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SPHINCTERS IN THE RAT PULMONARY VEINS. COMPARISON OF SCANNING ELECTRON AND TRANSMISSION ELECTRON MICROSCOPIC STUDIES**

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Abstract

The microvasculature of the rat lung was studied by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of vascular corrosion casts and tissue sections. Particular emphasis was placed on postcapillary venules, pulmonary venules and small pulmonary veins (small interlobular veins).

Casts of lung capillaries appeared inconspicuous with smooth surface. On the casts of pulmonary venules and small pulmonary veins, by contrast, series of narrow annular constrictions, present at regular distances of 20-25 μm, were seen. These constrictions may be drastic, narrowing down the caliber of the vessel up to 50 %. In the constrictions the marks of circularly running tubular structures were seen and were interpreted as being caused by circular bands of smooth muscle cells. Tissue sections of the corresponding vascular wall showed the presence of single or grouped smooth muscle cells which regularly formed myoendothelial junctions. These smooth muscle cells are interpreted as sphincters, responsible for the constrictions seen on cast preparations. Axon terminals were not found in spatial relationship to these sphincters. It is suggested that the described venous sphincters are governed by blood-borne and/or endothelium-derived substances and may significantly influence the blood flow.

Key Words: Venous sphincter, lung, microvascular corrosion casts, scanning electron microscope, transmission electron microscope, Mercox, smooth muscle cell, endothelial cell, rat.

**This work is dedicated to Professor Dr. Ernst WOLNER on the occasion of his 50th birthday.

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Introduction

Microvascular corrosion casting enables us to study the three-dimensional arrangement of the vascular bed. Further, it precisely shows the branching order of vessels and the spatial relationship between different vascular systems. This together with the luminal replica, which even reveals the surface morphology of the overlying endothelial cell nuclei and their border regions, furnish us with a promising new technique.

Using this method Schraufnagel (1987), Schraufnagel and Schmid (1988a, b), and Schraufnagel and Patel (1990), observed striking constrictions on pulmonary venous vascular casts of the rat. They interpreted these constrictions as being caused by venous sphincters. In order to prove whether or not these devices are under neural control, the animals were given a sharp head blow. Due to this procedure the furrows were deepened, which prompted the authors to suggest a nervously controlled mechanism. Konerding *et al.*, (1988) observed, on the surface of capillaries and small postcapillary venules in the chicken sciatic nerve, sphincter-like constrictions, without putting special emphasis on their structural details.

In vascular corrosion casting, no information is available concerning the detailed wall structure, since soft tissue is digested away. The study of sphincter-like structures, no doubt, needs precise information upon morphology and distribution of contractile elements and assumed nerve terminals governing them. Therefore, the present investigation combines vascular corrosion casting and tissue sectioning to correlate the different morphological features of pulmonary venous sphincters as they are observed.

Material and Methods

Vascular corrosion casting

Ten adult Sprague Dawley rats (200-250 g body weight) of both sexes were used for corrosion casting. The animals were anaesthetized with ether and the thorax and abdomen were opened by a median cut. A plastic catheter (Argyle 0.8 x 19 mm, Sherwood Medical,

St. Louis, MO, USA) connected to a two-way connector (LS-2,B, Braun-Melsungen, Germany) was introduced into the inferior vena cava at the level of the renal veins and the pulmonary circulatory system was rinsed (using manual pressure) with at least 80 ml 42 °C warm heparinized Tyrode solution (5,000 IU/l) until the efflux of the cannulated aorta (at the level of renal veins) was clear.

Sixteen ml Mercox CL-2B (Dainippon Ink and Chemicals, Tokyo, Japan) were mixed with 4 ml monomeric methylmethacrylate (Hodde, 1981) and immediately injected through the inferior vena cava. The animals were left at room temperature for 2 hours and then placed into a 60 °C water bath for final polymerization of the resin overnight. After solidification of the resin, lungs were dissected out, macerated in a 15% potassium hydroxide solution at 40 °C for 2 days or longer. Pulmonary vascular casts were cleaned in 5% formic acid for 30 minutes, rinsed in distilled water and finally frozen in a small volume of the latter. Some of the specimens were cut into 1-2 mm thick slices, using a specially adapted circular saw at -20 °C. All specimens were freeze-dried, mounted onto copper foils and fixed to specimen stubs with conductive silver paste, according to the method of Lametschwandtner *et al.*, (1980). The specimens were evaporated with carbon and gold for 3 seconds, then sputtered with gold for 600 seconds (Aharinejad *et al.*, 1989, 1990) and examined with a Cambridge Stereoscan 90B SEM, using an acceleration voltage of 15 kV.

