

Endothelial Plasmalemmal Vesicles Have a Characteristic Striped Bipolar Surface Structure

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ABSTRACT Capillary endothelial cells have a large population of small (65–80 nm diameter in transmission electron microscopy) vesicles of which a large fraction is associated with the plasmalemma of the luminal and abluminal side. We studied the fine structure and distribution of these plasmalemmal vesicles by high resolution scanning electron microscopy in cultured endothelial cells obtained from bovine adrenal cortical capillaries. Cell monolayers were covered with polylysine-coated silicon chips, split in high potassium buffer, fixed in aldehyde mixtures, and then treated with OsO₄ and thiocarbohydrazide. After critical point drying, the specimens were coated with a thin (<2 nm) continuous film of chromium. On the cytoplasmic aspect of the dorsal plasmalemmal fragments seen in such specimens, plasmalemmal vesicles appear as uniform vesicular protrusions ~70–90 nm in diameter, preferentially concentrated in distinct large fields in which they occur primarily as single units. Individual plasmalemmal vesicles exhibit a striped surface fine structure which consists of ridges ~10 nm in diameter, separated by furrows and oriented as meridians, often ending at two poles on opposite sides of the vesicles in a plane parallel to the plasmalemma. This striped surface structure is clearly distinct from the cage structure of coated pits found, at low surface density, on the same specimens. The cytoplasmic aspect of the plasmalemma proper is covered by a fibrillar infrastructure which does not extend over plasmalemmal vesicles but on which the latter appear to be anchored by fine filaments.

The permeability of blood capillaries provided with a continuous endothelium has been explained by convection and diffusion along intercellular junctions (1, 2) and transendothelial channels (3) and by transcytosis, i.e., vesicular transport across the endothelium via plasmalemmal vesicles (4–7). Available morphological and functional evidence supports transcytosis as an exchange mechanism across the endothelium of such capillaries (3, 8), but the surface and volume density of plasmalemmal vesicles (9), their relation to the plasmalemma proper and to one another, and especially their presence as free units within the endothelial cytoplasm are still debated (10–12; see, however, 13).

To gain additional information on plasmalemmal vesicles, we have adsorbed the plasmalemma of cultured endothelial cells to a solid substrate, split the cells to expose the cytoplasmic aspect of the adsorbed membrane, and examined it by high resolution scanning electron microscopy SEM¹ (14). The results indicate that plasmalemmal vesicles have a characteristic surface structure distinct from that of coated pits and plasmalemma proper. They also show that in the speci-

mens examined these vesicles occur primarily as single units rather than chains or dendritic structures. We assume that these findings will be of use in further studies on the physiological role of plasmalemmal vesicles and on the mechanisms involved in transcytosis.

Part of this work was presented at the 1984 meeting of the American Society for Cell Biology (15).

MATERIALS AND METHODS

Bovine adrenal cortex endothelial cells (BACE cells), the gift of Dr. M. Furie, Columbia University, NY, were grown to confluence on 30-mm diameter culture dishes coated with 1% gelatin. For transmission electron microscopy (TEM), the cell monolayer was washed once in phosphate-buffered saline (PBS) and fixed for 30 min at 22°C in 1.6% formaldehyde, 1% glutaraldehyde in 0.1 M Na cacodylate-HCl buffer, pH 7.0, containing 115 mM KCl, 5 mM MgCl₂, and 3 mM EGTA. The cells were subsequently treated first with 1% OsO₄ for 45 min at 4°C in the same buffer and then with 1% tannic acid in the same

¹ *Abbreviations used in this paper:* BACE cells, bovine adrenal cortex endothelial cells; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

buffer for 30 min at 22°C. The monolayer was then washed twice in 1% sodium sulfate, dehydrated, and embedded for thin sectioning.

