Differentiated Microdomains of the Luminal Plasmalemma of Murine Muscle Capillaries: Segmental Variations in Young and Old Animals

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ABSTRACT We investigated the luminal surface of the continuous endothelium of the microvasculature of the murine heart and diaphragm to find out whether it has differentiated microdomains. The probes were ferritin molecules, cationized to pl's 6.8, 7.15, 7.6, 8.0 and 8.4, which were introduced by retrograde or anterograde perfusion through the aorta or vena cava after the blood was removed from the vasculature. The pattern of labeling was analyzed by electron microscopy and assessed quantitatively by morphometry in arterioles, capillaries, and venules identified in bipolar microvascular fields in the diaphragm. The results showed that the plasmalemma proper was heavily but discontinuously labeled by all cationized ferritins (CF) used, the labeling being less extensive on the venular endothelium. CF had access as individual molecules to a fraction of the vesicular population opened on the luminal front of the endothelium. Plasmalemmal vesicle labeling increased from ~10 to ~25% as the pl decreased from 8.4 to 6.8. Vesicle labeling also increased with CF concentration in the perfusate. All CF binding sites were removed by pronase and papain. Heparinase and heparitinase caused only a slight reduction in CF labeling. Neuraminidase decreased the extent and density of labeling, especially on the plasmalemma proper of the venular endothelium; this decrease was particularly pronounced in old animals.

In work already published we demonstrated that the luminal surface of the endothelium of routine fenestrated capillaries has biochemically differentiated microdomains generated by a preferential distribution of anionic sites (26, 28) and glycosaminoglycans and sialoglycoproteins on the plasmalemma proper, and by heparan sulfate proteoglycans on fenestral diaphragms (32). In subsequent experiments, a comparable approach was used to map anionic sites on the continuous endothelium of the pulmonary microvasculature (22). Differentiated microdomains, comparable to those detected in fenestrated capillaries, were found associated with plasmalemmal vesicles and the plasmalemma proper. In addition, CF-detectable anionic sites were absent or present only at low density over large areas of highly attenuated, vesicle-free endothelium directly apposed to the alveolar wall.

In this paper we present the results of a new series of experiments carried out to investigate (a) the distribution of anionic sites on the continuous endothelium of murine muscle (heart and diaphragm) capillaries; (b) differences in the surface density of anionic sites among the endothelia of arterioles, capillaries, and venules in the diaphragm; (c) the effects of proteases, neuraminidase, and some glycosaminoglycans and sialoglycoproteins on the plasmalemma proper, and by heparan sulfate proteoglycans on fenestral diaphragms.
glycan-degrading enzymes on endothelial anionic sites; and (d) the effects of aging on the differential distribution studied in b.

MATERIALS AND METHODS

Animals

We used two groups of male Swiss albino mice. The first had 38 young animals (4 mo old), 20 to 28 g, and the second consisted of 15 old mice (23 mo), 45 to 53 g. The average life span of the Swiss mouse is 20 mo (35).

Reagents

Reagents were obtained from the following sources: neuraminidase (type VIII), papain (type III, X 2 crystallized), and pronase E (type XIV), from Sigma Chemical Co. (St. Louis, MO); heparinase from Seikagaku-Kogyo, Tokyo; and heparitinase from Dr. J. E. Silbert, Veterans Administration, Outpatient Clinic, Boston, MA. Tests for protease activity in the heparinase preparation were negative. According to the supplier, heparinase has no proteolytic activity and negligible lytic activities for glycosaminoglycans other than heparin sulfate. Native ferritin (Miles Laboratories Inc., Elkhart, IN) was cationized in the laboratory to the following pls: 6.8, 7.15, 7.6, 8.0, and 8.4 after the procedure of Danon et al. (9); CF of pl 8.4 was also obtained from Miles Laboratories Inc.

Experimental Procedures

GENERAL PROTOCOL: All experiments included the following steps, modified in some cases as indicated in the text or figure legends. (a) The vasculature was washed free of blood by retrograde perfusion for 5 min at a flow rate of 3 ml/min with Dulbecco's phosphate-buffered saline (DPBS) supplemented with 5% minimum essential medium amino acids and 14 mM glucose, gassed with 95% O$_2$ and 5% CO$_2$, and prewarmed to 37°C. (b) CF (used in two concentrations: 3 and 15 mg/ml and administered at 1 ml/100 g body wt) was introduced by perfusion and maintained in the vasculature for either 2, 5, or 10 min; CF perfusion was carried out either in a close circuit at a rate of 5 ml/min by connection of the abdominal aorta to the vena cava or aorta, respectively, as the outlet. (c) Excess CF was removed by perfusion with DPBS, as in a. (d) Fixation was carried through also by perfusion with 2% formaldehyde in either 0.1 M Na arsenate-HCl buffer or 0.1 M Na cacodylate-HCl buffer, pH 7.2.

