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

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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 mermbrane 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)[1] (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

[1] Abbreviations used in this paper: FSC, filipin-sterol complex; MEM, modified Eagle's medium; DMSO, dimethyl sulfoxide; CF, cationic ferritin; PSRS, preistomal ring of sterols.

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the lines of fusion between plasma membrane and plasmalemmal vesicles and its derivatives (channels and fenestrae) are surrounded by characteristic peristomal rings (annuli) 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 pI 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 feeding 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^{-1} for 5–6 min with PBS, pH 7.2, at 37°C, supplemented with 5% MEM amino acids, 14 mM glucose, and gased with 95% O_2 and 5% CO_2.

FIXATION BY PERFUSION: The vasculature was fixed in situ by perfusion with either 2.5% glutaraldehyde or a mixture of 1.5% glutaraldehyde and 2.5% formaldehyde in 0.1 M HCl–Na cacodylate or HCl-Na arsenate buffer pH 7.2–7.4, for 10 min.

ADMINISTRATION OF FILIPIN: Perfusion of same buffered fixative as above, containing 50 μM filipin (in 1% DMSO): the solution was maintained in the vasculature for 10, 15, 30, or 60 min.

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 endothelia, heart, lung, brain, and diaphragm: for fenestrated diaphragmed endothelia, pancreas, renal peritubular capillaries, and thyroid; for fenestrated nondiaphragmed endothelia, kidney glomerular capillaries; for sinusoid-fenestrated endothelia, adrenal cortex; for sinusoid endothelia, liver. Within these tissues, in addition to endothelia, 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 chromic 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% OsO_4 in 0.1 M HCl–Na arsenate or 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 0.5% galloylglucose (tannic acid) for 30 min at 22°C (39). After dehydration in graded ethanol and propylene oxide treatment, specimens were embedded in Epon-812. Sections cut with a diamond knife on an American Optical Ultracut Microtome (American Optical Corp., Buffalo, NY) or Reichert Ultramicrotome were stained with lead citrate. All preparations were examined with a Philips HM 400 electron microscope operating at 80 kV.

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 pI 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 peristomal 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 arenal 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 peristomal 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

