Endothelial Diaphragmed Fenestrae:
In Vitro Modulation by Phorbol Myristate Acetate

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Abstract. Cultured microvascular endothelial cells isolated from fenestrated capillaries have been shown to express many properties of their in vivo differentiated phenotype, yet they contain very few diaphragmed fenestrae. We show here that treatment of capillary endothelial cells with the tumor promoter, 4β-phorbol 12-myristate 13-acetate, induces more than a fivefold increase in the frequency of fenestrae per μm^2 of cell surface, as determined from a quantitative evaluation on freeze-fracture replicas. In quick-frozen, deep-etched preparations, the endothelial fenestrae appeared to be bridged by a diaphragm composed of radial fibers interweaving in a central mesh, as previously observed in vivo. These results indicate that diaphragmed fenestrae are inducible structures, and provide an opportunity to study them in vitro.

THE endothelium of visceral capillaries is perforated by numerous circular windows, or fenestrae, ~70 nm in diameter, which are usually closed (except in glomerular endothelium) by a thin, single-layered diaphragm (1, 9, 17, 20), and are believed to participate in the transcapillary exchange of substances between blood and tissues (18). The mechanism of formation of these openings is not known, nor is it known whether they are permanent specializations of the endothelial cell, or labile structures able to respond dynamically to environmental changes. Finding answers to these questions requires the development of a suitable in vitro model, in which fenestrae can be experimentally induced. Cultured microvascular endothelial cells isolated from fenestrated capillaries have been shown to express many properties characteristic of their in vivo differentiated phenotype (4, 6, 13), yet they contain very few fenestrations (4, 11). However, an increased number of transendothelial openings has recently been observed in endothelial cells grown on the extracellular matrix laid down by Madin-Darby canine kidney cells (11). In this report, we show that the tumor promoter, 4β-phorbol 12-myristate 13-acetate (PMA)[1], markedly enhances the formation of diaphragmed fenestrae in cloned bovine microvascular endothelial cells. This observation represents the first evidence for the modulation of endothelial fenestrae by a well-defined chemical signal, and provides an opportunity for the in vitro study of these specialized structures.

Materials and Methods

Bovine microvascular endothelial cells (BMEC) were isolated from bovine adrenal cortex according to Folkman et al. (5) and cloned as previously described (6). The cells were routinely subcultured in gelatin-coated tissue

1. *Abbreviations used in this paper:* BMEC, bovine microvascular endothelial cells; PMA, 4β-phorbol 12-myristate 13-acetate.

culture flasks (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) in minimal essential medium, alpha modification (Gibco Laboratories, Grand Island, NY) supplemented with 15% heat-inactivated donor calf serum (Flow Laboratories, Irvine, Ayrshire, Scotland), penicillin (500 U/ml), and streptomycin (100 μg/ml). For experiments, the endothelial cells were seeded either into 35-mm plastic dishes (Falcon Labware) (for thin section electron microscopy), or in 35-mm dishes containing 25-mm round plastic coverslips (Thermanox, Lux Scientific Inc., Newbury Park, CA) (for freeze-fracture), and grown to confluence before treatment with 20 ng/ml PMA (Sigma Chemical Co., St. Louis, MO).

For thin section electron microscopy, control and PMA-treated cultures were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After a rinse in cacodylate buffer, they were postfixed for 20 min in 1% osmium tetroxide in veronal acetate buffer, treated quickly (~1 min) with 0.1% tannic acid (Mallinckrodt Inc., St. Louis, MO) in 50 mM cacodylate buffer, pH 7.0, washed, stained en bloc with 0.5% uranyl acetate in veronal acetate buffer for 20 min, dehydrated in graded ethanols, and embedded in Epon. Thin sections were cut perpendicular to the plane of the monolayer and stained with uranyl acetate and lead citrate.

For freeze-fracture electron microscopy, cultures grown on plastic coverslips were fixed in glutaraldehyde as above, rinsed in cacodylate buffer, and freeze-fractured in a Balzers BAF 301 apparatus (Balzers High Vacuum Corp., Balzers, Liechtenstein) according to the method of Pauli et al. (16). Both thin sections and freeze-fracture replicas were examined in a Philips EM 300 or Zeiss EM 10 electron microscope.