For SEM, BACE cells were grown on gelatin-coated silicon chips (5 × 7 mm in size), transferred on the latter to a hypotonic cytosolic (H-C) buffer containing 70 mM KCl, 5 mM MgCl₂, 3 mM EGTA, and 30 mM Hepes, pH 7.0 (16), and then sandwiched with another polylysine-coated chip which adsorbed to their previously exposed "dorsal" plasmalemma. After separating the chips, the adsorbed plasmalemmal fragments and the complementary cell remnants were fixed in 1.6% formaldehyde, 1% glutaraldehyde in 70 mM KCl, 5 mM MgCl₂, 3 mM EGTA buffered to pH 7.0 with 0.1 M Na cacodylate-HCl (buffer F). They were then washed, treated for 30 min in 1% OsO₄, and washed again using the same buffer at each step. The specimens were subsequently treated for 10 min with saturated thiocarbonylhydrazide in 0.15 M KCl for 10 min, washed extensively with 0.15 M KCl, and reacted for 10 min with 1% OsO₄ in the same solvent to obtain a controlled osmium impregnation (17). After repeated water washes and dehydration in ethanol, the specimens were transferred into Freon 113, critical point dried in CO₂, coated with a 1–2-nm-thick continuous chromium film by high vacuum sputtering (18) and finally examined by SEM at 30 kV in the SE-I image mode (14) in a JEOL JFSM 30 provided with a cold field emission gun.

RESULTS

The cultured BACE cells grew on gelatin-coated dishes into a continuous monolayer and deposited a basement membrane-like material between their "ventral" membrane and the gelatin as seen by TEM (Fig. 1). They had many small vesicles (~65–80 nm in diameter) associated with both the dorsal and ventral plasma membrane (arrowheads) with some vesicles apparently free in the cytoplasm. Plasmalemmal vesicles appeared at the plasma membrane as single units or as chains or clusters of two to three units (arrows). Their number vastly outnumbered the population of clathrin-coated pits and vesicles. The ventral plasmalemma generally had more large bundles of thin filaments (stress fibers) associated with it (asterisk) than the dorsal plasmalemma. These structural characteristics were similar to those found *in vivo* in the cells of capillaries provided with a continuous endothelium. The endothelium of the bovine capillaries is fenestrated *in vivo*, but its ability to differentiate and maintain fenestrae is largely lost in culture (19).

Scanning electron micrographs of split BACE preparations revealed that large plasmalemmal fragments were detached from the free surface or dorsal plasmalemma of the cell presumably because this membrane adhered strongly to the polylysine-coated chip (Fig. 2) used for cell splitting. The cytoplasmic aspect of this membrane was routinely used for

SEM observations because it was covered by much fewer cytoplasmic remnants, i.e., cytoskeletal elements and membrane-bound subcellular components, than the opposite, ventral membrane. When unobscured by cytoplasmic elements the ventral vesicle populations closely resembled those seen on the dorsal membrane.

The dorsal membranes often appeared as intact flat sheets, which had the tendency to roll up and disintegrate near their periphery (Fig. 2*a*, arrowheads). At a higher magnification (Fig. 2*b*, asterisks) they exhibited areas in which large populations of associated vesicles were separated by vesicle-free regions covered by long filamentous structures (*f*). Within areas of high vesicle density (Fig. 2, *c* and *d*), it was apparent that there were two populations of vesicles associated with the membrane: (*a*) small vesicles with a diameter of 70–90 nm that were in majority and occasionally occurred in regular arrays, and (*b*) large vesicles (asterisks) with diameters of 130–190 nm scattered apparently at random. The small vesicles had the same size as plasmalemmal vesicles identified by TEM, but occurred primarily as single, isolated elements. Chains of two or three vesicles were rare (arrows) and complexes that could be dendritic structures of the type previously described (10–12) were only occasionally encountered (double arrows).

In between vesicles, the cytoplasmic aspect of the plasmalemma was covered by a tightly meshed reticular infrastructure made of randomly oriented strands or fibrils ~7–9 nm in diameter (Fig. 3*a*). Most vesicles protruded high above this network but strands were quite often seen connecting the protruding part of the vesicles with the infrastructure (arrows). Each fully raised vesicle was located in an area of ~150 nm in diameter which was relatively free of tightly meshed infrastructure (solid circle). The extent of vesicle protrusion above the plane of the infrastructure varied over a relatively wide range. Low protrusions or raised mounds ~70 to 100 nm in diameter were often encountered randomly distributed in between plasmalemmal vesicles (Fig. 2*c*, arrowheads). In addition, circular areas, ~150 nm in diameter, in which the fibrillar texture appeared more tightly meshed than the rest of the plasmalemmal infrastructure were sometimes encountered (see dashed circle in Fig. 3*a*).