ENZYME TREATMENT: After blood removal and before CF administration, the vasculature was perfused with the enzymes and under the conditions given in Table I. Enzyme treatment involved intermittent perfusion for 1 min at 5 min intervals at a rate of 3 ml/min to give the aggregate time indicated in Table I. At the end of this step, the enzymes were removed by flushing of the vasculature with DPBS under the same conditions as for blood removal (a under GENERAL PROTOCOL).

CONTROLS: In controls, the vasculature was perfused with DPBS or 0.1 M Na acetate buffer (pH 7.0) only, under the conditions given for each enzyme. To test for possible patching of anionic sites by CF, the order of the steps in the general protocol was changed as follows: after the blood was flushed with DPBS, the vasculature was fixed by perfusion (as in d above) for 4 min; quenching of residual aldehydes was carried out by continuous perfusion with 0.1 M glycine (~50 ml) for 15 min; and the quencher was washed out with DPBS (5 ml) before the perfusion with CF, which was carried out as in b above, was started. The rest of the procedure followed exactly the general protocol.

Tissue Processing for Electron Microscopy: At the end of the fixative perfusion, specimens were excised from the heart and from diaphragm regions provided with bipolar microvascular fields (27). The collected specimens were further fixed for 90 min by immersion in the same aldehyde mixture as in d, postfixed for 90 min at 4°C in 2% OsO$_4$ in 0.1 M Na arsenate-HCl buffer, pH 7.2, then treated en bloc with either 0.5% uranyl acetate or 0.5% tannic acid (galloylglucose) (25) for 30 min at 22°C, and then dehydrated and Epon embedded by standard procedures.

Electron Microscopy: Thin sections were cut on Sorvall (DuPont Instruments--Sorvall Biomedical Div., DuPont Co., Wilmington, DE) or Reichert ultramicrotomes (AO Reichert, Buffalo, NY), stained with uranyl acetate and lead citrate, and examined and micrographed under a Philips 301 or 400 electron microscope at primary magnifications ranging up to 50,000 times.

Morphometric Analysis: The luminal perimeters of the vessels examined were measured with a planimeter on electron micrographs of cross-sectioned, randomly collected vascular profiles. They were found to average 20 #m$_2$ for arterioles, 12 #m$_2$ for capillaries, and 42 #m$_2$ for venules. These perimeters, converted to surfaces under the assumptions of a mean section thickness of 60 nm, gave an average endothelial surface of 1.2 #m$^2$ for arterioles, 0.7 #m$^2$ for capillaries, and 2.52 #m$^2$ for venules, per vascular profile. In the bipolar microvascular fields of the diaphragm, the average number of vascular profiles examined for each experimental condition (from at least four different experiments) was 24 for arterioles, 36 for capillaries, and 34 for venules.

On the same micrographs, the fraction of the luminal perimeter labeled by CF was determined, and the CF density in the labeled areas of the luminal endothelial surface was assessed by CF particle counting. The figures obtained (CF per #m$^2$) should be considered relative rather than absolute values, given the uncertainties involved in converting CF counts on sections into CF density on surfaces. We assume, however, that the figures are useful for comparing CF surface density on different microvascular segments, since the errors should be comparable in all cases. Our approach may, in fact, minimize segmental differences, since counting may be more accurate at low CF surface density.

We determined the percentage of CF-labeled plasmaemal vesicles and coated pits was by counting (a) all vesicles and coated pits opened on the blood front or located within 100 nm from it, and (b) all elements in a labeled by CF particles.

RESULTS

The same general labeling pattern with CF of pl >7.6 was found on the luminal surface of the endothelium in the microvasculature of the heart and diaphragm, and similar variations in label extent and density were encountered in both microvascular beds. Such variations could be reliably established in specific areas of the diaphragm where the existence of bipolar microvascular fields allowed the identification of arterioles, capillaries, postcapillary (pericytic) vessels, and muscular venules on account of their location in the field, diameter of the vessels, and organization of their walls (27). For this reason, the results will be documented primarily for the microvasculature of the diaphragm. They were, however, the same in the myocardial vasculature, whenever the vessels could be reliably identified.