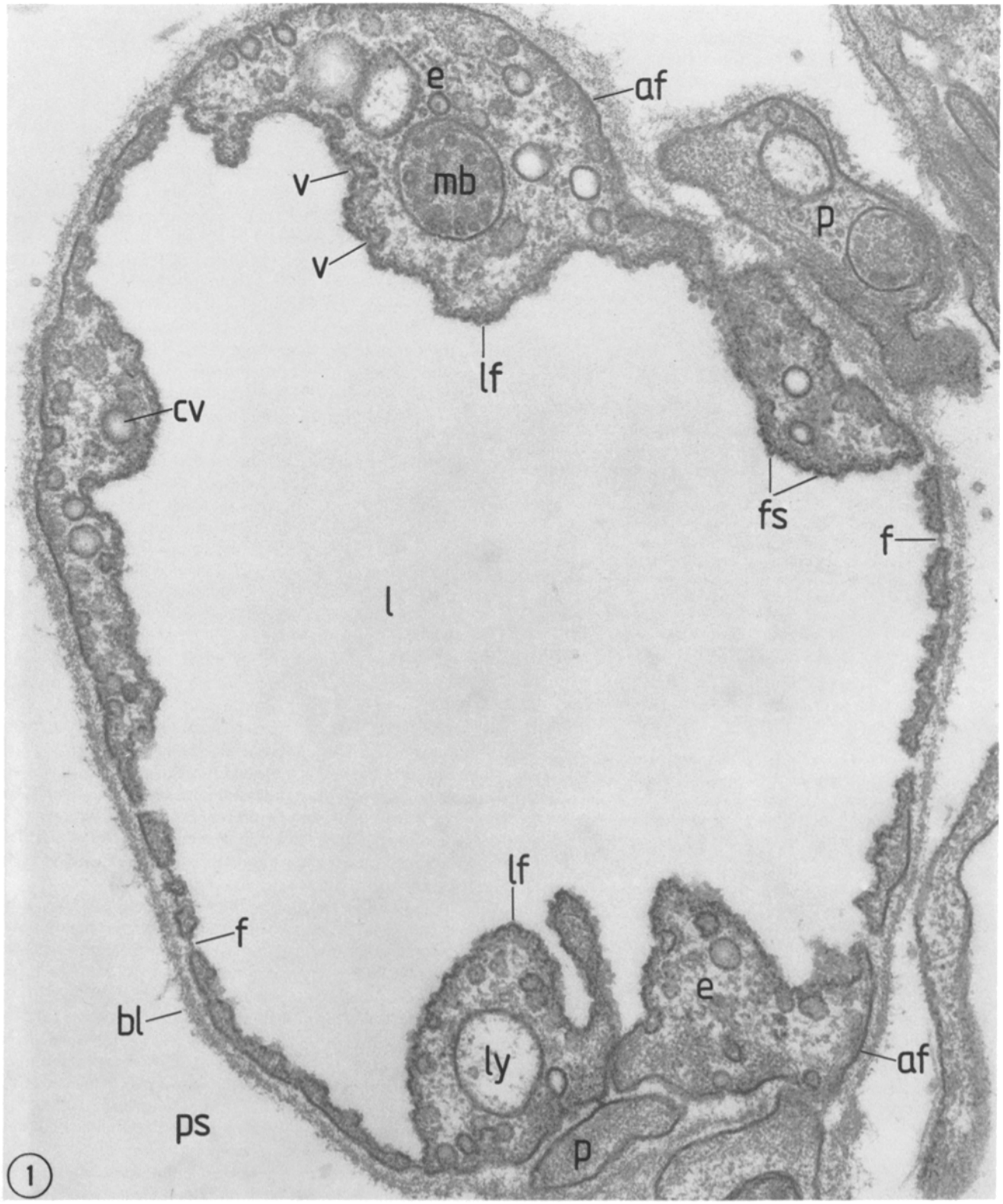
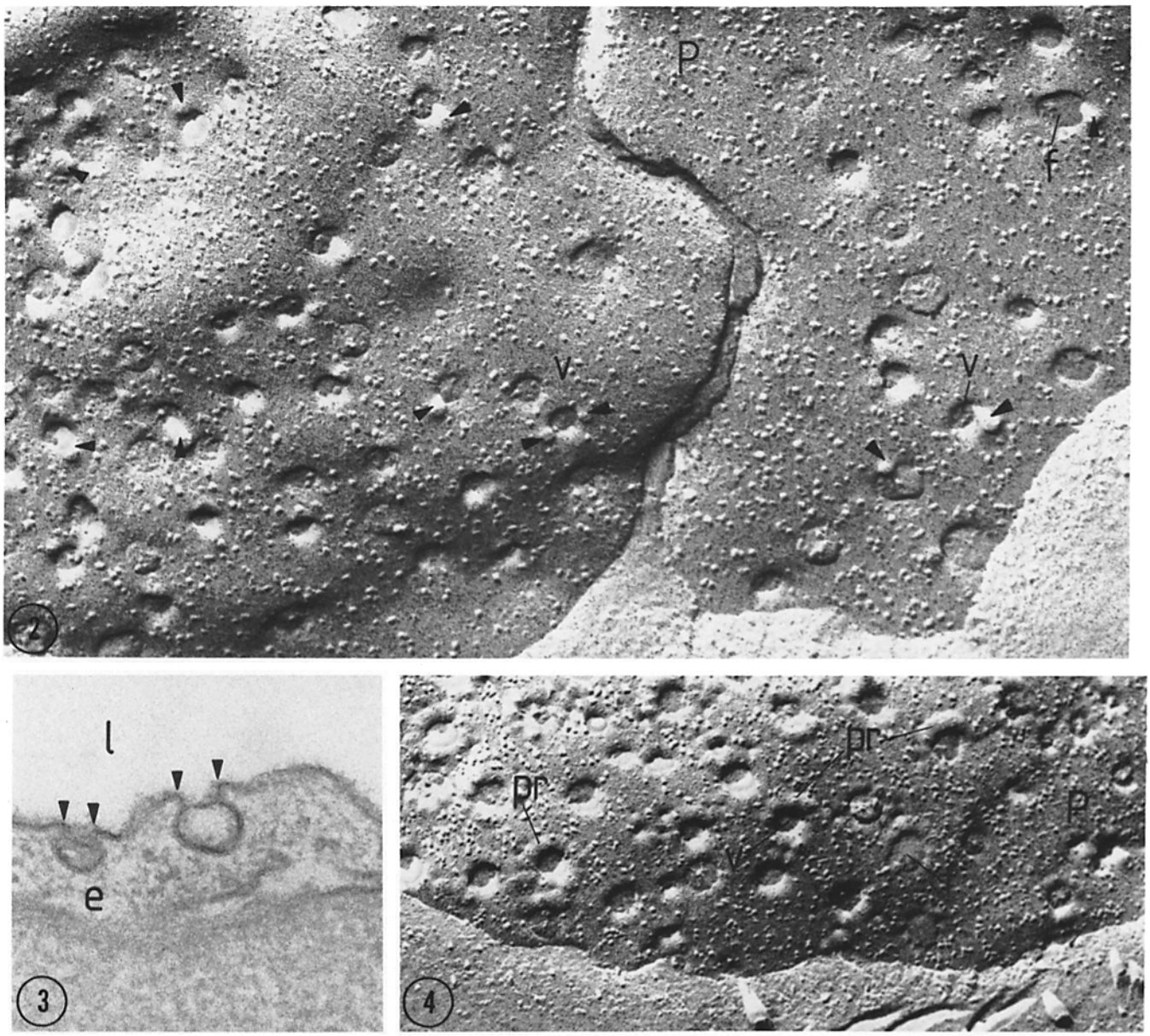


FIGURE 1 Mouse pancreas: blood capillary after 30-min exposure to 50 μM filipin in buffered glutaraldehyde. The luminal plasma membrane (*lf*) of endothelial cell (e) displays characteristic filipin-sterol complexes (*fs*), which also label luminal plasmalemmal vesicles (*v*) and, to a lesser extent, the abluminal plasma membrane (*af*). *l*, lumen; *f*, fenestra; *ly*, lysosome; *mb*, multivesicular body; *p*, pericyte; *ps*, pericapillary space; *cv*, coated vesicle. × 28,000.