Transmission electron microscopy

Five Sprague Dawley rats of both sexes were killed by an intraperitoneal injection of pentobarbital (100 mg/kg body weight). After thoracotomy the lungs were removed and small tissue blocks were cut from the inferior lobes and fixed by immersion into 2.5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4, for 2 hours. Specimens were rinsed in phosphate buffer and postfixation was performed in 1% osmium tetroxide in Michaelis buffer, pH 7.2, for 2 hours. Dehydration was done in a series of graded ethanols and finally in propylene oxide. Embedding was performed in Epon 812. Semi-thin sections were stained with alkaline Toluidine blue 0; thin sections were double stained with methanolic uranyl acetate and alkaline lead citrate. The specimens were studied with a Zeiss EM9 electron microscope.

Results

In order to correlate the scanning electron (SEM) and transmission electron microscopy (SEM) findings, results from similar samples are presented on the same plates: Figures 1 to 3 show micrographs of pulmonary venules; observations from small pulmonary vein and postcapillary venule are illustrated in Figures 4-7.

Figure 1. Corrosion cast of a pulmonary venule of about 100 μm diameter. Two unbranched bands of surface constrictions are indicated by small arrows, two additional less conspicuous indentations are seen in between. Note imprints of endothelial cell nuclei on the surface of the venular cast (arrowheads), particularly in the upper half of this figure. Bar = 50 μm.

Figure 2. Transmission electron micrograph showing part of the wall of a pulmonary venule with endothelial lining (arrowhead) and a single smooth muscle cell (S) beneath. This smooth muscle cell is situated within a layer of elastic fibers (E). The cross-section profile of this smooth muscle cell corresponds in size to the cross-section of constriction bands seen on the venular cast in Fig. 1. Bar = 2 μm.

SEM observations

Venous vessels are identified as they are interspersed in the casted pulmonary capillaries without any closer spatial relationship to airways. The venous vascular tree begins with postcapillary venules (10 to 25 μm diameter) which merge to form pulmonary venules (25 to 200 μm diameter), the latter join small pulmonary veins (interlobular veins, 200 to 1,000 μm diameter). Into the surface of all these venous casts reliefs of endothelial cell nuclei are indented (Fig. 1) together with bands of circular constrictions. On the surface of smaller veins the constrictions occur solitarily (Fig. 1), whereas larger veins show groups of furrows (Fig. 3). The individual grooves are unbranched and according to their shape and dimensions they may well correlate to smooth muscle cells. The depth of constrictions varies: they may be just flat (Fig. 1) or more drastic, so reducing the diameter of the vessel to about half of its original luminal diameter. The entire course of constrictions cannot be observed easily unless they are exposed by slicing the corrosion casts with a saw. In this manner the frequency of constrictions is underestimated, they are only observed by chance if the sectioned area offers a favorable situation. Hence, venous casts, the smallest possible, were dissected free from the adhering vasculature, so to enable us to study the constrictions selectively (Fig. 6). By this method, however, only larger veins can be prepared, where numerous constrictions are also present. These flatter impressions are numerous, run circularly or spirally, giving the cast surface a zig-zag pattern. The dimension of these furrows once again correspond to the dimension of smooth muscle cells.

