of plasmalemmal vesicles and fenestrae, but they were absent from the rim of the large discontinuities characteristic for these types of endothelia (Figs. 8 and 9). In all endothelia examined, no comparable annuli of FSC were observed around coated pits (Fig. 10), phagocytic vacuoles invaginated from the plasma membrane (not illustrated) or on the plasmalemma proper. At early time intervals (10 and 15 min) there was a certain degree of variation in the extent of plasma membrane labeling by FSC (compare Figs. 5 and 6), that may be due to differences in the accessibility of the perfusate to various capillaries.

At 30 min or more, filipin gained access also to the abluminal front of endothelial cells as well as to the other neighboring elements such as pericytes (some were labeled even at 15 min; see Fig. 6) and smooth muscle cells. At these time intervals, FSC were scattered randomly on large areas of endothelial plasma membrane, which displayed a rather generalized labeling by FSC; the latter also labeled the membrane of phagocytic vacuoles, plasmalemmal vesicles, transendothelial channels, and some intracellular organelles. The FSC distribution on the latter is not detailed in this paper. The membranes of coated pits and coated vesicles lacked FSC (Figs. 5, 7, 10).

After 60-min exposure to the filipin-glutaraldehyde solution, the response of the endothelial cell plasmalemma to filipin was rather uniform on both the luminal and abluminal surfaces, including the parajunctional zones, and focally the interstrand junctional areas as well. FSC were absent from communicating (gap) junctions and from the crevices devoid of particles of endothelial junctions in venules. Coated pits remained devoid of FSC. Rings of FSC were never found on plasma membrane without being associated with one of the opening types mentioned above. FSC overlie membrane regions rich in intramembrane particles (Fig. 8). No FSC were observed on vesicle or fenestral diaphragms.

Plasma membrane of pericytes and smooth muscle cells occurring in the vessel wall of postcapillary and muscular venules, respectively, at their early stage of filipin lesions (30 min), displayed characteristic peristomial rings of FSC around the openings of their plasmalemmal vesicles (pericytes) (Fig. 6) and sarcolemmal vesicles (smooth muscle cells) (Fig. 7). After 60-min exposure to filipin, when cells located deeper in the specimen (stroma cells and epithelial cells) showed also extensive filipin-induced lesions, the plasma membranes of pericytes, and smooth muscle cells still remained only poorly (10-20% of their surface) labeled by FSC. These occurred
almost invariably aggregated into clusters with rare solitary lesions (Figs. 6 and 7).

A striking difference was observed as regards the sidedness of FSC: while in the endothelial cell plasma membranes they were preferentially located on the P faces, in smooth muscle cells the protrusions occurred mostly on the E faces (compare Figs. 5 and 7).

Observations mentioned above were made on freeze-fracture...
FIGURES 2-4  Mouse thyroid capillary: early stages in the formation of peristomal rings of sterols (pr). Fig. 2: After 10-min exposure to the filipin-glutaraldehyde solution, the first filipin-sterol complexes (arrowheads) appear in the rims of plasmalemmal vesicles (v) and fenestrae (f). P, P face. Fig. 3: In thin-sectioned specimens, the peristomal rings (arrowheads) can be detected as almost symmetrical protrusions in the rims of vesicle stomata. e, endothelial cell. Fig. 4: At 15 min, the peristomal rings of sterols (pr) display FSC in various numbers. f, fenestra, v, plasmalemmal vesicle, P, protoplastic face. Fig. 2, x 68,000; Fig. 3, x 73,000; Fig. 4, x 66,000.

ture replicas and were confirmed by thin sections which also showed that preservation of cells and membranes was satisfactory after filipin treatment.

Experiments in which the filipin administration was preceded by perfusion with CF showed that the regions of PSRS were usually devoid of CF-detectable anionic sites (not illustrated). CF, however, labeled rather uniformly the plasma membrane regions affected by filipin.

No significant differences in the distribution of PSRS were recorded between various endothelia examined. At late time intervals (30 to 60 min), FSC were in general equally distributed on the plasma membranes of the luminal and abluminal fronts of endothelial cells.

DISCUSSION

The salient findings of this study are: (a) Short exposure to low concentration of filipin results in the appearance on the plasma membrane of capillary endothelium of characteristic FSC, some of which form rings around the openings of many plasmalemmal vesicles, channels, and fenestrae. (b) These PSRS are absent from the rims of coated vesicles, phagocytic vacuoles, and large discontinuities in the endothelium of sinusoid capillaries (Fig. 11). (c) Comparable PSRS are also found in the plasma membrane of pericytes and smooth muscle cells. (d) The polarity of filipin-induced protrusions occurring mostly on the P faces of endothelial plasmalemma.
is opposite in the membrane of smooth muscle cells where FSC as well as PSRS appear preferentially, and in much lower incidence, on the E faces. (e) The region of PSRS is devoid of cell surface anionic sites detectable with CF.

By its position, the luminal plasma membrane of endothelial cells is quickly and directly accessible to solutions perfused intravascularly. That made possible a rapid and progressive penetration of filipin that allowed the detection of the location and configuration of a particular pool of cholesterol which, probably by its position, is readily available for complexing with filipin. The subsequent FSC formed are organized as annuli or rings surrounding many of the openings of plasmalemmal vesicles and fenestrae. At the time of the appearance of PSRS, FSC are very scarce on the rest of luminal plasma membrane of endothelium. At later time intervals (up to 60 min) when plasmalemma proper is heavily labeled with FSC, the PSRS, though present, are indistinguishable from the rest of filipin-induced protrusions. This initial heterogeneous labeling of plasmalemma was probably not due to problems of penetration, since the antibiotic had free access to the entire luminal plasmalemma that allowed a rapid filipin-sterol association into these structures. We consider, however, that F labeling only represents a qualitative evidence for sterol topographical inhomogeneity and does not permit evaluation of the absolute sterol (or cholesterol) content of a certain membrane region.