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-frac-



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*, protoplasmic face. Fig. 2, × 68,000; Fig. 3, × 73,000; Fig. 4, × 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

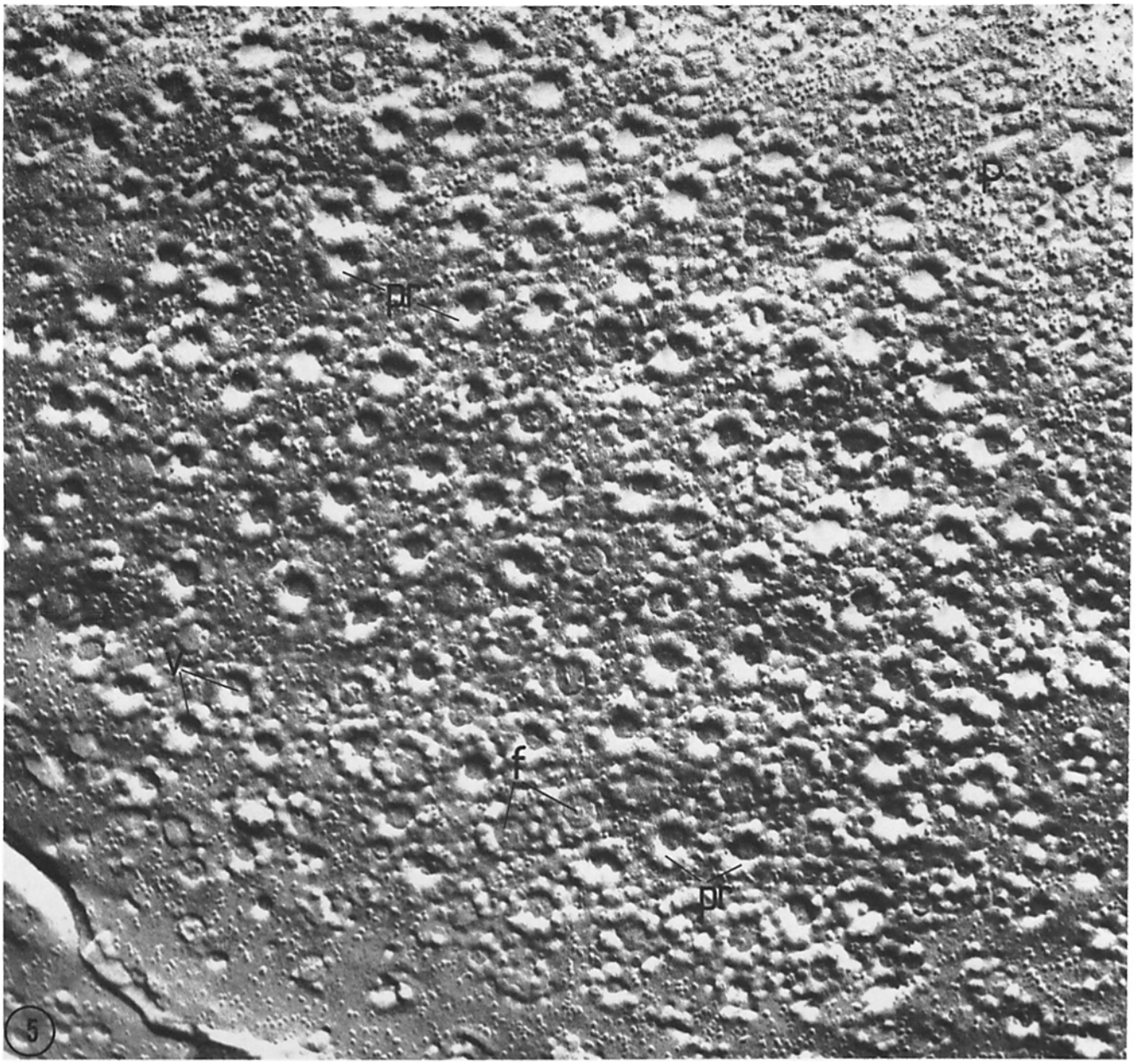


FIGURE 5 Mouse pancreas capillary, 15 min after exposure to filipin-glutaraldehyde solution. Stomata of plasmalemmal vesicles (*v*) and fenestrae (*f*) are surrounded by peristomal rings of sterols (*pr*). *P*, P face labeled by FSC. × 70,000.