The cytoplasmic aspect of the plasmalemmal vesicles, identifiable only at high magnifications (Fig. 3), was found to have

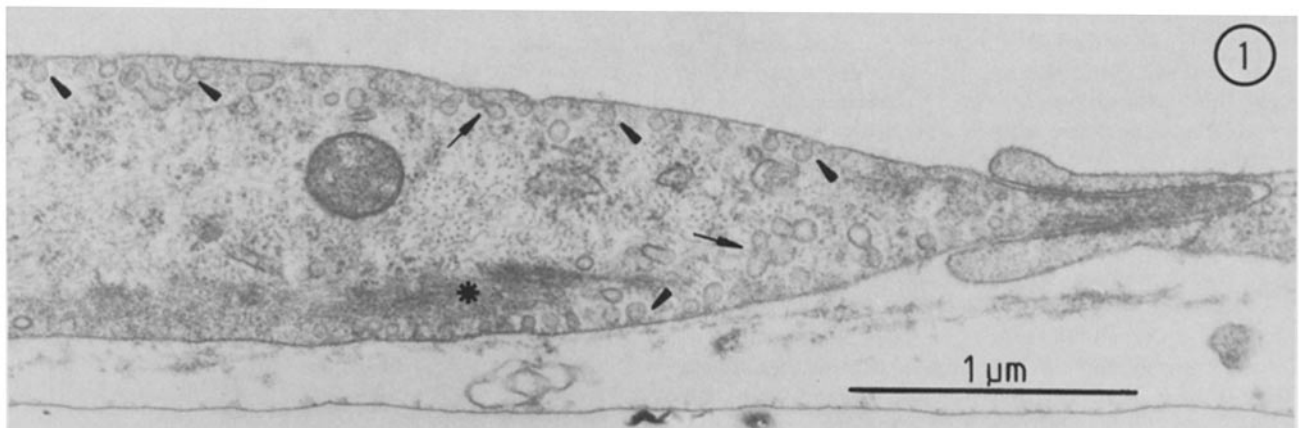


FIGURE 1 TEM of a cross section through a monolayer of cultured bovine endothelial cells (derived from the capillaries of the adrenal cortex). Plasmalemmal vesicles (arrowheads) are associated with both the ventral and dorsal domains of the plasmalemma apparently as single units, but chains and "dendritic" vesicular structures (arrows) are also present. Thick bundles of fine fibrils (stress fibers) are seen next (asterisk) to the ventral plasmalemma. × 37,000.