A quantitative evaluation of the frequency of endothelial fenestrae in BMEC cultures was carried out as follows on freeze-fracture replicas. In each of six distinct experiments (passages 15-21 after cloning) three different replicas of control BMEC and BMEC treated with PMA for 3 d were separately recovered on 150-mesh, square-hole copper grids (hole side, 117 μm) (Veco, Solingen-Hoehscheid, West Germany). On each of the three grids, two holes completely filled with an uninterrupted expanse of plasma membrane fracture faces were randomly selected, and all clearly identifiable fenestrae present in each grid hole were systematically photographed and counted. In this way, the number of fenestrae/μm^2 of cell surface could be calculated in each experimental condition.

Results and Discussion

By phase contrast microscopy, confluent BMEC cultures appeared as a monolayer of closely apposed, slightly elongated
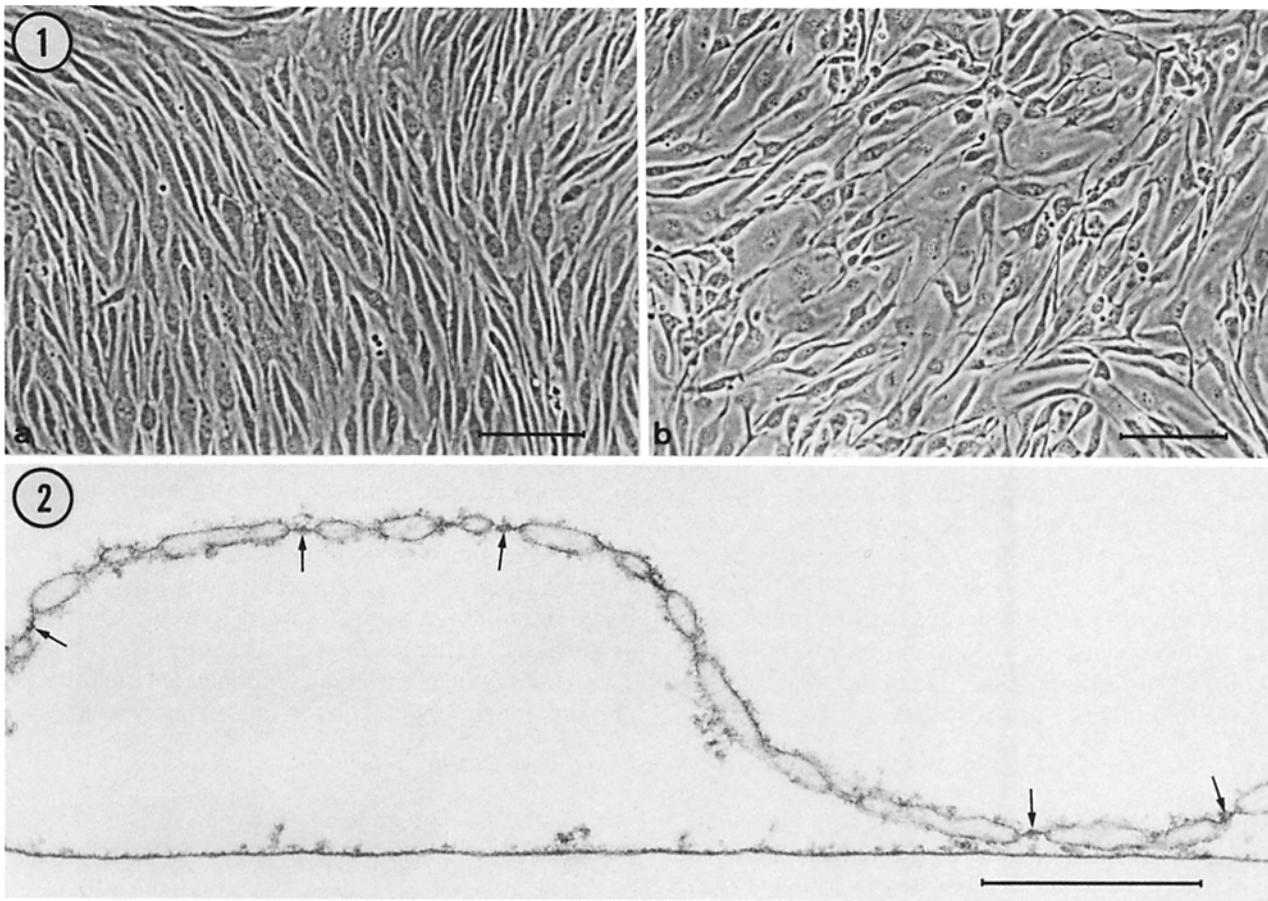
Figures 1 and 2. (Fig. 1) Effect of PMA on BMEC morphology (phase contrast). (*a*) Control BMEC form a monolayer of closely apposed, slightly elongated cells. (*b*) BMEC after 1 d of treatment with PMA. The cells have an irregular shape with refractile cell borders and long cell processes. Bars, 100 μm. (Fig. 2) Portion of a highly attenuated region from a PMA-treated BMEC (thin section perpendicular to the bottom of the dish). The endothelial cell cytoplasm is perforated by numerous fenestrae that are bridged by a thin diaphragm with a central knob (*arrows*). Bar, 0.5 μm.