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 their assumed derivatives, the transendothelial channels 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

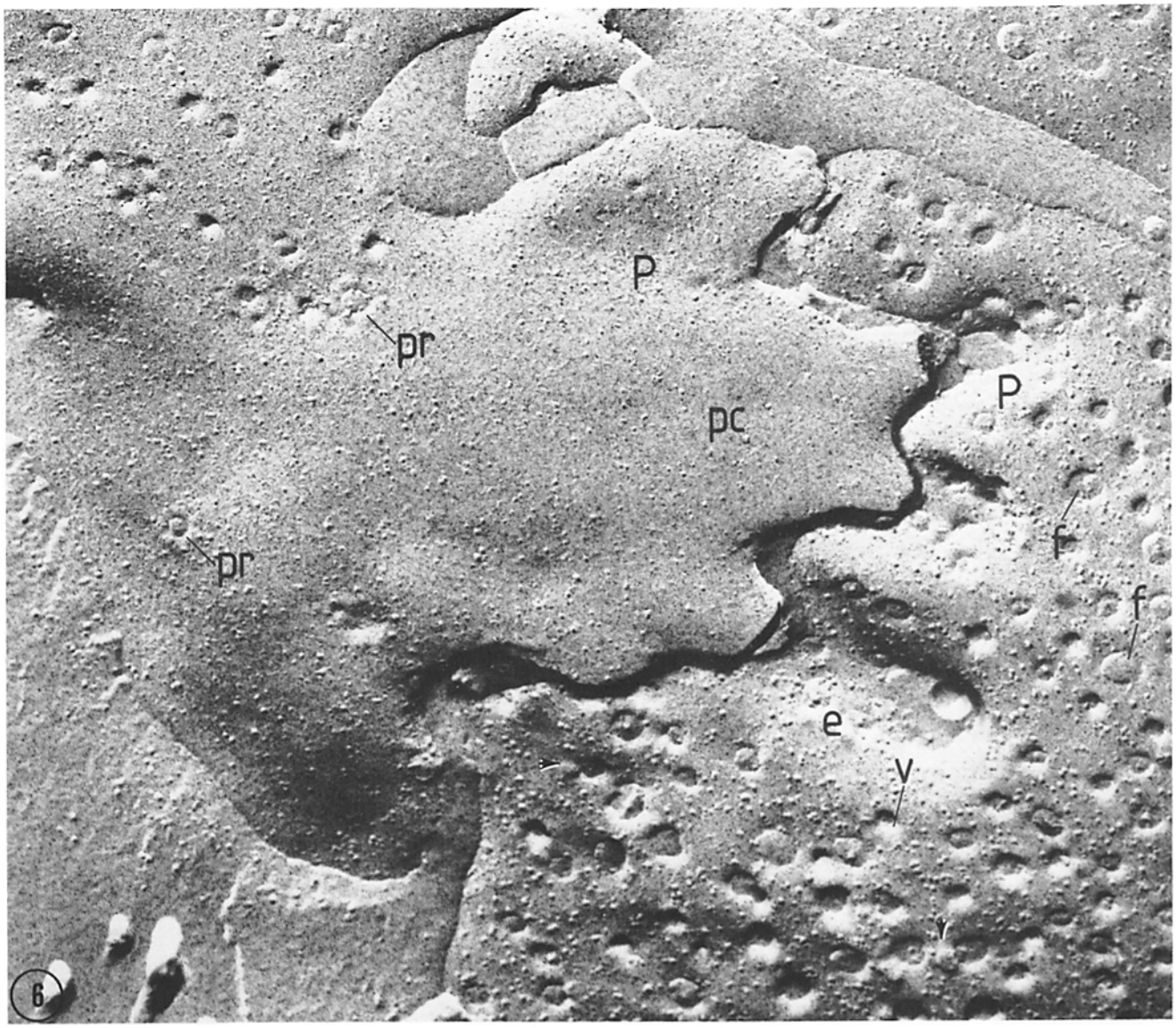


FIGURE 6 Mouse thyroid capillary, 15 min after exposure to filipin-glutaraldehyde. This freeze-fracture replica exhibits the cleaved plasma membranes of an endothelial cell (e) and a pericyte (*pc*). Filipin-sterol complexes (arrowheads) can be seen on the rims of few openings of plasmalemmal vesicles (*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

