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Endothelial plasmalemmal vesicles as elements in a system of branching invaginations from the cell surface

(endothelium/tannic acid/vesicular transport)

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ABSTRACT **In electron microscopy studies of the endothelial vesicles in frog mesenteric capillaries, an accidental observation was made concerning vesicular organization. When tannic acid was added to already fixed tissue, the mordant reached apparently free vesicles in the cytoplasm under conditions in which vesicular movement was excluded and in which the impermeability of the cell membranes was preserved. This indicates a spatial continuity between the vesicles and the cell exterior. It is proposed that cytoplasmic vesicles in endothelial cells are elements of branching, permanent or semipermanent invaginations of the plasmalemma.**

Palade discovered numerous vesicles in the capillary endothelium and suggested that they ferry small amounts of material between blood and the pericapillary space (1). This picture implies that the vesicles go through a cycle. They are temporarily attached to the cell surface, where they appear as invaginations of the plasmalemma. In this state the vesicular content equilibrates with the extracellular fluid. Subsequently, the vesicles pinch off from the surface and move through the cytoplasm to the opposite cell front where the vesicular membrane fuses with the plasmalemma and the content is discharged to the cell exterior (2). The process is assumed to be bidirectional. Subsequent observations have been interpreted in support of the above hypothesis of "vesicular transport" (3–11). Although some authors have expressed doubts about the hypothesis (12–16), it nevertheless stands as a generally accepted idea, explaining, in particular, macromolecular transport in capillaries.

During an ultrastructural study of endothelial vesicles in frog mesenteric capillaries we made an accidental observation that indicates that almost all vesicles are members of a system of invaginations of the plasmalemma. By using tannic acid after aldehyde and osmium fixation to increase membrane contrast, we observed in many cases a connection between vesicles deep in the cytoplasm and the surface. This observation invites the proposal that the cytoplasmic, plasmalemmal membranes in endothelial cells, rather than being organized as freely moving vesicles, represent elements in an organized system of a more permanent or semipermanent nature.

The picture we propose reconciles several conflicting interpretations of the behavior of macromolecular tracers in endothelia.

MATERIALS AND METHODS

The experiments were carried out on mesenteric capillaries of eight frogs (*Rana temporaria*), each weighing approximately 40 g. The animals, which had been kept for 6–8 months at 4°C, were anesthetized by exposure to 5% urethane for about 5 min until mouth respiration had stopped.

Preparation for Electron Microscopy. The frog mesenteries were fixed *in situ* by injecting 8–10 ml of 1% formaldehyde and 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) into the abdominal cavity. After 10 min the mesenteries were excised and left for 20 hr in the above fixative. After a buffer rinse, the tissue was postfixed in 2% OsO_4 in 0.1 M cacodylate buffer for 2 hr. In order to increase membrane contrast, the mesenteries were treated with 1% tannic acid (Mallinckrodt) in 0.05 M sodium cacodylate buffer for 45 min (17) or impregnated with 2% aqueous uranyl acetate for 15 hr. The tissue was dehydrated in a graded series of ethanol and embedded in Epon. Thin sections (silver to grey) were cut with a diamond knife on an LKB Ultratome and poststained with lead for 5 min. Electron micrographs were taken with a Zeiss 10 B electron microscope operated at 60 kV. The magnification was calibrated with a carbon replica of an optical grating (568 lines per mm).

Quantitative Evaluation of Vesicles. The capillaries chosen for investigation had an average diameter of 12 μm. Cross sections of 42 capillaries were sampled for quantitative measurements. The entire capillary wall in each section was photographed at an electronic magnification of ×25,000, giving a total of 650 exposures. On average, 14–15 pictures were necessary to cover the entire circumference of a single capillary.

All the vesicles were counted and their diameter (distance between the electron-lucent lines of the membranes) was measured. The area of the endothelial cytoplasm was measured with a Zeiss planimeter by two persons working independently. Volume density of vesicles was expressed as the ratio of the area covered by vesicles [number $\times \pi \times$ (average radius)^2] to the area of the total cytoplasm. This led to some overestimation because the section thickness was large compared with the average diameter of the vesicles [Holmes effect (18)].