cells (Fig. 1 *a*). As previously described (7, 12), within 2-6 h after the addition of 20 ng/ml PMA, the endothelial cells became more refractile and took on an irregular shape with long cell processes (Fig. 1 *b*). In thin sections perpendicular to the culture plane, BMEC treated for 3 d with PMA showed numerous highly attenuated cytoplasmic regions containing diaphragmed fenestrae (Fig. 2). In contrast, both highly attenuated regions and diaphragmed fenestrae were seen only in rare instances in control BMEC cultures maintained in normal medium during the same time period. To quantitate these observations, we turned to the freeze-fracture technique, which has the advantage of providing large *en face* views of the plasma membrane. In freeze-fracture replicas of both control (Fig. 3) and PMA-treated BMEC (Figs. 4 and 5), fenestrae appeared as circular depressions (on the P-face) or elevations (on the E-face), which could easily be distinguished from plasmalemmal vesicles (caveolae) by their larger diameter, their shallow, flat floor, and their characteristic occurrence in clusters, as is observed in vivo (15, 19). As Table I shows, PMA treatment induced more than a fivefold increase in the frequency of the fenestrae/μm^2 of cell surface.

Besides clearly recognizable fenestrae, freeze-fracture replicas of PMA-treated cells also disclosed localized intramembrane particle clearings (Fig. 6 *a*), and circular grooves sur-

Table I. Frequency of Fenestrae in Control and PMA-treated BMEC

|	Total cell surface examined	Total number of fenestrae	Number of fenestrae/100 μm^2
	μm^2		
Control BMEC	492,804*	5,319	1.08 ± 0, 19[‡]
PMA-treated BMEC	492,804*	30,208	6.13 ± 1, 60[‡]

BMEC (passage 15-21) were grown to confluency on plastic coverslips and further incubated for 3 d in either normal medium or medium containing 20 ng/ml PMA. The quantitative evaluation of the frequency of fenestrae was carried out on freeze-fracture replicas as described in Materials and Methods. The values of number of fenestrae/100 μm^2 represent the mean ± SEM of six distinct experiments.
* Surface of 36 grid holes (see Materials and Methods).
[‡] $P < 0.01$ (Student's *t* test).

rounding flat membrane disks (Fig. 6 *b*), which we interpret as putative steps in the process of pore formation. Most importantly, quick-frozen, deep-etched and rotary-shadowed preparations allowed the visualization of fenestral diaphragms. The diaphragms were composed of radial fibers interweaving in a central mesh (Fig. 7), as observed in endothelial cells in vivo (1).

