

Contractile Proteins in Pericytes.

I. Immunoperoxidase Localization of Tropomyosin

NANCY C. JOYCE, MARCY F. HAIRE, and GEORGE E. PALADE

Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT In these studies we have compared the relative amounts and isoforms of tropomyosin in capillary and postcapillary venule pericytes, endothelial cells, and vascular smooth muscle cells in four rat microvascular beds: heart, diaphragm, pancreas, and the intestinal mucosa. The results, obtained by *in situ* immunoperoxidase localization, indicate that (a) tropomyosin is present in capillary and postcapillary venule pericytes in relatively high concentration; (b) the tropomyosin content of pericytes appears to be somewhat lower than in vascular smooth muscle cells but higher than in endothelia and other vessel-associated cells; and (c) pericytes, unlike endothelia and other nonmuscle cells, contain detectable levels of tropomyosin immunologically related to the smooth muscle isoform. These results and our previous findings concerning the presence of a cyclic GMP-dependent protein kinase (Joyce, N., P. DeCamilli, and J. Boyles, 1984, *Microvasc. Res.* 28:206–219) in pericytes demonstrate that these cells contain significant amounts of at least two proteins important for contraction regulation. Taken together, the evidence suggests that pericytes are contractile elements related to vascular smooth muscle cells, possibly involved, as are the latter, in the regulation of blood flow through the microvasculature.

Pericytes are polymorphic cells closely associated with the walls of capillaries and postcapillary venules. They have a cell body which is often highly elongated, and multiple branching foot processes which partially encircle the vessel wall. Pericytes can be distinguished from other perivascular cells, such as adventitial fibroblasts, by their location within the basement membrane of the vessels, and by the close apposition of the tips of their processes to the underlying endothelium. Electron microscopic studies have identified pericytes in a number of tissues and organs (10, 12, 20, 36). They may, in fact, be common to all microvascular beds, although their relative frequency and distribution appear to vary from one microvascular bed to another (32, 33).

The function of pericytes is still unclear; however, morphologic evidence suggests that they may be contractile cells related to vascular smooth muscle. Numerous microfilaments form a continuous plate in the adluminal cytoplasm of their cell body and extend from it into the foot processes, where, in the more distal segments, they fill the cytoplasm to the relative exclusion of other subcellular components. In addition, the foot processes have densities that appear similar to the attachment plaques and dense bodies of smooth muscle cells (29, 32, 34). The presence of such structures within circumferentially oriented pericyte processes suggests a poten-

tial contractile function that may contribute to the regulation of blood flow in the microvasculature (12, 19, 24, 37).

Since the morphologic findings mentioned above are simply suggestive, biochemical evidence is needed to establish the presence of significant amounts of essential contractile and regulatory proteins in these cells as a necessary basis for a contractile function. Actin has already been identified in pericytes (12, 14, 21, 35), and, in the companion paper, we present evidence for the presence in these cells of myosin in relatively high concentration (18).

Pericytes and vascular smooth muscle cells are related by a similar topography in the wall of the vessel (1), and a biochemical relationship between the two cell types is suggested by the immunocytochemical localization in both cells of cyclic GMP-dependent protein kinase (17), an enzyme believed to function in the regulation of smooth muscle cell contractility (13, 28, 30), and of the intermediate filament proteins desmin and vimentin (9). To explore this relationship further and to provide evidence for the presence in pericytes of additional contraction-associated proteins, we extended the inquiry to tropomyosin, a protein considered essential for the regulation of cellular contractility. We took advantage of the fact that this highly conserved molecule exists in several isoforms, apparently distributed preferentially to specific cell types (7,

8, 11, 15, 16). Using two antitropomyosin antibodies with different isoform specificities, we showed that (a) pericytes of both capillaries and postcapillary venules contain relatively high levels of tropomyosin; (b) the tropomyosin concentration in pericytes appears to be somewhat lower than that in vascular smooth muscle cells but higher than in endothelia and other vessel-associated cells; and (c) pericytes, unlike endothelia and other nonmuscle cells, contain tropomyosin immunologically related to that of smooth muscle cells.