General Labeling Patterns

Over the entire luminal surface of the microvasculature, i.e., in arterioles (Fig. 1), precapillary sphincters (Figs. 2 and 3), capillaries (Figs. 4–6), and venules (Figs. 7 and 8), the plasmalemma proper was labeled by clusters of CF particles. We assume, however, that the figures are useful for comparing CF density on different microvascular segments, since the errors should be comparable in all cases. Our approach may, in fact, minimize segmental differences, since counting may be more accurate at low CF surface density.

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Table I

| Enzyme  | Source          | Concentration | pH | Temperature °C | Time min |
|---------|-----------------|---------------|----|----------------|----------|
| Neuraminidase | Clostridium perfringens | 0.5–2 U/ml | 5.5 | 37 | 30–60 |
| Heparinase      | Flavobacterium heparinum | 0.5–1 mg/ml | 7.0 | 35 | 20–40 |
| Heparitinase    | Flavobacterium heparinum | 5 U/ml     | 7.0 | 40 | 45 |

* Observations were made primarily on the bipolar microvascular fields of the diaphragm.

* Neuraminidase and crude heparitinase were dissolved in DPBS whereas heparitinase was dissolved in 0.1 M sodium acetate buffer.

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FIGURE 1 Terminal arteriole with a discontinuous monolayer of smooth muscle cells (sm). The luminal surface of the endothelium is labeled with CF, pl 8.4, in a discontinuous pattern. The cationic probe penetrates the beginning of the intercellular spaces (arrows) but does not reach the subendothelial layer. The inset shows the characteristic pattern of endothelial labeling, e.g., discontinuous patches of CF on the plasmalemma proper, and an absence of bound CF on plasmalemmal vesicles (v), stomata diaphragms (s), and infundibula leading to vesicles. Note the unstained elastica interna (el) and the heavy concentration of filaments in the endothelium (e). L lumen, × 14,000; (inset) × 87,000.

The rather striking difference between the heavy labeling of plasmalemmal vesicles. In many cases, CF clusters ranging in size from 50 to 300 nm were separated by small unlabeled areas of the plasmalemma proper, and, in almost all cases, the surface labeling extended for a variable distance along the luminal parts of the intercellular spaces (Figs. 1 and 6) without reaching beyond the level of intercellular junctions. In certain areas, CF formed a single or double planar lattice on the luminal plasmalemma (Fig. 5, inset), suggesting that the interacting anionic molecules were locally tightly packed (~15 nm apart) and protruded at least 20 nm above the plane of the plasmalemmal bilayer.

In contrast to the plasmalemma proper, the membrane of the large majority of plasmalemmal vesicles open to the luminal front of the endothelium was not labeled by CF (Figs. 1, inset, 3, 5 and 7). A minority of vesicular profiles contained, however, one, two, or (rarely) more than two CF molecules (Fig. 9), which appeared individually scattered within the profiles of the corresponding vesicles rather than clustered on the inner aspect of the membranes of the latter.

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Figures 2 and 3 Fig. 2: Precapillary sphincter at a capillary branching from an arteriole. As in Fig. 1, the endothelium is extensively but discontinuously labeled by CF, pl 8.4. Smooth muscle cells (sm) cover as a monolayer part of the arteriolar endothelium. Two layers of muscle cells appear to be present at the level of the precapillary sphincter (sm1 and sm2). The adventitia of the vessel includes numerous collagen bundles (c), a Schwann cell (sc) with amyelinated nerve fibers, and a fibroblast with an extremely long lamellar pseudopodium (l); another long lamellar pseudopodium appears at Ip (the latter elements are assumed to represent the "veil cells" of the light microscope literature. e, endothelium, l, lumen, mf, skeletal muscle fiber. x 9,000. Fig. 3: Enlarged area of one side of the precapillary sphincter demonstrating the discontinuous labeling of the endothelium by CF and the absence of labeling of most plasmalemmal vesicles opened on the luminal front. A myoendothelial junction appears at mj. Note the complex layering of the basement membranes of the endothelium and smooth muscle cells (arrows). x 34,000.

of the plasmalemma proper by CF clusters and the light or absent labeling of the plasmalemmal vesicles by individual or small groups of molecules was valid for the entire microvasculature (Figs. 1, 3, 5, 7, 9). In most cases, infundibula leading to vesicular introits were also not labeled (Figs. 5 and 9), and CF bound to the stomatal diaphragms of neither plasmalemmal vesicles (when present) nor transendothelial channels (Fig. 8).