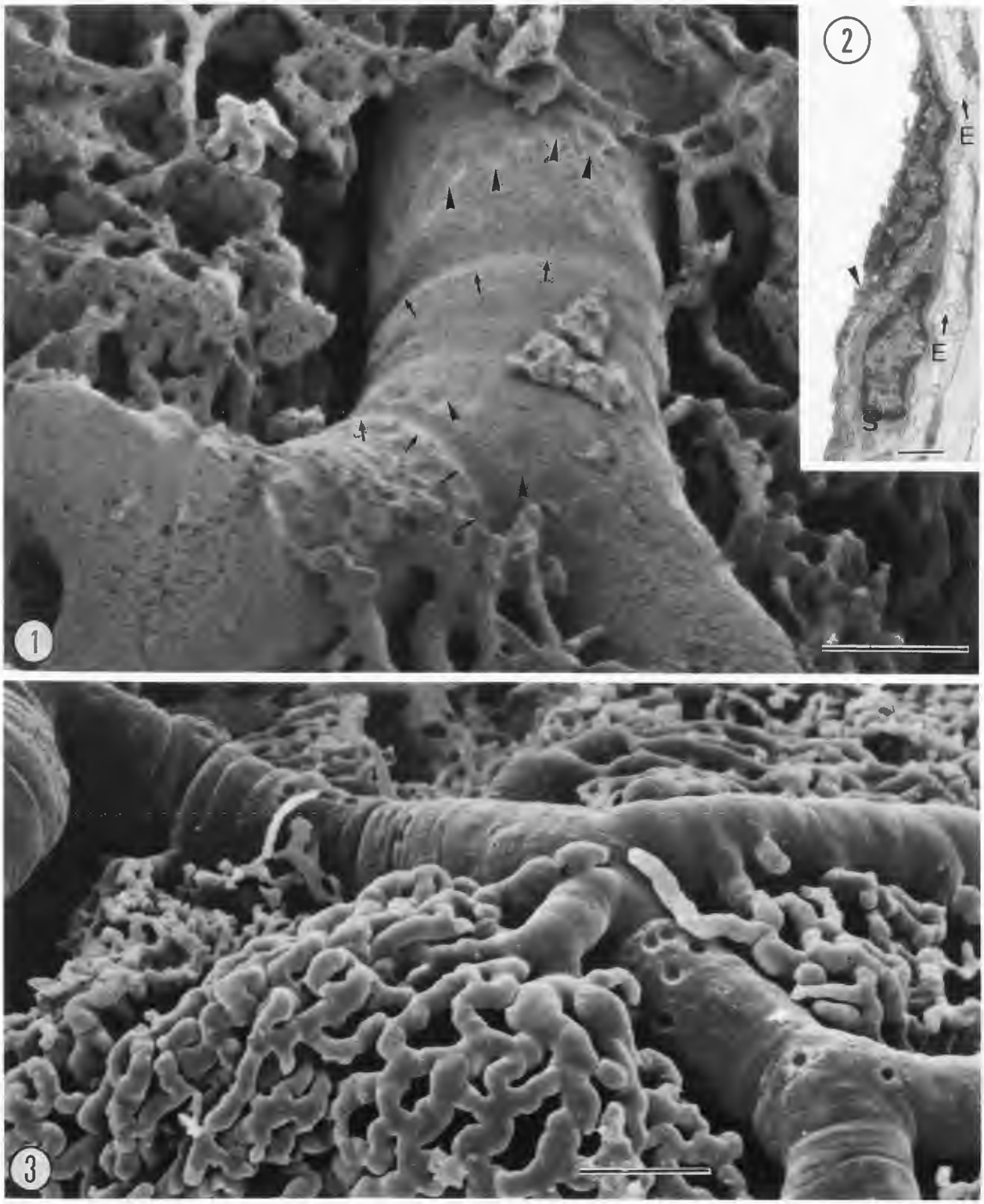

Fig. 3. Corrosion cast of pulmonary venules of about 100 μm diameter, which show multiple deep (left segment of the horizontally running venule and the venule at the top) and flat (the right segment of the horizontally running venule) constriction bands which might be caused by circularly running grouped smooth muscle cells (see also Figs. 6 and 7). Bar = 50 μm.