RESULTS

The endothelium contained a large amount of intracytoplasmic membrane profiles that appeared in the sections as vesicles either surface-bound or apparently free. The average diameter was 68 nm; (SD = 16 nm and n = 7061); 12.5% and 12.3% opened directly to the blood front and to the tissue front, respectively. Thus, 75% of the structures were confined to the interior of the cell, where they appeared either isolated or as clusters of two to five fused vesicles. The average vesicular density was 12.1% (SD = 3.0% and n = 41), corresponding to approximately 550 vesicles per μm^3. The frequency appeared to vary randomly throughout the cytoplasm. The average density in the nuclear region and in the peripheral cytoplasm was the same. The majority of the structures were circular, but elliptical and irregular forms occurred (Fig. 1). Transendothelial channels were not observed in 700 sections.

These general findings agree with observations on mammalian capillaries (19–22).

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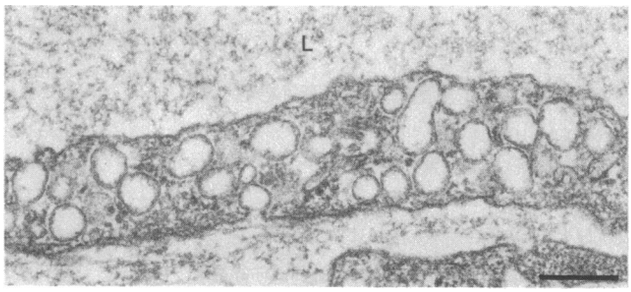

FIG. 1. Endothelial segment from frog mesenteric capillary. The cytoplasm contains numerous vesicles of variable size and shape. Some vesicles are surface-bound, others were apparently free in the cytoplasm, either isolated or mutually fused. L, capillary lumen; bar, 0.2 μm.

Interpretation of the Endothelial Plasmalemmal System. The picture that led us to propose a different model for the organization of the plasmalemmal vesicles in endothelial cells is illustrated in Fig. 2. This shows a section of capillary that after fixation was treated with tannic acid. Contrary to our intentions, the membrane contrast was not augmented uniformly throughout the section. The blood cells and the luminal plasmalemma appeared with low contrast. However, the pericapillary space, including the interendothelial clefts, was filled with an electron-dense material. The outer plasmalemma clearly defined a border between regions with high and low electron density. Apparently the tannic acid solution had not permeated the entire section. What is important is the abrupt stoppage of tannic acid at the outer cell membrane, signifying a region where the impermeability of the cell membranes to tannic acid was preserved. The fact that intracytoplasmic, apparently free vesicles were labeled indicates a continuity between the interstitial space and their interior. This would normally have been interpreted as indicative of vesicular transport, but, with the technique used, labeling occurred in fixed tissue so that movement of surface-bound, labeled vesicles into the interior of the cell was out of the question.

A solution to this apparent enigma is offered by the conspicuous bush-like structures also found in Fig. 2. Here, a continuity between cell exterior and a branching system of invaginated plasmalemmal membranes is seen. If what normally appears to be isolated intracytoplasmic vesicles are rather part of a vesicular complex communicating with the cell surface then labeling of "isolated" vesicles would occur even in fixed tissue. With this interpretation, the finding that half of the cytoplasmic vesicles were labeled and the other half were unlabeled indicates that there are two different sets of structures that invaginate from either the luminal or the basal cell surface (Fig. 3) in a manner reminiscent of the T-system in striated muscle fibers (23).

The many apparently free, but labeled vesicles are explained by the fact that electron micrographs are projections of very thin sections of tissue and they would therefore rarely contain an entire vesicle-cluster system. Thus, the connection between the surface and a vesicle located centrally in the cytoplasm will frequently be out of the plane of section.

In order to illustrate the picture we suggest, a simple three-dimensional model was built (Fig. 4). We want to stress that this organization does not exclude the existence of isolated vesicles, but insofar as the present findings are typical, their number would be small.

Studies with ultrathin serial sectioning of tannic acid-treated capillaries have corroborated the interpretation given here, because almost all vesicles had connections with the surface in three-dimensional reconstructions of the sections (unpublished results).

DISCUSSION

The concept of a racemose, vesicular organization, which we propose, raises the question whether the structures might result from fixation artifacts. It cannot definitely be excluded that membrane fusions occur after application of fixative (24), which would create the picture we have seen. However, in retrospect, several earlier investigations with macromolecular tracers support the notion that vesicles are elements in permanent or semipermanent invaginations.