Segmental Differences

In young animals: As illustrated by Figs. 1 to 8 and shown by the data in Table II, the percentage of the luminal perimeter covered by the cationic probe was equally high in arterioles and capillaries but lower in pericytic venules, primarily because CF clusters were smaller and more widely spaced on the luminal plasmalemma of the venular endothelium. The density of the labeling, i.e., the number of CF particles per μm² decreased from arterioles and capillaries to pericytic venules and appeared to be particularly low for muscular venules (Fig. 8).

Plasmalemmal vesicles were much less extensively labeled than plasmalemma proper and most CF molecules appeared to label their contents rather than their membrane (Fig. 9).

The percentage of vesicles labeled with CF, pl 8.4 (Table III), was low in arterioles (~10%), slightly higher in capillaries (~12%), and highest in venules (~17%). In the capillary endothelium, vesicular labeling was found to be a function of the pl of the probe (Table IV): it increased markedly (from 12 to 26%) with decreasing pl (from 8.4 to 6.8). It was also affected by CF concentration in the perfusate, but the response was not proportional: it increased by only a small factor (~1.5 times) when the concentration of the cationic probe was raised five times (Table IV).

Most of the coated pits were heavily labeled by CF, pl 8.4, on the endothelium of capillaries and arterioles (Table V and Fig. 9); but a relatively large fraction (>40%) of morphologically identical pits was not labeled on the endothelium of pericytic venules, irrespective of the anterograde or retrograde direction of CF perfusion.

In old animals: The extension of the survey to old mice showed no decrease in percent perimeter labeled by CF for arterioles and capillaries (Fig. 10) but revealed a remarkable decrease by ~50% of the corresponding figure for venules (Table II and Fig. 11). The decrease affected also the density of CF labeling, which dropped in venules by ~35% (Table II).

Vesicular labeling appeared to be moderately increased in
old animals, especially in the venular endothelium (Table III). Coated pits, however, were found to be less extensively labeled in the venular endothelium of old animals, the corresponding values for arterioles and capillaries being similar in the two age groups (Table V).

Effect of Prior Fixation on CF Labeling

To test the possibility that CF acts as a polyvalent ligand and causes patching of the anionic sites of the luminal plasmalemma, the probe was also introduced after fixation followed by quenching, as described in Materials and Methods. The same general pattern was obtained, i.e., reduced labeling or no labeling of plasmalemmal vesicles and their infundibula, and extensive labeling of the plasmalemma proper, except that labeled patches appeared to be smaller and generally limited to one or two CF layers (Fig. 12a). The perfusion of the probe without previous quenching (or with insufficient quenching) resulted in heavy uniform labeling of the entire endothelium and in more extensive labeling of the plasmalemmal vesicles (Fig. 12b).

CF Labeling after Enzyme Perfusion

Neuraminidase: In young animals, perfusion with neuraminidase before CF resulted in a general but unequal reduction of both the extent and density of labeling of the plasmalemma proper. The reduction was moderate for arterial and capillary endothelia (Table II and Fig. 6) but more pronounced for venular endothelia (Table II). The pattern of labeling of the plasmalemmal vesicles was not affected by this treatment, and the same applied for the labeling of coated pits (Tables III and V).

Neuraminidase affected almost as extensively the labeling of the plasmalemma proper of the venular endothelium in old mice (Fig. 13). The percentage of luminal perimeter labeled by CF dropped from 32% in controls to 18% after neuraminidase treatment, and the density of label (CF × micrometers was squared) concomitantly reduced by >50% (Table II), to give the lowest values recorded in this survey.

Pronase and Papain: After perfusion with either pronase E or papain carried out only in young animals, CF did not bind to the luminal aspect of the plasmalemma proper.
Figure 5: High magnification of the endothelium in a sector of a blood capillary. The plasmalemma proper is discontinuously decorated with CF patches, one or two molecules thick. (Note the regular spacing of the CF molecules in the layer(s) immediately adjacent to the plasmalemma [long arrows].) The plasmalemmal vesicles and the infundibula (i) leading to their openings are usually not labeled, but individual CF molecules are seen in the content of some plasmalemmal vesicles (short arrows). (Inset) The micrograph illustrates the crystalline lattice formed by CF molecules in certain areas on the surface of the plasmalemma proper (between the short arrows), the regular spacing of the lattice at ~3 nm from the plasmalemma, and the presence of two CF, pl 8.4, layers in certain parts of the lattice (long arrows), bm, basement membrane, mf, skeletal muscle fiber, p, pericyte, ps, pericapillary space. × 86,000. (Inset) × 100,000.

and appeared to label less extensively the plasmalemmal vesicles throughout the entire microvasculature. The basal laminae (basement membranes) of the vessels were extensively disorganized, which suggests that during perfusion the proteases gained access to the pericapillary spaces.