In our previous study on the effect of PMA on BMEC
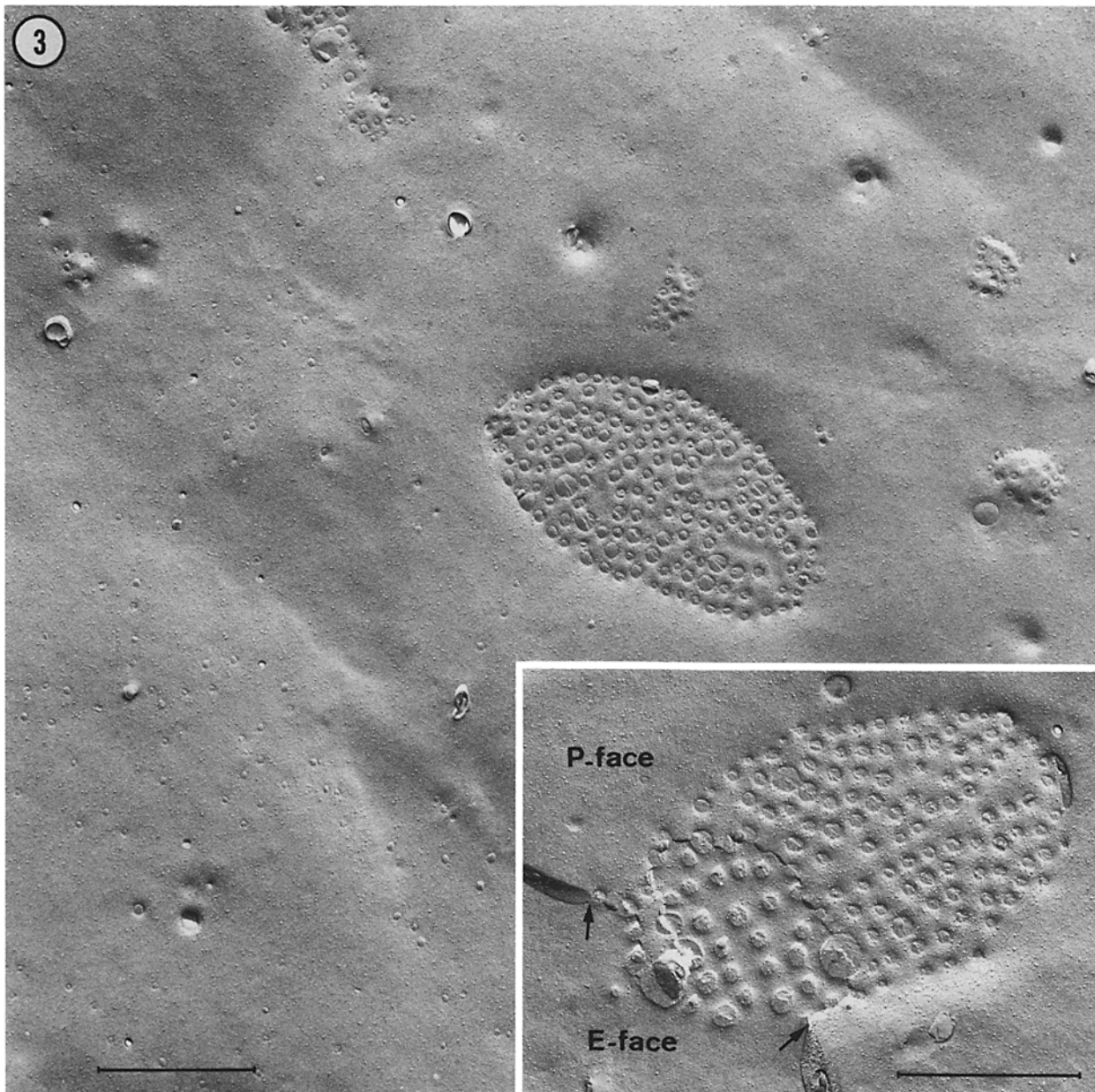
Figure 3. Freeze-fracture replica of a control BMEC culture. Fenestrations occur in clusters and are recognized as circular depressions on the plasma membrane (*P-face*). (*Inset*) P- to E-fracture-face transition showing the matching between the circular depressions on the P-face and the corresponding elevations on the E-face. Notice the sharp reduction in the thickness of the cytoplasm at the level of the cluster (*arrows*). Bar, 1 µm.

invasion of collagen gels (12), diaphragmed fenestrae were noticed in sections of PMA-treated BMEC, whereas they appeared virtually absent in control endothelial cells. Subsequent observations on BMEC grown on conventional plastic substrata also showed an apparent increase in the frequency of fenestrae after PMA treatment. However, it was difficult to obtain reliable quantitative data on the magnitude of the effect of PMA from the examination of thin sections. This difficulty prompted us to carry out a quantitative evaluation on freeze-fracture replicas, which allowed us to examine very large expanses of cell membranes. Freeze-fracture replicas, besides clearly revealing the clustered distribution of the fenestrae in the plane of the plasma membrane, enabled us to

demonstrate a more than fivefold increase in the number of endothelial fenestrae/μm^2 of cell surface in response to PMA. Our recent observations that PMA is also able to stimulate *de novo* formation of fenestrae in cultured endothelial cells from the human umbilical vein and the calf pulmonary artery (T. Lombardi et al., manuscript in preparation) demonstrate that this effect is not peculiar to the particular clone of bovine microvascular endothelial cells we have used.