TEM observations

The wall of veins we were interested in, consisted mainly and over long distances of an endothelial lining, a basement membrane and an adhering connective tissue sheath. Numerous specific Weibel-Palade bodies are regularly seen in the endothelial cells; the connective tissue sheath is composed of collagen fibrils together with elastic material (Fig. 4). Sometimes pericytes occur, the cytoplasmic processes of which are interposed between endothelial cells and their basement membrane. The pericytes processes cause folds of the endothelial layer, leading it to protrude towards the venous lumen (Fig. 5). Occasionally single true smooth muscle cells are present even in the wall of postcapillary venules and pulmonary venules (Fig. 2). Figures 1 and 2 show corresponding situations: circular constrictions on the surface of a casted pulmonary venule (Fig. 1) and a solitary smooth muscle cell in a TEM section of a similarly sized venule (Fig. 2). These smooth muscle cells become more numerous and the amount of elastic material in the adventitional connective tissue sheath increases, as the venous vessels get larger in caliber. In small pulmonary veins (interlobular veins), the smooth muscle cells show a tendency to link together to form a muscular cuff composed of 5-10 circular smooth muscle cells (Fig. 7). Grouped circular constrictions on the cast of a small pulmonary venule are seen in Fig. 3 and grouped smooth muscle cells in the wall of a similarly sized vessel in the sectioned specimen are shown in Fig. 7. It should be stressed that these grouped smooth muscle cells are situated inside the elastic lamina of the venous wall (Fig. 7). Beginning and ending of muscular cuffs is abrupt. The venous wall, in its course before and after the cuffs, merely consists of endothelium and adventitial connective tissue.

No vegetative nerve terminals were found in the wall or beneath the wall of pulmonary venules or small pulmonary veins of the rat. In particular, no vegetative nerves were seen in spatial relationship to single or grouped smooth muscle cells suspected as venous sphincters.

Discussion

For our studies we used the same casting protocol as suggested by Schraufnagel and Patel (1990). In analogy to these authors, we cannulated the inferior vena cava as the perfusion site and used pre-warmed Tyrode solution for lavage. The resulting casts were sufficient, i.e., the pulmonary vascular bed was completely filled, substantiated by the absence of blind ended vascular casts. Arteries, capillary networks and the venous segments were found as described (Ohtani, 1980; Schraufnagel and Patel, 1990; Schraufnagel and Schmid, 1988a, b). In addition, we regularly found constrictions on venous casts surface, which had the same appearance as described by Schraufnagel and Patel (1990) in their animals which received a sharp head blow during casting, although our animals had not received such a treatment. This observation confirms the published data by Ohtani (1980), where similar constrictions were illustrated, but not more comprehensively addressed. However, in the observations of Schraufnagel and Patel (1990) the constrictions were impressively deepened during their experimental procedure, other casts (animals without head blow) showed only insignificant grooves. This phenomenon was interpreted as a reply of nervously controlled contractile elements (sphincters) upon an appropriate stimulus. In our interpretation, by contrast, a sharp head blow is not obligely necessary to intensify the functioning of sphincters, it is rather an unphysiologic stimulus. Several other factors which may stimulate the contractile elements have to be considered. (1) Handling of the organs during surgical procedures; (2) Thermal stimuli (lavage fluid, room temperature, instruments); (3) Osmotic stimuli (saline?); (4) Monomeric methylmethacrylate, fixative; and (5) Perfusion pressure.

In addition, our tissue sections did not show any nerve terminals in sphincter regions or in close proximity, a finding which confirms the observations of Rhodin (1978). Most probably blood borne substances, such as catecholamines, serotonin or thromboxane, or local endothelium-derived substances, e.g., endothelin, endothelial derived relaxing factor, or prostaglandines, may govern the sphincters *in vivo*.

The advantage of combined TEM and SEM studies in analyzing venous sphincters is almost evident (Aharinejad *et al.*, 1990). A series of criteria should be considered as guide lines to correlate surface details on casts with their complimentary structures in tissue sections: (1) Correlation of dimensions; (2) Correlation of localization; (3) Correlation of shape; (4) Correlation of frequency; and (5) Combination of the above criteria.