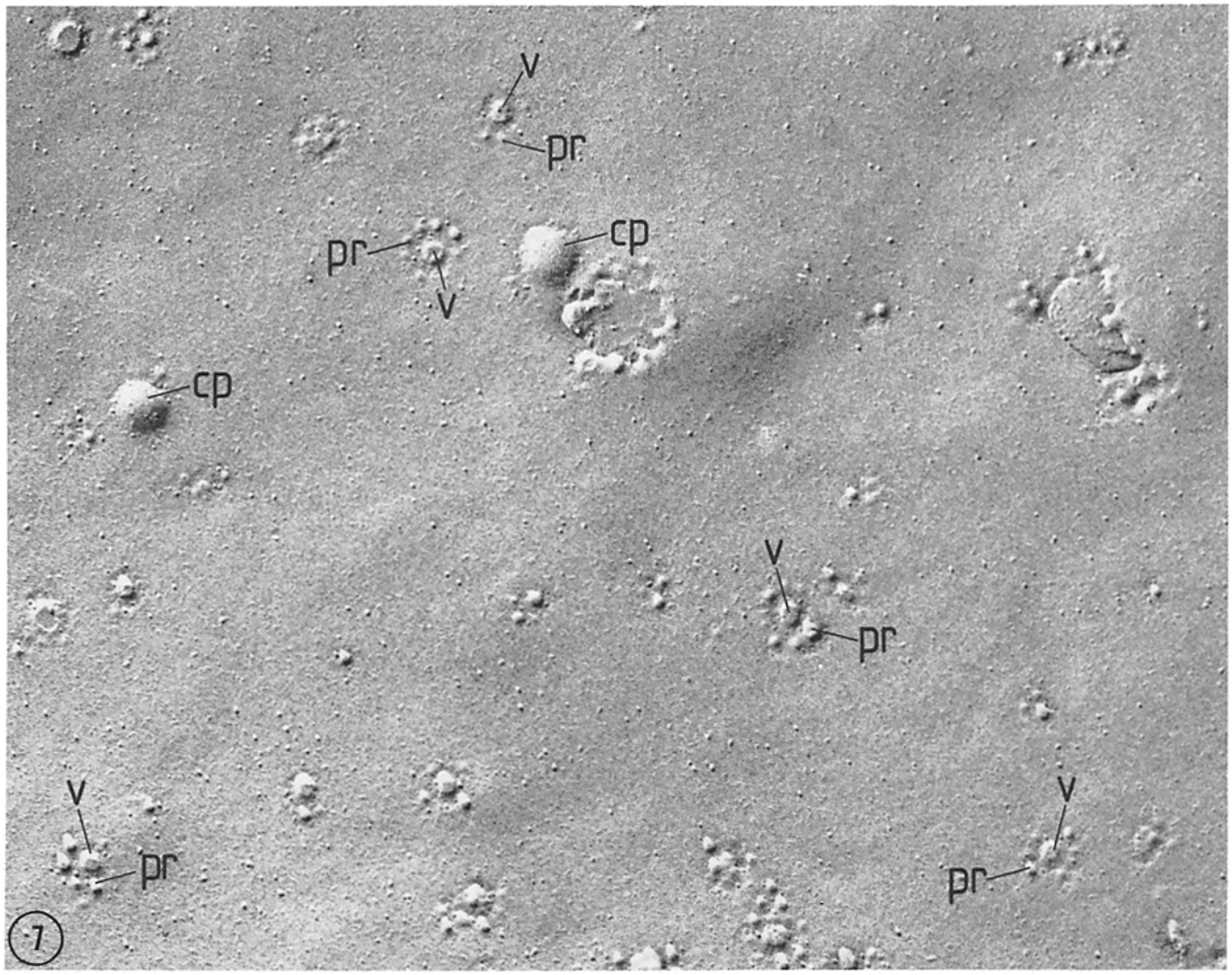


FIGURE 7 Mouse pancreas: ectoplasmic face (*E*) of a cleaved plasma membrane of a smooth muscle cell of a muscular venule. Peristomal rings of sterols (*pr*) can be seen forming around the stomata of sarcolemmal vesicles (*v*). Such features are absent from the periphery of coated pits (*cp*); the plasmalemma in general is poorly labeled by filipin-induced protrusions. × 40,000.

also be caused by a change in the radius of curvature of membrane (21).