Our results, together with the recently reported effect of extracellular matrix substrata (11), provide direct evidence that fenestrae are inducible structures, and may explain the reported appearance of fenestrations in the continuous endothelium of some microvessels in abnormal situations (8,
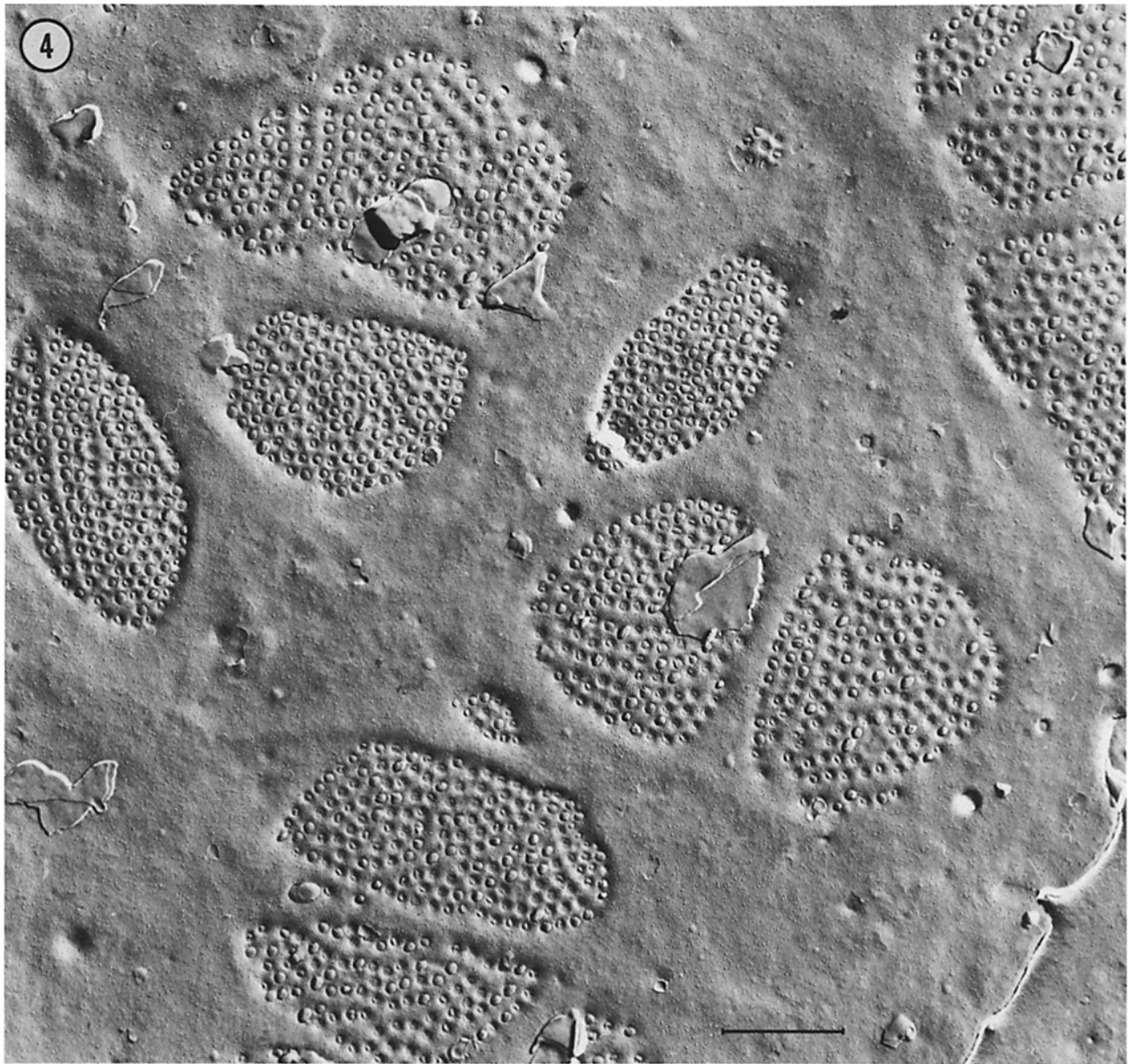
Figure 4. Freeze-fracture replica of a PMA-treated BMEC culture showing numerous clusters of fenestrae (P-face). Bar, 1 μm.