In previous studies it has been demonstrated that endothelial cell membrane contains biochemically differentiated microdomains of glycoconjugates (40–46) and domains of lipids (20). The newly described PSRS can be considered as "sterol microdomains" transiently formed at the line of fusion and
FIGURE 6 Mouse thyroid capillary, 15 min after exposure to filipin-glutaraldehyde. This freeze-fracture replica exhibits the cleaved plasma membranes of an endothelial cell (e) and a pericyte (pc). Filipin-sterol complexes (arrowheads) can be seen on the rims of few openings of plasmalemmal vesicles (v) and fenestrae (f), or they almost form complete peristomal rings of sterols (pr). P, protoplasmic face. × 66,000.

Fission between plasma membrane and membranes of plasmalemmal vesicles and their derived channels and fenestrae. PSRS are absent from the rims of features resulting from the invagination of plasma membrane, coated pits, and phagocytic vacuoles, as well as from the margins of large discontinuities of endothelia of sinusoid capillaries.

It appears that PSRS circumscribe tiny membrane areas with a selective exclusion of some membrane components. PSRS probably impart a certain rigidity to plasmalemma at their level, and many represent means of lateral phase separation between two fused membranes (15, 26, 33, 49), plasma membrane, and vesicle membrane, thus preventing randomization of their constituents (41, 42). The observations reported suggest that PSRS contribute to a lesser extent in the maintaining of the sharp bending of the openings since they are virtually absent from the rims of large discontinuities occurring in the endothelium of sinusoid capillaries. PSRS do not reveal as much a difference in the local concentration of sterols but rather a special intramembrane topography of sterol molecules that renders them more easily accessible for complexing with filipin.

The findings suggest that also in the endothelial cell, membranes involved in various types of endocytosis do not react in the same way with filipin (29); thus, such vesicles or vacuoles may have a different cholesterol content and probably different mechanisms of formation. Membranes involved in phagocytosis and receptor-mediated endocytosis lack PSRS, whereas membranes involved in transcytosis (41, 42) do have such means of lateral phase separation. Lateral phase separation of lipids was demonstrated in liquid-liquid and liquid-gel mixed systems, and cholesterol may play a role in such phase separations (34). Lateral phase separation could
FIGURE 7 Mouse pancreas: ectoplasmic face (E) of a cleaved plasma membrane of a smooth muscle cell of a muscular venule. Peristomal rings of sterols (pr) can be seen forming around the stomata of sarcolemmal vesicles (v). Such features are absent from the periphery of coated pits (cp); the plasmalemma in general is poorly labeled by filipin-induced protrusions. × 40,000.

It seems unlikely that PSRS are the result of a redistribution of sterol molecules under the influence of filipin, since this has concomitantly access to the entire luminal plasma membrane. The polarity of filipin-induced deformations in the membrane of the endothelial cell (mostly on P faces) is opposite that in the membranes of pericytes and smooth muscle cells (predominantly on E faces). This asymmetry could be due to the unequal partitioning of sterols (including cholesterol) between the two leaflets of these membranes. If the assumption that FSC are diverted from the richest leaflet of plasma membrane (11) is correct, that may indicate that in the endothelial cell the inner half contains more cholesterol than the outer half, whereas in smooth muscle the opposite is true. The inner leaflet in particular is continuously and extensively exposed to fusion-fission with plasmalemmal vesicles. In general, FSC are seven to ten times more abundant in the plasmalemma of endothelial cells than in that of pericytes and smooth muscle. These findings show that various cell types present in the microvascular wall are similar with regard to the existence of PSRS, but are heterogeneous with respect to the degree of filipin labeling and the sidedness of FSC (i.e., endothelium vs. smooth muscle), which may indicate a transmembrane asymmetry in cholesterol distribution (4, 5, 8).

The absence of anionic sites in the zone of the PSRS may be part of the simplification of the chemical composition in the area of membrane fusion (2, 7, 22, 33), which includes redistribution of proteins, cholesterol (15-17, 26) and probably anionic groups bearing glycoconjugates (40-46).

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A preliminary account of part of the findings reported in this paper was presented at the First European Congress on Cell Biology, Paris, July, 1982 (47).
FIGURES 8–10  Figs. 8 and 9: Mouse liver sinusoid capillaries, 15 min after filipin-glutaraldehyde treatment: endothelial cell plasma membranes display FSC (fs) on the rims of plasmalemmal vesicles (v) and fenestrae (f), but not around the large discontinuities (d). P, protoplasmic face rich in intramembrane particles but poorly labeled by filipin-induced protrusions. x 72,000. Fig. 10: Mouse diaphragm, at 30 min after filipin-glutaraldehyde treatment: endothelium (e) of a muscular venule displays FSC (fs) on the plasma membrane of both cells fronts as well as on most plasmalemmal vesicles (v), but not on coated pits (cp). Filipin had also reached and affected the subjacent smooth muscle cell (sm). bl, basal lamina; l, lumen. x 42,000.

FIGURE 11  Localization FSC in relation to various features opened on the cell surface of capillary endothelia. FSC occur as peristomal rings around the openings of plasmalemmal vesicles (v), transendothelial channels (c), and fenestrae (f), and are practically absent from the rims of surface openings of features such as phagocytic vacuoles (ph), coated pits (cp), and large discontinuities (d) displayed by the endothelium of sinusoid capillaries.
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