HEPARITINASE AND HEPARINASE: Perfusion with these enzymes, which degrade sulfated glycosaminoglycans with the specificities indicated in Materials and Methods, did not affect to a detectable extent the pattern and the intensity of labeling of the plasmalemma proper and of the plasmalemmal vesicles in any segment of the microvasculature. The only effect recorded was a slight reduction in the CF-labeling of coated pits on the venular endothelium (Table V). The inquiry was not extended to old animals.

DISCUSSION

Our results indicate that the luminal surface of the continuous endothelium of the microvasculature of the diaphragm and the myocardium has a distribution pattern of anionic sites similar to that already established for the continuous endothelium of lung capillaries (20, 22) and for the nonfenestrated areas of the endothelium of visceral capillaries (26, 28). In all of these cases, the luminal surface of the endothelium is characterized by a high concentration of bound CF, ascribed to a commensurately high concentration of anionic sites on the plasmalemma proper, and by the absence or scarcity of CF binding to the membrane of the plasmalemmal vesicles (open on the blood front) and to their associated infundibula and stomatal diaphragms (when present). Transendothelial channels made up of two (or more) vesicles are less easily recognized and more complex in structure than are the one-vesicle channels provided with two stomatal diaphragms characteristic of the capillary and the venular endothelium of the visceral fenestrated microvasculature. When detected, as in Fig. 8, the transendothelial channels of the continuous endothelium have similar surface properties: neither their membranes nor their stomatal diaphragms are labeled by CF.

In the fenestrated capillaries of the pancreas and intestinal mucosa, the percentage of vesicles labeled by CF, pl 8.4, is <10% (28). In the microvascular endothelium of the diaphragm and heart, CF has access to slightly more of the plasmalemmal vesicles open to the luminal front. In these vesicles, however, CF is present as individual molecules (singly or in small numbers) and appears to be scattered within the vesicles rather than bound in clusters to their membranes. In the endothelium of these capillaries, the extent of vesicular labeling increases as a function of the pl and concentration of the probe: it is ~10% for CF, pl 8.4, and increases to ~25% when the pl decreases to 6.8. Native ferritin, pl ~4.5, has access to most of the vesicle population in both fenestrated (6) and continuous capillaries (3). Ferritins of intermediate pl have not been tested. The plasmalemmal vesicles of the
Figures 6 and 7  Fig. 6: Capillary endothelium in the diaphragm of a young mouse; specimen perfused with neuraminidase before administration of CF, pH 8.4. The CF binding pattern is not visibly altered, except that there is less order of CF particles within the patches (long arrows) and that the latter are less clearly defined. Some plasmalemmal vesicles are labeled by individual or small clusters of CF molecules (short arrow). Note the binding of CF to the cell membrane along the adluminal part of the intercellular space (is) and the high concentration of microtubules (mt) in the endothelial cytoplasm. bm, basement membrane. e, endothelium. l, lumen. × 64,000. Fig. 7: Pericytic venule in the diaphragm of a young mouse. The labeling pattern is similar to that found in capillaries and arterioles except that the patches (arrows) are smaller, less regular, and more widely separated by stretches of unlabeled plasmalemma. Plasmalemmal vesicles (v) and their infundibula are not labeled. The heavily labeled structure at s is probably a recess of the luminal surface. e, endothelium. l, lumen. p, pericytes. × 60,000.

Fenestrated endothelium of these vessels were also shown to be heavily labeled by neutral macromolecules such as dextrans and glycogens (24).

As shown in our previous studies, the mouse diaphragm has characteristic bipolar microvascular fields in which the sequential segments of the microvasculature can be easily and reliably identified (27). We took advantage of this situation and surveyed the distribution of anionic sites on the endothelium of arterioles, capillaries, and venules. The general pattern of anionic site distribution is the same in all segments. The extent and density of the sites appear to be the same in arterioles, precapillary sphincters and capillaries, but both parameters are reduced to lower values on the venular endothelium. Our unpublished observations on other microvascular beds suggest that this is a trend.