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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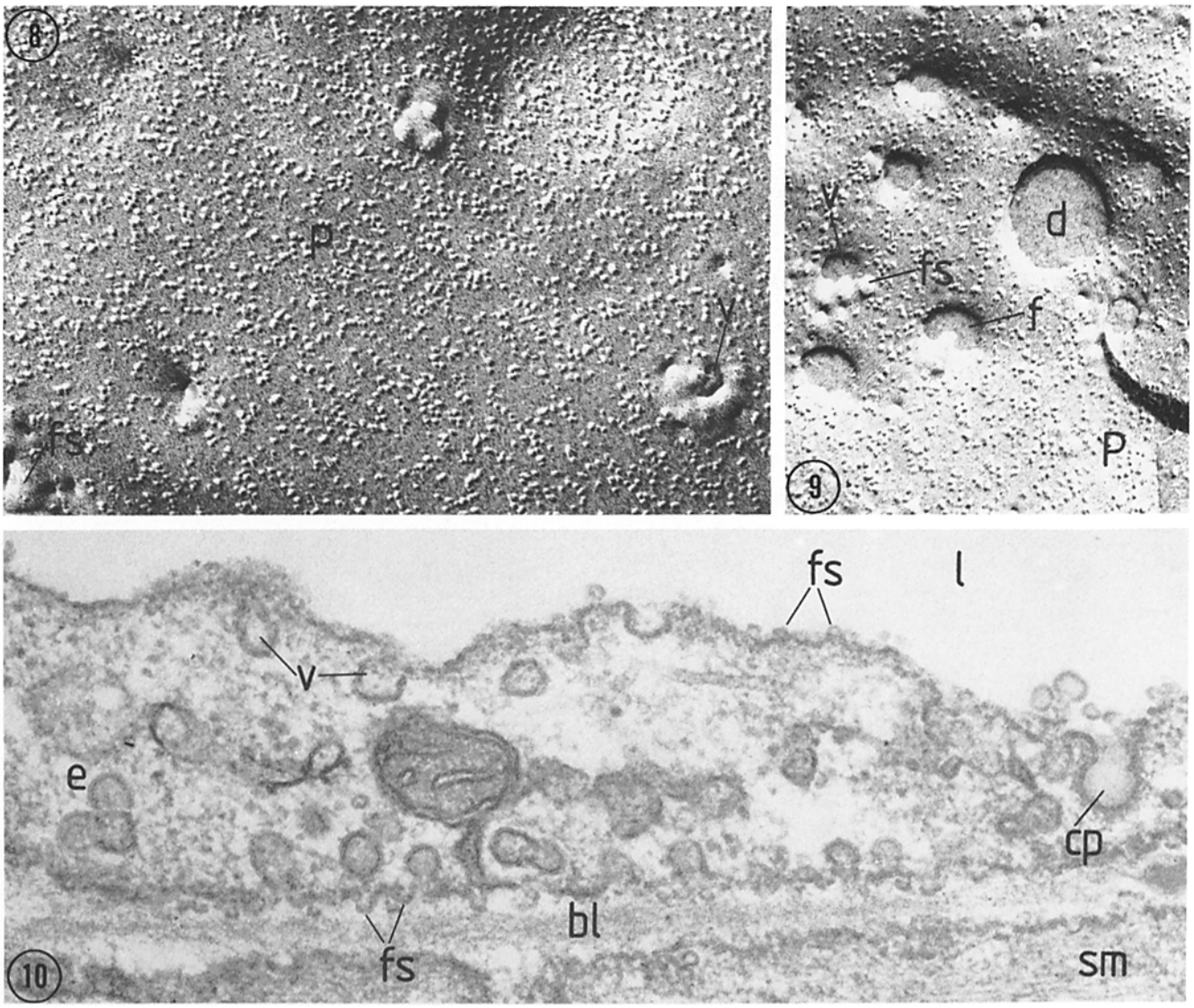

FIGURES 8–10 Figs. 8 and 9: Mouse liver sinusoid capillaries, 15 min after filipin-glutaraldehyde treatment: endothelial cell plasma membranes display FSC (*fs*) on the rims of plasmalemmal vesicles (*v*) and fenestrae (*f*), but not around the large discontinuities (*d*). *P*, protoplasmic face rich in intramembrane particles but poorly labeled by filipin-induced protrusions. × 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. × 42,000.

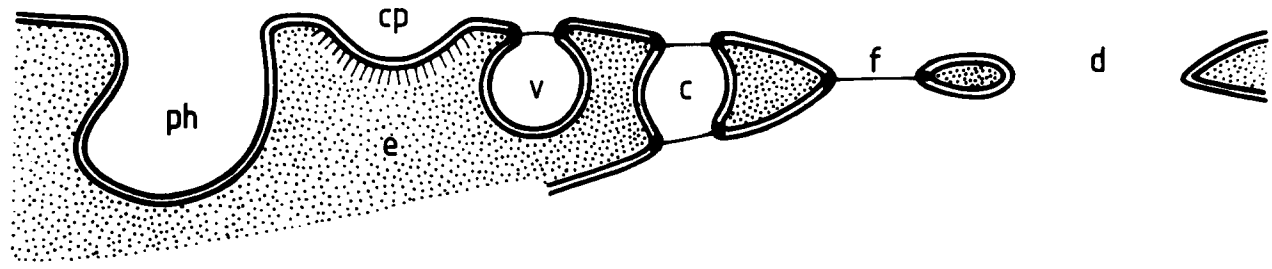

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FIGURE 11 Localization FSC in relation to various features opened on the cell surface of capillary endothelia. FSC occur as peristomal rings around the openings of plasmalemmal vesicles (*v*), transendothelial channels (c), and fenestrae (*f*), and 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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REFERENCES