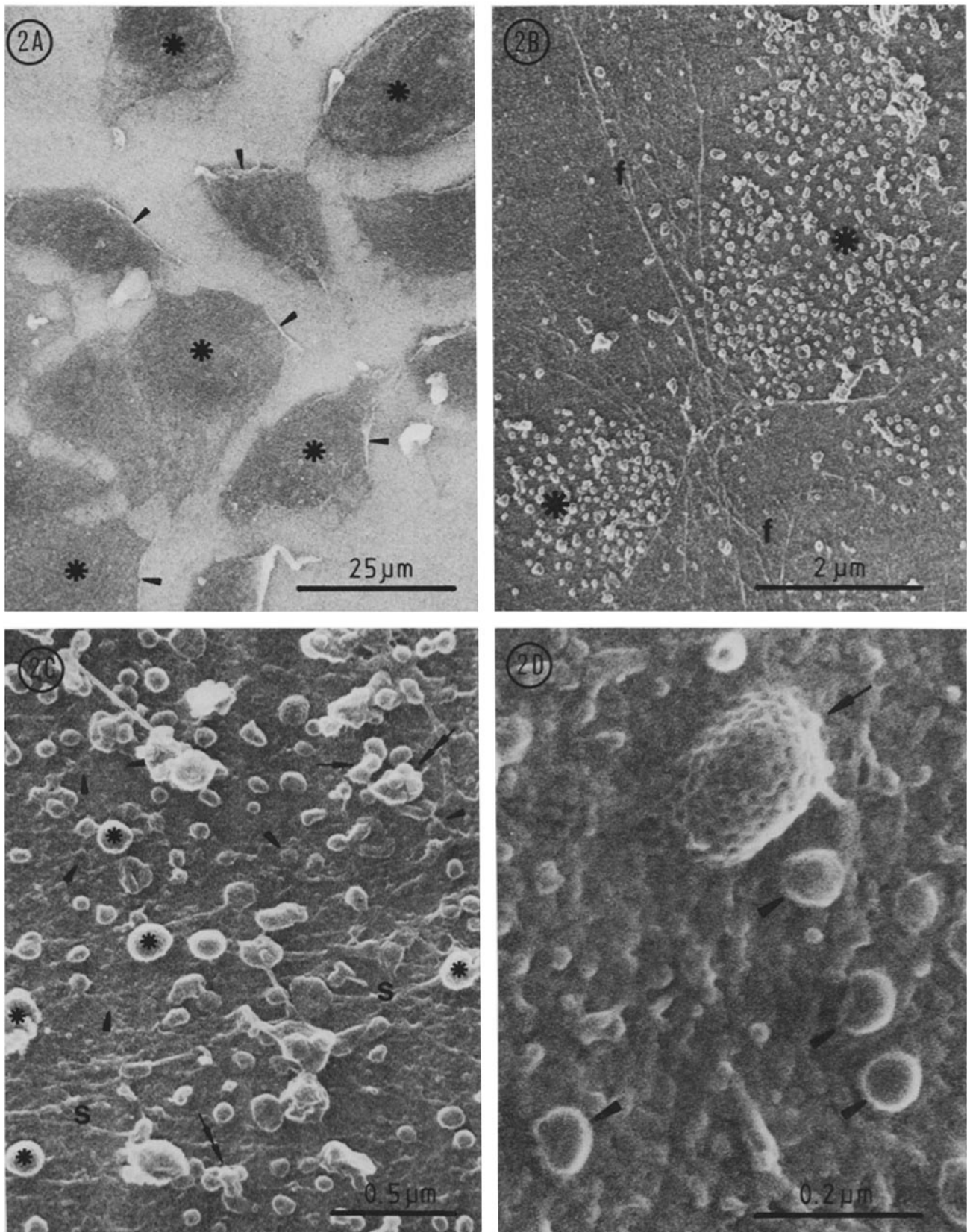


FIGURE 2 Fragments of dorsal plasmalemma detached upon adhesion to polylysine-coated silicon chips. (A) The adhering membrane fragments (asterisks) extend over relatively large areas. Their free margins have the tendency to roll over (arrowheads). $\times 1,100$. (B) Clusters of plasmalemmal vesicles (asterisks) separated by remnants of stress fibers (*f*) appear on the cytoplasmic aspect of the membrane fragments. $\times 15,000$. (C) The clusters are comprised of mostly individual plasmalemmal vesicles, a few short chains (arrows), and a few vesicular aggregates (double arrows) which may represent dendritic structures. Low protruding vesicles and domes are marked by arrowheads. Larger coated vesicles (asterisks) are randomly distributed. The fibrillar infrastructure under the plasmalemma proper is clearly visible at *s*. $\times 45,000$. (D) The vesicular clusters consist of a few coated pits, 200–250 nm in diameter (arrow), and many plasmalemmal vesicles 60–90 nm in diameter (arrowheads), most of which protrude high above the cytoplasmic aspect of the plasmalemma. At this magnification, fine surface structural details cannot be recognized. $\times 150,000$.