A survey of the microvasculature showed that the extent and density of anionic sites on the venular endothelium are considerably reduced in old mice as compared with young animals.

As in our previous studies (32), we explored the surface chemistry of the endothelium by perfusing the vasculature with proteases, neuraminidase, heparinase, and heparitinase before exposing the luminal plasmalemma to the cationic probes. Broad specificity proteases (papain and pronase) effectively prevented CF binding, presumably by removing all anionic sites from the luminal surface of the endothelium. These results indicate that the main contributors of acidic sites on the luminal surface are sialoglycoproteins and proteoglycans rather than sialylglycolipids. Neuraminidase perfusion reduced the extent of labeling of the venular endothelium, and the reduction was particularly marked in old animals. After heparitinase and heparinase perfusion, the reduction was less pronounced, which suggests that sulfated proteoglycans are not major contributors of anionic sites to the luminal surface of the continuous microvascular endothelia we have examined. Yet their occurrence in higher concentration than suggested by the data in Table II cannot be ruled out; they may be present but inaccessible, under our experimental conditions.
conditions, to the glycosaminoglycan-degrading enzymes we have tested.

The microvasculature of the myocardium and diaphragm is structurally representative of most of the microvascular beds of the organism. They have in common an attenuated but continuous (nonfenestrated) endothelium, provided with a large population of plasmalemmal vesicles (18, 37) and with simplified intercellular junctions (18, 23). Vascular beds of this type have been used extensively in the past for physiological studies on capillary permeability. As a result, current concepts, theories, and hypotheses that pertain to this important topic rely primarily on data obtained on this type of microvasculature (14, 20). The results reported in this paper add new and potentially significant elements to our understanding of the organization of these vessels. They show that access of CF to plasmalemmal vesicles in this type of endothelium depends on the pI of the tracer and establish quantitatively that the concentration of anionic sites on the luminal surface of the endothelium is lower in old than in young animals. In both cases, anionic site concentration decreases from arterioles and capillaries to venules, being minimal on the venular endothelium of old animals. Our findings may apply to most, if not all, capillaries provided with a continuous endothelium; hence, similar differentiated microdomains may exist on the luminal surface of their endothelium, but their existence remains to be confirmed by further experimental work.

The differentiated microdomains detected on the luminal surface of capillaries provided with a continuous endothelium are simpler and less diversified than their counterparts already studied in fenestrated capillaries. They are reduced, in fact, to the plasmalemma proper, plasmalemmal vesicles, their infundibula and their stomatal diaphragms (when present), transendothelial channels (when present), and coated pits.
**TABLE II**

| Experimental conditions          | % of luminal plasmalemma labeled by CF | CF/μm² endothelial surface |
|----------------------------------|----------------------------------------|-----------------------------|
|                                  | Arterioles | Capillaries | Venules | Arterioles | Capillaries | Venules |
| Young animals (4 mo)             |            |            |         |            |            |         |
| Control perfusion                | 76 ± 12    | 81 ± 9     | 61 ± 7  | 4,110 ± 510| 3,620 ± 330| 2,960 ± 310|
| Neuraminidase perfusion          | 61 ± 7     | 68 ± 8     | 43 ± 4  | 3,220 ± 350| 2,810 ± 320| 2,120 ± 160|
| Heparinase perfusion             | 60 ± 8     | 78 ± 10    | 55 ± 7  | 3,700 ± 380| 3,200 ± 410| 2,720 ± 180|
| Heparitinase perfusion           | 78 ± 14    | 76 ± 14    | 58 ± 9  | 4,040 ± 480| 3,710 ± 440| 3,040 ± 330|
| Old animals (23 mo)              |            |            |         |            |            |         |
| Control perfusion                | 73 ± 7     | 79 ± 11    | 32 ± 5  | 3,770 ± 440| 3,960 ± 410| 1,880 ± 200|
| Neuraminidase perfusion          | 60 ± 6     | 62 ± 9     | 18 ± 4  | 3,060 ± 390| 2,940 ± 400| 840 ± 170 |

The aggregate perimeters surveyed amounted to 480 μm for arterioles, 216 μm for capillaries, and 1,428 μm for venules. The total areas used for CF particle counting were 36 μm² for arterioles, 10.8 μm² for capillaries, and 76.5 μm² for venules. In each experimental condition, the extent of CF labeling was the same irrespective of the anterograde or retrograde direction of the perfusion. Values are ± SD.