Use of these criteria supports the mentioned hypothesis that constrictions in cast preparations correspond to or are even caused by single or grouped smooth muscle cells, although the involved stimuli are not completely understood as yet. The question arises as to how the grooves on cast preparations are caused. They may be due to an active contraction of contractile cells and/or the result of a passive resistance hold against luminal pressure (e.g., by contractile cells as well as connective tissue bundles). This question remains open when only cast preparations are studied. However, when combined TEM and SEM studies assure the corresponding presence of smooth muscle cells, it becomes plausible that the latter are responsible for the furrows seen in casts. Furthermore, if smooth muscle cells are identified, an active contraction mechanism has to be favored.

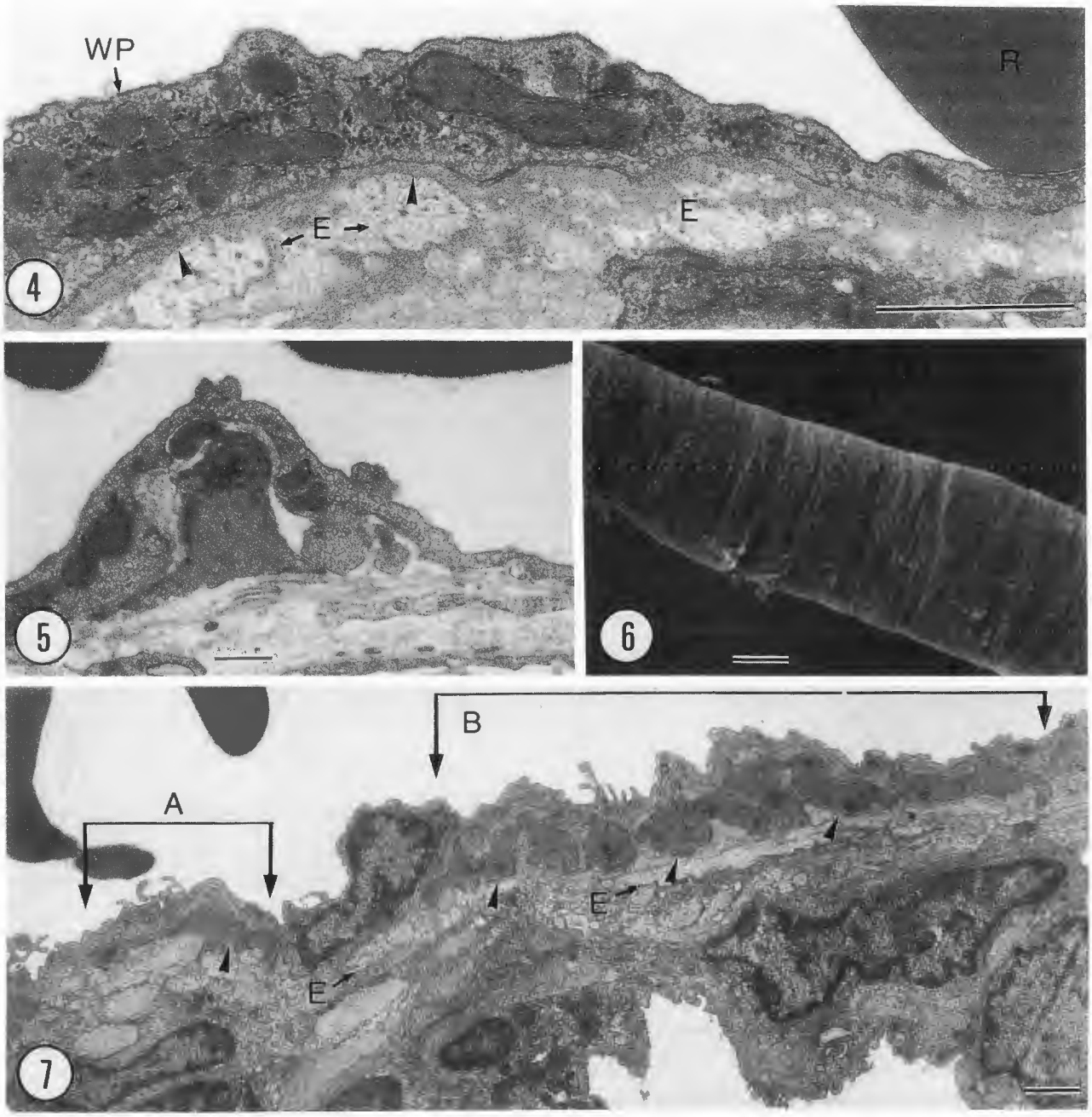

Figure 4. Transmission electron micrograph showing the wall of a small pulmonary vein. The endothelial layer rests upon a basal lamina (arrowheads) which in turn is placed upon a net of elastic fibers (E). Pericytes or smooth muscle cells are not seen. Endothelial cells regularly contain remarkable amounts of specific Weibel-Palade bodies (WP). "R" indicates a red blood cell. Bar = 1 μm.