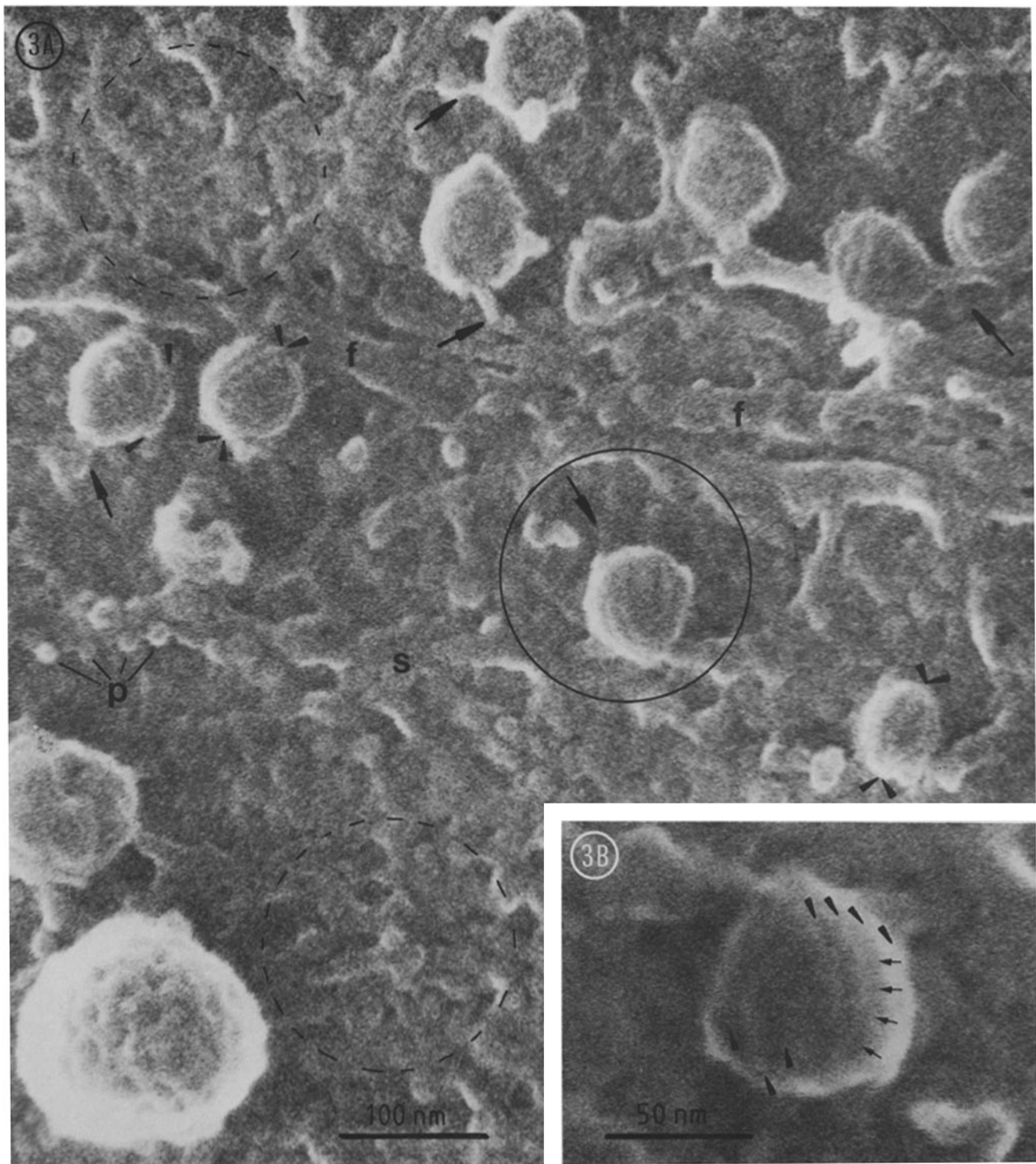


FIGURE 3 High magnification images of the cytoplasmic aspect of plasmalemmal vesicles. (A) Vesicles attached to the plasmalemma are surrounded by areas relatively free of tightly meshed fibrillar infrastructures (solid circle), but fine fibrillar strands (arrows) connect their surface to the infrastructure of the plasmalemma proper. The infrastructure consists of fibrillar elements (*s*), apparently randomly distributed although preferentially oriented fibrillar arrays are also found (*f*). The bright spots (*p*) represent fibril remnants oriented perpendicularly to the rest of the infrastructure. The dashed circles indicate areas of different texture from the rest of the plasmalemmal infrastructure. The plasmalemmal vesicles have a characteristic striped structure (arrowheads). $\times 250,000$. (B) The structure of the plasmalemmal vesicles consists of meridian ridges (arrowheads) which converge at two opposite poles and are separated by furrows marked by linear species of dimples (arrows). The 1–2-nm thick layer of chromium does not have a resolvable structure and is visualized as a distinct 1–2-nm wide bright line that follows the contour of each topographic detail. $\times 500,000$.