* Measurements and counts were made primarily on the bipolar microvascular fields of the mouse diaphragm.

But, in both types of endothelia, microdomains are created primarily by large differences in local anionic site concentrations.

**Functional Implications**

As in the other vascular beds investigated, most plasmalemmal vesicles (up to 80–90%) were found to have few or no anionic sites detectable by CF, pl 8.4. CF of this pl had, however, access to a small fraction (~10–15%) of the total vesicle population opened to the luminal front of the endothelium, and this fraction increased when the pl of the probe approached neutrality or went beyond it. Native ferritin, pl 4.6, is known to have access to a large fraction of plasmalemmal vesicles opened on the luminal front in a mammalian (rat diaphragm) (3) and amphibian (frog mesentery) (8, 36) capillaries. (In frog mesenteric capillaries, plasmalemmal vesicles take up and transport cationic ferritin of pl > 10.5 [8].)

Taken together these findings support our assumption that plasmalemmal vesicles represent a preferential carrier for neutral and anionic proteins. As already established, almost all plasma proteins are anionic (1). The size limit beyond which the net charge of the molecule becomes a significant factor in permeability remains to be established. The evidence is still fragmentary and inconclusive.

Leukocyte diapedesis is known to occur preferentially in the postcapillary venules of the microvasculature (17, 21). The process probably depends on specific cell–cell interactions, but the relatively low density of anionic sites on the venular endothelium may facilitate the initial local attachment of leukocytes to the endothelial plasmalemma.

**Integration of Our Findings in the Existing Literature**

A continuous endothelium provided with a large but variable population of plasmalemmal vesicles lines most of the...
vasculature of the body from the aorta to the venae cavae. Given the wide variation in local conditions along this large circuit, it is unlikely that our findings on the striated muscle microvasculature apply to all continuous endothelia. In fact, the observations so far recorded in the literature suggest considerable variation rather than uniformity. For instance, the endothelia of the thoracic aorta of the guinea pig (33, 34), rat (10), and rabbit (15) were found to bind uniformly CF (of unspecified pI) over the entire luminal surface; microdomains were not detected; patching of CF-decorated sites occurred as a function of time in unprocessed specimens; and rather massive vesicular transport of clustered CF molecules to the tissue front of the endothelium occurred upon long (30 min), con-

**TABLE III**

| Labeling of Plasmalemmal Vesicles by CF (pI 8.4) in the Microvascular Endothelium (Mouse Diaphragm) |
|-------------------------------------------------|--------------------------------------------------|---------------------------------|
| Experimental conditions | Arteriole | Capillaries | Venules |
| Young animals* | | | | |
| Control perfusion | 10 ± 4 | 12 ± 3 | 17 ± 3 |
| Neuraminidase perfusion | 13 ± 2.5 | 10 ± 1.5 | 15 ± 2 |
| Old animals* | | | | |
| Control perfusion | 15 ± 2 | 14 ± 2.5 | 21 ± 5 |
| Neuraminidase perfusion | 12 ± 2.5 | 13 ± 3 | 22 ± 6 |

The sampling is described under morphometric analysis (see Materials and Methods). The data refer to vesicles open on the luminal front of the endothelium or located within 100 nm therefrom. For each experimental condition, the average numbers of counted vesicles were arterioles, 410; capillaries, 2,100; and venules, 1,050. The average length of aggregated vascular profiles examined was 200 μm. Values are ± SD.

* 4-mo-old mice.
* 23-mo-old mice.

**TABLE IV**

| Effects of CF Concentration and CF Isoelectric Point on the Labeling of Plasmalemmal Vesicles in the Continuous Endothelium of Muscle Capillaries (Mouse Diaphragm)* |
|-------------------------------------------------|--------------------------------------------------|---------------------------------|
| CF concentration | 6.8 | 7.4-7.15 | 8.4 |
| CF pI | | | |
| mg/ml | | | |
| 10 | 26 ± 5 | 17 ± 3 | 12 ± 3 |
| 50 | 34 ± 7 | 21 ± 4 | 18 ± 5 |

The sampling used to collect the data is given in Materials and Methods (morphometric analysis). In each case, 850 plasmalemmal vesicles were counted. The figures are percentages (± SD) of labeled vesicles in the total population of vesicles opened on the luminal front of the endothelium or located within 10 nm therefrom.

* Experiments carried out in young (4-mo-old) mice.