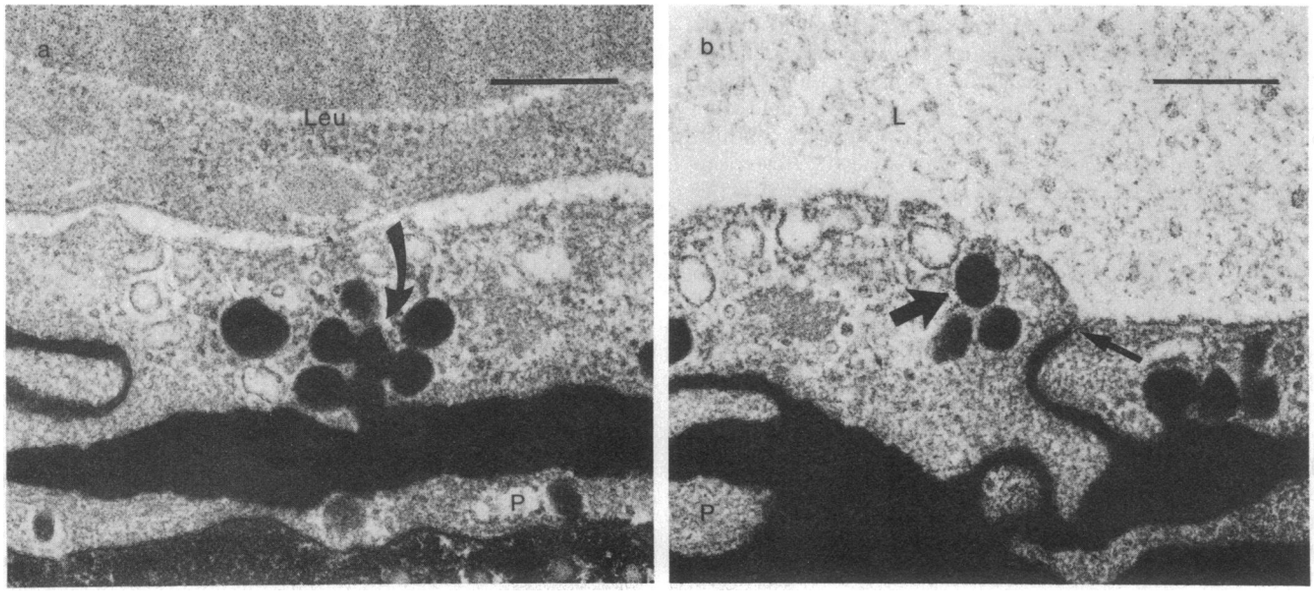

FIG. 2. Endothelium from frog mesenteric capillary. Tannic acid treatment of the tissue resulted in a very intense staining of the pericapillary space. (*a*) The curved arrow indicates a cluster of fused vesicles communicating with the abluminal surface. Leu, leucocyte; P, pericyte; bar, 0.2 μm. (*b*) The content of a vesicle near the luminal surface of the endothelium (large arrow) has the same electron density as the pericapillary space. Small arrow indicates a punctate junction at the luminal end of an interendothelial cleft, preventing electron dense material from reaching the lumen. L, lumen; P, pericyte; bar, 0.2 μm.

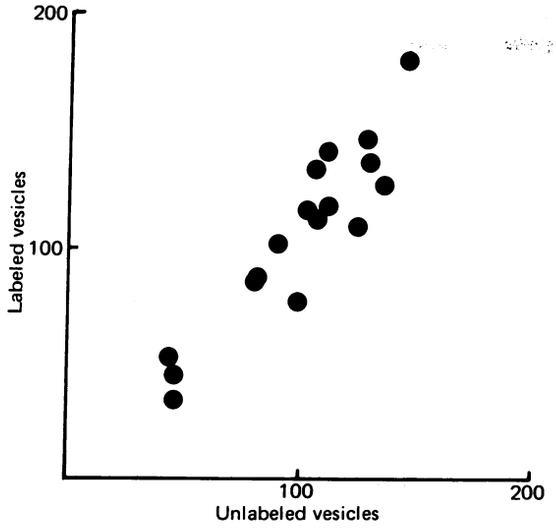

FIG. 3. Correlation between labeled and unlabeled vesicles. Each point represents the counting from one capillary cross section. Out of a total of 3473 vesicles, 52% were labeled.

First, our model implies that transport of macromolecular tracers into the interior of the vesicular system occurs by diffusion driven by a concentration gradient. A picture suggesting such a gradient has been observed by Johansson (25) and Clough and Michel (26), who, in shortlasting experiments with ferritin, found declining tracer concentration within vesicles situated at increasing distance from the site of labeling that took place from the tissue side and from the luminal side, respectively.