a distinct fine surface structure which consisted of ridges (Fig. 3*a*, arrowheads) oriented as meridians separated at a spacing of ~10 nm by furrows along which were arranged in series dimples of ~10 nm (Fig. 3*b*, arrows). The meridian ridges appeared to converge at two opposite poles located in a plane parallel to the plasmalemma proper. The poles appeared to have a similar orientation within groups of clustered vesicles. In projection many vesicles appeared to have, in part, polygonal profiles, and the filamentous strands mentioned above were usually attached to the profile vortices.

Examination of the larger (130–250 nm) vesicular protrusions at higher magnifications revealed a surface structure identical to that of clathrin-coated pits and vesicles (Figs. 2*d* and 3*a*). The size and geometry of the surface elements of the cage structures were similar to those reported by others in negatively stained (20) or rotary-shadowed specimens (21, 22) but the diameter of the strands appeared to be larger.

Plasmalemmal vesicles have, therefore, a uniform and characteristic surface structure that can be readily distinguished from that of clathrin coated pits and vesicles and from that of the plasmalemma proper. In addition, they differ from coated pits due to their clustered distribution on the plasmalemma and to their interactions with the cell membrane infrastructure.

DISCUSSION

In the preparation procedure used for our SEM specimens, the dorsal plasmalemma of cultured endothelial cells is adsorbed (and thereby immobilized) to a solid substrate, i.e., the polylysine-coated silicon chip (23). Thereafter, the cells are split; most subcellular components are removed with the ventral cell moieties; part of the cytosolic proteins are extracted in the high potassium buffer; and the cytoplasmic aspect of the cell membranes is exposed before chemically fixing the specimens with cross-linking aldehydes. Mobility of molecules and structures in the plane of the adsorbed membrane may still be maintained, and artefacts involving plasmalemmal vesicles are not entirely excluded, but it can be assumed that cross-linking of cytosolic proteins and relocation of plasmalemmal vesicles are less extensive than in specimens directly fixed in toto in glutaraldehyde–formaldehyde mixtures. The preponderance of single plasmalemmal vesicles over the vesicular chains (4) or dendritic structures (10–12) described by others probably reflects the specific order in which specimen splitting, extraction, and fixation are carried out in our preparation procedure. We assume that vesicle redistribution is minimized because of the immobilization of the membrane fragments and also because the membrane reservoir represented by the cytoplasm and its organelles is removed with the ventral cell moieties.

The procedure described can be modulated to determine the effects of fixing or rapid freezing before splitting. Such comparative studies may not reveal faithfully the true *in vivo* situation, but they may indicate trends of further changes undergone during chemical fixation (13).

Coating the specimens with a thin chromium film and then viewing them in the SEM SE-I image mode reveals fine topographic detail which can be visualized at high magnifications without the disadvantage of large deposits of metal crystals that may generate excessive decoration effects. The SEM imaging used in our studies is quite different from conventional SEM methods since it uses a high resolution

signal contrast able to reveal macromolecular details at a 2–10-nm level (14, 17). The image contrasts are generated with thin (<2 nm) continuous films of metal which do not decorate but coat evenly the surface features. With such films, true topographic contrasts are imaged down to the level of the smallest recognizable details (18). On clathrin coats, for instance, metal is deposited not only on elevated surfaces or ridges where platinum crystals preferentially grow, but also on the sides of the triskelion assemblies. This condition probably explains the apparent increase in width of the lattice strands regularly seen in our micrographs. The SE-I images used in this study could not be digitally summed and reconstructed so that a certain noise level, characteristic for this high magnification imaging mode, is present.