**FIGURES 10 and 11**  Fig. 10: Capillary in the diaphragm of an old mouse that shows the discontinuous CF, pI 8.4, decoration of the plasmalemma and the absence of labeling of plasmalemmal vesicles (v). i, endosome marked by a large lipoprotein particle, x, branched chain of vesicles opened on the abluminal front of the endothelium. bm, basement membrane. e, endothelium. l, lumen. p, pericyte; pc, pericapillary space. × 82,000. Fig. 11: Pericytic venule in the diaphragm of an old mouse: discontinuous CF labeling of the luminal surface in patches separated by relatively long stretches of unlabeled plasmalemma. Note the high concentration of intermediary filaments in the endothelial cell (between arrowheads). × 60,000.
tinuous exposure to the cationic probe. CF transport across the aortic endothelium was more active in old animals (10). An identical CF binding pattern was found for the endothelium of the vena cava (34). By contrast, in the continuous endothelium of the murine microvasculature we have studied, CF binding was discontinuous, microdomains were present, and there was no evidence of transepithelial transport over the short periods involved in our experimental protocol; vesicular labeling was found to affect a small fraction of the plasmalemmal vesicle population which increased with decreasing CF pl and CF concentration.

CF, pl 8.8 to 10.9, bound also uniformly to the exposed surface of cultured human endothelium (umbilical vein); microdomains were not observed, but vesicular uptake of CF followed by degradation (rather than transcytosis) was detected (19).

The endothelium of rabbit carotid arteries was treated with Vibrio cholerae neuraminidase by Görög et al., who found that this enzyme treatment increases the adhesion of circulating platelets (12) and the uptake of low density lipoproteins and fibrinogen (13).

CF was found to label the luminal plasmalemma of capillary endothelium in the lung (20, 22). Finally, CF of unspecified pl was shown to bind to the luminal surface of the endothelium in amphibian mesenteric capillaries (7, 36), to have access to plasmalemmal vesicles open on the blood front, and to be transported across the endothelium by such vesicles. Native anionic ferritin did not bind to the luminal surface but had access to more luminal plasmalemmal vesicles (8, 16) and was transported less efficiently than CF across the endothelium (8, 36). Labeling of luminal vesicles is usually interpreted as the first step in transendothelial transport of ferritin either by shuttling vesicles (3, 18) or fusion of vesicles followed by mixing of their contents (8).

Vesicular transport across the endothelium has been questioned, however, on the basis of morphological evidence, i.e., three-dimensional reconstructions from serial sections, which indicate that in the specimens examined there are almost no free vesicles in the endothelial cytoplasm (4, 5, 11). The underlying (but not validated) assumption is that fixation does not change the distribution of vesicles in the endothelium. Experimental data indicate that there is vesicular transport across the microvascular endothelium in the perfused

| Percentage of Coated Pits Labeled by CF (pl 8.4) in Sequential Segments of Microvascular Endothelium* |
|-------------------------------------------------|------------------|------------------|------------------|
| Experimental conditions | % of detected coated pits labeled by CF | Arterioles | Capillaries | Venules |
|-------------------------|--------------------------------|--------|------------|--------|
| Young animals | | | | |
| Control | 72 ± 6 | 81 ± 9 | 56 ± 6 |
| Neuraminidase | 74 ± 7 | 84 ± 7 | 52 ± 8 |
| Heparinase | 68 ± 6 | 78 ± 8 | 41 ± 5 |
| Heparitinase | 68 ± 8 | 77 ± 9 | 49 ± 7 |
| Old animals | | | | |
| Control | 75 ± 9 | 76 ± 10 | 44 ± 9 |

The sampling used for these measurements is indicated in Materials and Methods (MORPHOMETRIC ANALYSIS). In each case, 35-60 coated pits were counted. Values are ± SD.

* Countings made primarily on the bipolar microvascular fields of the mouse diaphragm.

**Figure 12** Postfixation labeling with CF (pl 8.4, 10 mg·ml⁻¹) of capillary endothelium with (a) and without (b) quenching by perfusion with 0.1 M glycine before the introduction of the probe. Note the heavy continuous labeling of the endothelial surface in the unquenched specimen and the detection of characteristically differentiated microdomains after quenching. e, endothelium. l, lumen. (a) × 60,000; (b) × 70,000.

**Figure 13** Venular endothelium in the diaphragm of an old animal. After perfusion with neuraminidase, CF, pl 8.4, labeling (arrows) is decreased primarily as a result of the appearance of large unlabeled plasmalemmal areas. f, fibroblast. l, lumen. p, pericyte. × 45,000.
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