Figure 5. Transmission electron micrograph of a pericyte process on the endothelial lining of a postcapillary venule, causing a fold to protrude into the lumen. Such a fold should appear as a furrow on the vascular cast. Bar = 1 μm.

Figure 6. Isolated cast of a small pulmonary vein of about 320 μm diameter. Multiple flat constriction bands cause a zig-zag pattern on the cast surface. Bar = 100 μm.

Figure 7. Single (bracket A) and grouped (bracket B) smooth muscle cells (arrowheads) beneath the endothelial layer of a small pulmonary vein. These smooth muscle cells are situated between endothelium and an elastic layer (E). The endothelial surface above them irregularly outlines the vascular lumen. Bar = 2 μm.

In this connection, pericytes in venous walls have also to be considered as probable candidates for causing the constrictions seen in cast preparations. Shallow constrictions on the surface of capillaries and postcapillary venules have been already described by Aharinejad *et al.*, (1990) in the exocrine pancreas of the rat and in the peri- and endoneural plexuses of the sciatic nerve in chickens (Konerding *et al.*, 1988). In the pulmonary microvascular bed, however, deeper constrictions were observed on pulmonary venules and small pulmonary veins also. These pulmonary sphincters on larger vessels therefore have to be clearly distinguished from those on vessels with small diameter. Their role has to be discussed in relation to active contraction and passive resistance, as dealt with above. In both cases the ramified shape of pericytes is difficult to be correlated to the unramified crests on the casts surface. If pericytes are considered as candidates to cause luminal impressions (Fig. 5), two possibilities could be supposed. First, the pericyte might not be ramified; second, it contains only a main bundle of filaments which either contracts actively or withstands luminal pressure passively. This would mean that the shape of pericytes in small pulmonary veins is unusual and deserves further investigation. Since the contractility of pericytes is generally not accepted, the occurrence of constrictions in vessels which are provided with pericytes would favor the hypothesis that pericytes hold a passive resistance against increased pressure. In our opinion smooth muscle cells are the most probable candidates for venous sphincters in the rat lung, particularly if the relative paucity of pericytes is considered.

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Discussion with Reviewers

M.A. Konerding: Why didn't you perfuse a fixative prior to the injection of the casting medium?

Authors: In this study the existence and precise architecture of venous sphincters were addressed. A fixative, e.g., glutaraldehyde, may cause pulmonary edema [Bachofen H, Ammann A, Wangensteen D, Weibel ER (1982) Perfusion fixation of lungs for structure-function analysis: credits and limitations. J. Appl. Physiol. **53**, 528-533]. On the other hand, the fixative may be an additional stimulus for contractile cells. Realizing these facts, we tried to study the morphology of venous sphincters, avoiding any other stimuli except for the probable one of the resin; since previous studies showed that methylmethacrylate can stimulate venous sphincters (Aharinejad *et al.*, 1990).

M.A. Konerding: Did the authors also find circular constrictions on the surface of casted bronchial veins?

Authors: Although pulmonary veins were the main subjects of our study, we did observe sphincters on the surface of bronchial veins. These constrictions were often of an impressive depth and predominantly localized on larger bronchial veins.

M.A. Konerding: You describe a narrowing of the luminal caliber of up to 50%. Does this already have hemodynamic consequences?

Authors: The role of venous sphincters as devices involved in the control of blood flow is generally accepted (Aharinejad *et al.*, 1990; Shraufnagel and Patel, 1990). In our opinion a reduction of the luminal caliber up to 50% certainly has hemodynamic influences; the precise mechanisms and effects, however, deserve further physiological experiments.