1. Andrews, L. D., and A. I. Cohen. 1979. Freeze-fracture evidence for the presence of cholesterol in particle-free patches of basal disks and the plasma membrane of retinal rod outer segments of mice and frogs. *J. Cell Biol.* 81:215–228.
2. Bearer, E. L., and D. S. Friend. 1982. Modifications of anionic-lipid domains preceding membrane fusion in guinea-pig sperm. *J. Cell Biol.* 92:604–615.
3. Bittman, R. 1978: Sterol-polyene antibiotic complexation: probe of membrane structure. *Lipids.* 13:686–691.
4. Bittman, R., L. Blau, S. Clejan, and S. Rottem. 1981. Determination of cholesterol asymmetry by rapid kinetics of filipin-cholesterol association: effect of modification in lipids and proteins. *Biochemistry.* 20:425–432.
5. Blau, L., and R. Bittman. 1978. Cholesterol distribution between the two halves of the lipid bilayer of human erythrocyte ghost membranes. *J. Biol. Chem.* 253:8366–8368.
6. Chavez, B., A. Martinez-Palomo, M. Cereijido, and I. Meza. 1981. Polarity of filipin-sterol complexes in the plasma membrane of cultured and natural epithelia. *J. Cell Biol.* 91(2, Pt. 2):275*a*. (Abstr.)
7. Cohen, F. S., and M. H. Akahas. 1982. The nature of the region of contact between fusing membranes. *Biophys. J.* 37:26*a*. (Abstr.)
8. Cullis, P. R., and B. De Kruyff. 1979. Lipid polymorphism and the functional role of lipids in biological membranes. *Biochim. Biophys. Acta.* 559:399–420.
9. De Kruyff, B. 1976. Lipid-sterol interactions in membranes. *In* Lipids, R. Paoletti, G. Porcellati, and G. Jacini, editors. Raven Press, New York. 1:169–179.
10. Demel, R. A., and B. De Kruyff. 1976. The function of sterols in membranes. *Biochim. Biophys. Acta.* 457:109–132.
11. Elias, P. M., D. S. Friend, and J. Goerke. 1979. Membrane sterol heterogeneity. Freeze-fracture detection with saponins and filipin. *J. Histochem. Cytochem.* 27:1247–1260.
12. Friend, D. S. 1980. Freeze-fracture alterations in guinea-pig sperm membranes preceding gamete fusion. *Soc. Gen. Physiol. Ser.* 34:153–165.
13. Friend, D. S. 1982. Plasma membrane diversity in a highly polarized cell. *J. Cell Biol.* 93:243–249.
14. Fujita, H., K. Iashimura, and M. Matsuda. 1981. Freeze-fracture images of filipin-sterol complexes in the thyroid follicle epithelial cell of mice with special regard to absence of cholesterol at the side of micropinocytosis. *Histochemistry.* 75:57–64.
15. Gibbson, G. F., K. A. Mitropoulos, and N. B. Myant. 1982. Biochemistry of Cholesterol. Elsevier Biomedical Press, Amsterdam, New York. 303–341.
16. Hope, M. J., K. R. Bruckdorfer, C. A. Hart, and J. A. Lucy. 1977. Membrane cholesterol and cell fusion of hen and guinea-pig erythrocytes. *Biochem. J.* 166:255–263.
17. Horwitz, A. F., A. Whight, P. Ludwig, and R. Cornell. 1978. Interrelated lipid alterations and their influence on the proliferation and fusion of cultured myogenic cells. *J. Cell Biol.* 77:334–357.
18. Jain, M. K. 1975. Role of cholesterol in biomembranes and related systems. *Curr. Topics Membr. Transp.* 6:1–57.
19. Karnovsky, M. J. 1982. Intramembranous cytochemistry: a perspective. *Lab. Invest.* 46:637–639.
20. Karnovsky, M. J., A. M. Kleinfeld, R. L. Hoover, and R. D. Klausner. 1982. The concept of lipid domains in membranes. *J. Cell Biol.* 94:1–6.
21. Lenz, B. R., Y. Barenholz, and T. E. Thompson. 1976. Fluorescence depolarization studies of phase transitions and fluidity in phospholipid bilayers. II. Two-component phosphatidyl-choline liposomes. *Biochemistry.* 15:4529–4537.
22. Lucy, J. A. 1978. Mechanisms of chemically induced cell fusion. *Cell Surf. Rev.* 5:267–304.
23. Montesano, R. 1979. Inhomogeneous distribution of filipin-sterol complexes in smooth muscle cell plasma membrane. *Nature (Lond.).* 280:328–329.
24. Montesano, R., A. Perrelet, P. Vassalli, and L. Orci. 1979. Absence of filipin-sterol complexes from large coated pits on the surface of culture cells. *Proc. Natl. Acad. Sci. USA.* 76:6391–6395.
25. Montesano, R., P. Vassalli, A. Perrelet, and L. Orci. 1980: Distribution of filipin-cholesterol complexes at sites of exocytosis—a freeze-fracture study of degranulating mast cells. *Cell Biol. Int. Rep.* 4:975–984.
26. Myant, N. B. 1981. Sterols in Biological Membranes. W. Heinemann Medical Books. London. 317–337.
27. Norman, A. W., A. M. Spielvogel, and R. G. Wong. 1976. Polyene antibioticsterol interaction. *Adv. Lipid Res.* 14:127–170.