10, 21, 22). Although phorbol esters are not physiologically occurring substances, they have been shown to mimic the effects of endogenous mediators by activating a key enzyme in signal transduction, protein kinase C (14). The induction of diaphragmed fenestrae, together with previously described effects, such as stimulation of protease secretion (7) and formation of capillary-like tubes inside collagen matrices (12), indicate that phorbol esters are able to profoundly modify the differentiation program of endothelial cells, as has been shown for other cell types (2, 3).

The availability of simple in vitro systems in which fenestrae can be readily induced makes it possible to study the

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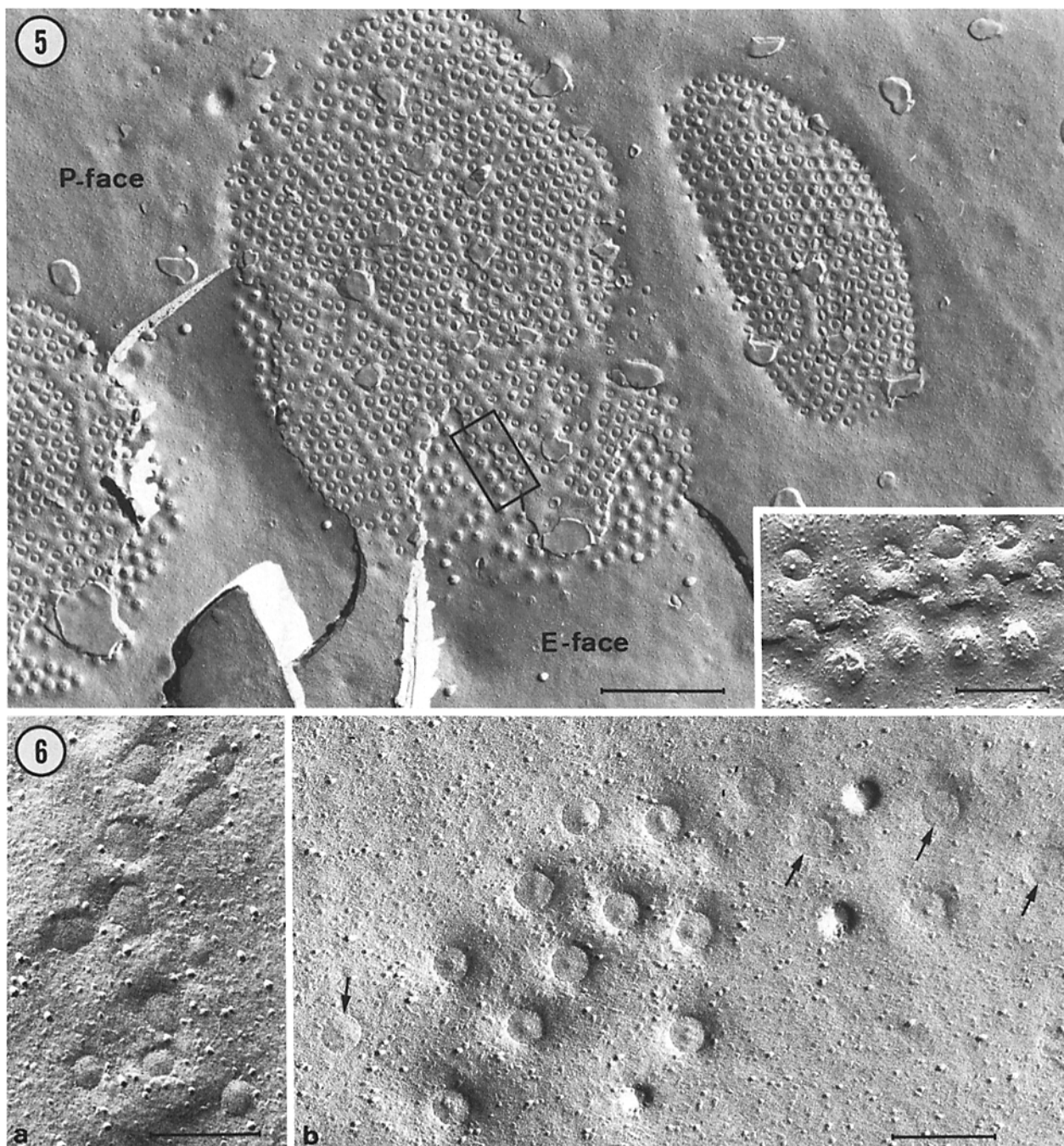
Figures 5 and 6. (Fig. 5) Large clusters of tightly packed fenestrae in a PMA-treated BMEC culture. Bar, 1 µm. (*Inset*) Higher magnification of the region of fracture-face transition outlined by the rectangle. Notice the matching between the circular depressions on the P-face and the circular elevations on the E-face. Bar, 0.2 µm. (Fig. 6) Presumptive steps in the formation of fenestrae. (*a*) Rounded particle-free patches in the plasma membrane of a PMA-treated endothelial cell (*P-face*). (*b*) Circular grooves surrounding flat membrane disks (*arrows*). Bars, 0.2 µm.