Second, experiments of longer duration with proteinaceous tracers on capillaries with low macromolecular permeability indicate that the vesicles are rather static structures. Pietra *et al.* (13), studying lung capillaries observed that 20 min after the injection of hemoglobin this tracer only appeared in vesicles close to the luminal surface. Labeling of apparently free vesicles was considered to be a consequence of communication with the capillary lumen out of the plane of section. Third, Williams and Wissig (27), in muscle capillaries, showed that only the luminally placed vesicles were labeled with horseradish peroxidase at short times. This situation did not change for 10–15 min. Then the tracer began to appear in the interstitium and finally all vesicles were labeled, the abluminal with the same tracer concentration as the interstitial.

The fact that tracers of increasing molecular size appear in the pericapillary space of muscle capillaries with variable delays suggests diffusion rather than vesicular transport, which should result in more equal transport rates. The smallest tracer, microperoxidase, appeared after 30–40 sec (10, 28), cytochrome *c* and myoglobin appeared after 1 min (6, 29), horseradish peroxidase appeared after 5–16 min (24, 30) and ferritin appeared after 10 min (4).

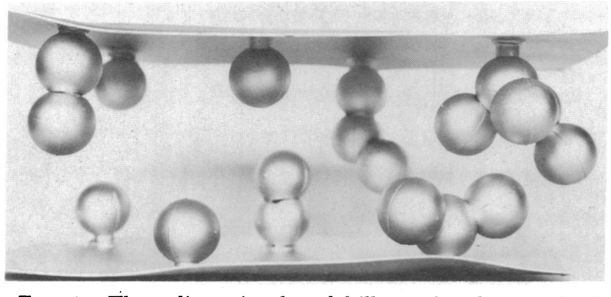

FIG. 4. Three-dimensional model illustrating the organization of the endothelial vesicles as proposed in the text. The plates indicate the plasmalemma. The spheres represent vesicles as elements of clusters communicating with the endothelial surfaces.

Addition of tracers to *fixed* capillaries in heart or striated muscle resulted in pictures that were surprisingly similar to those found after administration of tracer *in vivo* (14, 31). Karnovsky (31) thus commented, "It is noteworthy that many of the vesicles fill with lanthanum, indicating that most of these seen in a section are opening to either the luminal or the basal surface within or without the plane of section. This could also explain much of the vesicular staining seen with the peroxidase technique." We obtained similar results in preliminary experiments in which fixed capillaries were perfused with ruthenium red or horseradish peroxidase. The tracers were found in the capillary lumen, luminal caveolae, and cytoplasmic free vesicles. Because of low and variable tracer densities in the capillaries, a rigid quantification of labeled versus unlabeled vesicles was not possible. However, a large proportion of the apparently free vesicles were labeled in support of the idea that there exists a luminal racemose system of vesicles corresponding to the abluminal one demonstrated with tannic acid in the present series.

Florey's observation (12) that labeling of "free" vesicles with ferritin was unaffected by anoxia and metabolic poisons has always been puzzling because it implies that energy-requiring processes such as fusion and fission of membranes are not instrumental in vesicle labeling. The observation lends support to the presence of a vesicular system communicating with the surface.

Rippe *et al.* (16), in a study of transcapillary transport of tracer albumin at 37°C and 13–15°C, found that the transfer did not stop at the lower temperature at which vesicular dynamics supposedly were arrested. They take this as evidence that vesicular transport is not responsible for albumin transfer across muscle capillaries.

The system of permanent plasmalemmal invaginations may occasionally be involved in transendothelial transport. Thus, the view, formulated most clearly by Simionescu, Simionescu, and Palade (32), that chains of fused vesicles may connect the two endothelial surfaces is not incompatible with our interpretation of the system. It still remains a possibility that two oppositely situated invaginated clusters in close proximity might sometimes lead to fusion of neighboring, centrally located membranes, thus creating a transcellular channel.

An important physiological implication of the revised view of endothelial vesicles to discuss is that of protein transport. If vesicular transport is absent or of very limited capacity, protein transport must occur by diffusion and convection through a large pore system as suggested by Grotte (33). This idea was later extended by Bill (34), who found that macromolecules were mainly transported through "leaks" in the venous ends of the microcirculation.

To the extent that large pores or gaps do occur, they could be situated *between* endothelial cells as large widenings of the junctions at discrete points and *within* endothelial cells in the form of fenestrae or transendothelial channels formed by fusion of the central parts of the racemose invaginations as described above.

Although our revised picture of the organization of endothelial plasmalemmal vesicles leaves an unanswered question about the morphological identification of protein-transporting structures in capillaries, it indirectly supports the notion that all transcapillary solute transport occurs by passive processes in accordance with the original pore theory (35).

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