With these SEM procedures, we were able to detect a distinct surface structure consisting of polarized, meridian ridges and intervening furrows which gives a characteristic “striped” appearance to the cytoplasmic aspect of the plasmalemmal vesicles. Since no other membrane or membrane domain described so far in endothelial cells appears to have a similar surface structure, this finding provides a specific morphological marker for endothelial plasmalemmal vesicles. It indicates that their membrane is structurally—and presumably chemically—different from both coated pits and plasmalemma proper, although it shares with the latter a continuous polar lipid bilayer. It rules out their derivation by random invaginations from the plasmalemma, and raises the question as to the means by which endothelial cells generate and maintain differentiated microdomains in the continuity of their surface membrane. The new finding adds a structural feature to the growing list of distinct properties of the membrane of endothelial plasmalemmal vesicles, i.e., the paucity or absence of anionic sites detected by cationized ferritin binding (24, 25), the high concentration of lectin receptors (26), and the presence of a specific antigen, detected by its monoclonal antibody (27) only on vesicles and not on the adjacent plasmalemma proper.

It is noteworthy that the surface structure described in this paper was not detected by TEM on endothelial plasmalemmal vesicles, although the latter have been extensively studied in many laboratories. The presence of dense ridges was, however, occasionally recorded on equivalent vesicular structures in smooth muscle fibers (28, 29). This observation validates the inherent advantage of high resolution SEM for studying the surface structure of cellular membranes. Further work by high resolution SEM is needed to find out whether the new surface structure is found on all plasmalemmal vesicles, irrespective of cell type, or is limited to those of endothelial cells.

Our observations concerning the cytoplasmic surface structure of coated pits and coated vesicles agree in general with results obtained on negatively stained specimens (20) and on replicas of the cytoplasmic surface of the plasmalemma after rotary shadowing with platinum–carbon (21). This comparison of results, obtained by procedures which differ in the means by which they generate contrast, shows that our technique reveals the same general morphology as the other methods mentioned above; in addition, it provides a comparative control for our new findings on the structural characteristics of plasmalemmal vesicles.

The cytoplasmic surface of the plasmalemma proper is covered by a fibrillar meshwork which does not extend over the plasmalemmal vesicles. The latter appear to be located in

domains relatively free of fibrillar infrastructure but often seem to be connected to it by single strands (Fig. 3a, arrows). The low domes seen in Figs. 2c and 3a may represent membrane microdomains from which plasmalemmal vesicles arise by a process similar to that involved in the generation of coated pits and coated vesicles. Their presence, which has not been noted in fixed endothelial cells, and the scarcity of dendritic structures and chains formed by plasmalemmal vesicles, appear to be structural features that precede chemical fixation, but are extensively modified by the latter. Further work is needed to define the surface structure of the domes, to find out to what extent they are related to circular areas like the one in Fig. 3a (dashed outline), and to elucidate in appropriate detail the process of plasmalemmal vesicle formation.

We assume that the fibrillar infrastructure associated with the plasmalemma proper maintains the distinct chemistry of this part of the cell membrane by restrictive interactions with the latter's integral membrane proteins. Spectrin, fodrin, and actin have been detected by immunofluorescence in bovine aortic endothelial cells (Heltianu, C., I. Bogdan, E. Constantinescu, and M. Simionescu, manuscript submitted for publication; see also reference 30) and nonmuscle isoforms of myosin and actin have been found by immunoperoxidase procedures in the endothelial cells of the microvasculature of the rat (31), but the localization of all these proteins at the microdomain level remains unknown. A similar stabilizing function is probably performed by the clathrins and associated proteins that form the geodesic cages of coated pits and coated vesicles. The molecules that generate the meridian ridges on plasmalemmal vesicles may have a similar role.

In principle, our procedure, which makes the cytoplasmic aspect of the plasmalemma and associated vesicles easily accessible to macromolecular reagents, could be used to define the chemistry of the domain-specific infrastructures via gold-labeled antibodies. Such an approach may yield information about molecules involved in vesicular interactions. If vesicles become free in the cytoplasm at one time, they may associate with a filamentous system for targeting to another membrane. It is this complex system of membrane-vesicle-filaments that needs to be understood to be able to manipulate the number of vesicles attached to the plasmalemma, to correlate this number with the uptake of macromolecules by cells *in vitro*, and perhaps eventually with transcytosis *in vivo*.

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