molecular organization of the diaphragm that closes these fenestrae and their mechanism of formation.
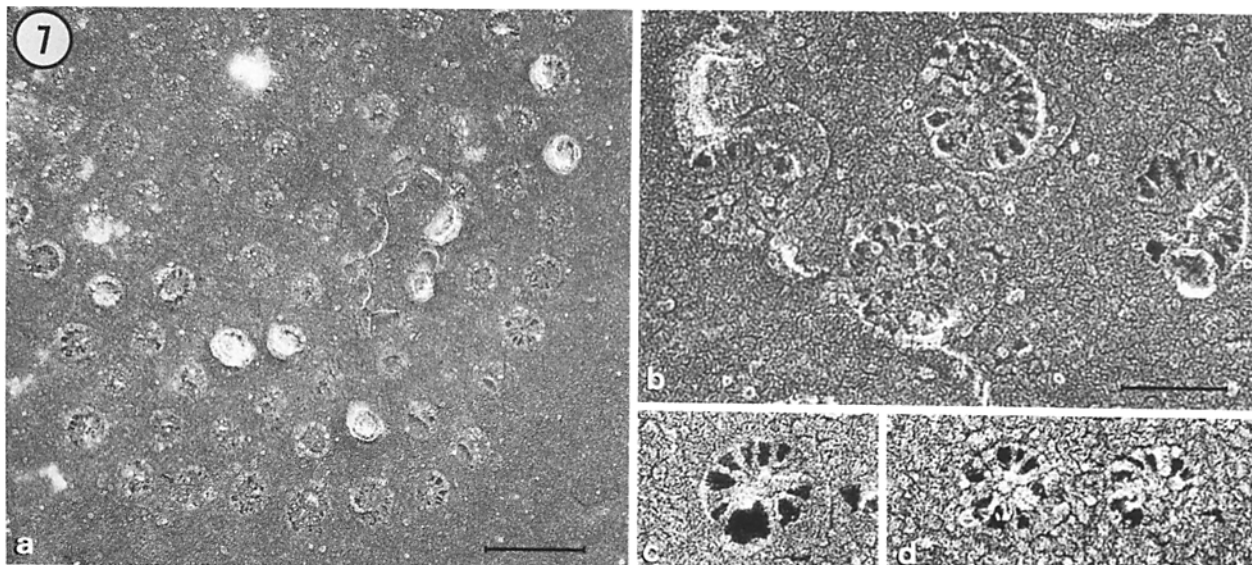
Figure 7. Structure of fenestral diaphragms in quick-frozen, deep-etched and rotary-shadowed preparations (1) of PMA-treated BMEC cultures. (*a*) Survey of a cluster of diaphragmed fenestrae. Bar, 0.2 μm. (*b–d*) Details of fenestral diaphragms composed of radial fibers interweaving in a central mesh. Bar, 0.1 μm.

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