28. Orci, L., R. Montesano, and D. Brown. 1980: Heterogeneity of toad bladder granular cell luminal membranes distribution of filipin-sterol complexes in freeze-fracture. *Biochim. Biophys. Acta.* 601:443–452.
29. Orci, L., R. G. Miller, R. Montesano, A. Perrelet, M. Amherdt, and P. Vassalli. 1980. Opposite polarity of filipin-induced deformations in the membrane of condensing vacuoles and zymogen granules. *Science (Wash DC).* 210:1019–1021.
30. Orci, L., D. Brown, M. Amherdt, and A. Perrelet. 1982. Distribution of intramembrane particles and filipin-sterol complexes in plasma membrane of kidney. I. Corpuscle of Malpighi. *Lab. Invest.* 46:543–553.
31. Papahajdopoulos, D., G. Poste, and B. E. Schaeffer. 1973. Fusion of mammalian cells by unilamellar lipid vesicles: influence of lipid surface charge, fluidity and cholesterol. *Biochim. Biophys. Acta.* 323:23–42.
32. Papahajdopoulos, D. 1976. The role of cholesterol as a membrane component: effects on lipid-protein interactions. *In* Lipids. (R. Paoletti, G. Porcelatti, and G. Lacini, editors. Raven Press, New York. 1:187–196.
33. Poste, G., and A. C. Allison. 1973. Membrane fusion. *Biochim. Biophys. Acta.* 300:421–465.
34. Rechtenwald, D. J., and H. M. McConnel. 1981. Phase equilibria in binary mixtures of phosphatidylcholine and cholesterol. *Biochemistry* 20:4505–4510.
35. Robinson, J. M., and M. J. Karnovsky. 1980. Evaluation of the polyene antibiotic filipin as a cytochemical probe for membrane cholesterol. *J. Histochem. Cytochem.* 28:161–168.
36. Rothman, J. E., and L. Lenard. 1977. Membrane asymmetry. *Science (Wash. DC).* 195:743–753.
37. Severs, N. J. 1981. Plasma membrane cholesterol in myocardial muscle and capillary endothelial cells. Distribution of filipin-induced deformations in freeze-fracture. *Eur. J. Cell Biol.* 25:289–299.
38. Severs, N. J. 1982. Comparison of the response of myocardial muscle and capillary endothelial nuclear membranes to the cholesterol probe filipin. *J. Submicrosc. Cytol.* 14:441–452.
39. Simionescu, N., and M. Simionescu. 1976. Galloylglucoses of low molecular weight as mordant in electron microscopy. I. Procedure and evidence for mordanting effect. *J. Cell Biol.* 70:608–621.
40. Simionescu, N., and M. Simionescu. 1978. Differentiated distribution of anionic sites on the capillary endothelium. *J. Cell Biol.* 79(2, Pt. 2):59*a*. (Abstr.)
41. Simionescu, N. 1979. The microvascular endothelium: segmental differentiations, transcytosis, selective distribution of anionic sites. *Adv. Inflammation. Res.* 61–70.
42. Simionescu, N. 1981. Transcytosis and traffic of membranes in the endothelial cell. *In* International Cell Biology: 1980–1981. H. G. Schweiger, editor. Springer-Verlag, Berlin. 657–672.
43. Simionescu, N., M. Simionescu, and G. E. Palade. 1981. Differentiated microdomains on the luminal surface of capillary endothelium. I. Preferential distribution of anionic sites. *J. Cell Biol.* 90:605–613.
44. Simionescu, M., N. Simionescu, and G. E. Palade. 1981. Differentiated microdomains on the luminal surface of the capillary endothelium. II. Partial characterization of their anionic sites. *J. Cell Biol.* 90:614–621.
45. Simionescu, M., N. Simionescu, and G. E. Palade. 1982. Differentiated microdomains on the luminal surface of capillary endothelium: distribution of lectin receptors. *J. Cell Biol.* 94:406–413.
46. Simionescu, M., N. Simionescu, and G. E. Palade. 1982. Preferential distribution of anionic sites on the basement membrane and the abluminal aspect of the endothelium in fenestrated capillaries. *J. Cell Biol.* 95:425–434.
47. Simionescu, N., F. Lupu, and M. Simionescu. 1982. Annuli of membrane sterols surround the openings of vesicles and fenestrae, in capillary endothelium. *Biol. Cell.* 45:236. (Abstr.)
48. Tillack, T. W., and S. C. Kinsky. 1973. A freeze-etch study of the effects of filipin on liposomes and human erythrocyte membranes. *Biochim. Biophys. Acta.* 323:43–54.
49. Van Venetie, R., and A. J. Verkereij. 1981. Analysis of the hexagonal II phase and its relations to lipidic particles and the lamellar phase. A freeze-fracture study. *Biochim. Biophys. Acta.* 645:262–269.
50. Verkeleij, A. J., D. De Kruyff, R. A. Gerritsen, and L. L. M. Demel. 1973. Freeze-etch electron microscopy of erythrocytes, *Acholeplasma laidlarvii* cells and liposomal membranes after the action of filipin and amphotericin B. *Biochim. Biophys. Acta.* 291:577–581.