MATERIALS AND METHODS

Antibodies

Two affinity-purified IgGs were used in these studies. One, a rabbit anti-pigeon gizzard tropomyosin IgG, was a kind gift of Dr. Jan DeMey, Janssen Pharmaceutical Research Laboratories, Beerse, Belgium; the other, an anti-bovine brain tropomyosin IgG (raised also in rabbits) was obtained from Dr. Anthony Bretscher, Cornell University, Ithaca, NY. To test the isoform specificity of these antitropomyosins, extracts of rat brain and intestinal smooth muscle were prepared by incubation of the tissues for 30 min at 100°C in 2.5% sodium dodecyl sulfate, 2.5% 2-mercaptoethanol, 10 mM sodium phosphate, pH 7.4, as in reference 3. The extracts were then electrophoresed on 10% Laemmli polyacrylamide gels. The Coomassie Blue-staining pattern of each extract is shown in Fig. 1, lanes 1 and 2. The position of nonmuscle tropomyosin subunits (M_r 30,000) in the brain extract and of smooth muscle tropomyosin subunits (M_r 35,000) in the intestinal smooth muscle extract was determined by calculation of the molecular weight of the Coomassie Blue-stained bands relative to standards run on the same gel. The polypeptides were electrophoretically transferred to nitrocellulose (Schleicher & Schuell, Keene, NH), and individual nitrocellulose strips were incubated in 0.16 μ g/ml of nonimmune IgG, anti-brain or anti-gizzard tropomyosin IgG. The presence of bound IgG was detected by incubation of the strips in 125 I-Protein A (New England Nuclear, Boston, MA) and subsequent autoradiography. The results of this experiment are shown in Fig. 1, lanes 3–8. No binding was observed when the transferred extracts were incubated with nonimmune IgG (Fig. 1, lanes 3 and 4). Anti-brain tropomyosin bound both nonmuscle (Fig. 1, lane 5) and smooth muscle tropomyosin isoforms (Fig. 1, lane 6), indicating its lack of isoform specificity. Anti-gizzard tropomyosin recognized the smooth muscle tropomyosin isoform in the smooth muscle extract (Fig. 1, lane 8) and a low intensity band equal in electrophoretic mobility to smooth muscle tropomyosin in the brain extract (Fig. 1, lane 7). This band may represent a small amount of smooth muscle tropomyosin contributed by the brain vasculature. Nonmuscle tropomyosin was not recognized by this antibody, which suggests its specificity for the smooth muscle isoform. Further evidence for the smooth muscle specificity of anti-gizzard tropomyosin was obtained in immunoperoxidase localization studies. This IgG was unreactive with skeletal and cardiac muscle as well as nonmuscle cells.

Tissue Preparation

FIXATION: The vasculature of Sprague-Dawley rats (male retired breeders) was perfused with oxygenated Dulbecco's phosphate-buffered (pH 7.4) saline (PBS) (Gibco Laboratories, Grand Island, NY) at room temperature and the tissues were lightly fixed by perfusion for 10 min at room temperature with 4% formaldehyde in PBS (freshly prepared from paraformaldehyde). This step was followed by immersion of excised specimens in the same fixative for 20 min at room temperature. Small blocks cut from heart, diaphragm, intestine (mainly jejunum), and pancreas were rinsed in PBS and then incubated for 1 h on ice in 10% dimethylsulfoxide (in PBS) as a cryoprotecting agent, before the blocks were frozen in liquid nitrogen-cooled isopentane.

PROCESSING OF CRYOSTAT SECTIONS: Cryostat sections \sim 30 μ m thick were collected in 0.15 M glycine in half-strength PBS (270 mOsmol) and incubated in the same buffer for 30 min at room temperature to quench residual free aldehydes. The sections were then permeabilized by incubation for 1 h on ice in PBS supplemented with 0.3% Triton X-100, 0.015 M glycine (to provide additional quenching), and 0.1% bovine serum albumin (to prevent nonspecific protein adsorption). The same buffer was used throughout the experiments as an antibody diluent and washing medium. All incubation and washing steps were performed at 0–4°C in a rotating apparatus to improve access of reagents to the tissue and to minimize structural artifacts.

ANTIBODY INCUBATIONS: Tissue sections were incubated for 18–24 h in 8 μ g/ml of either anti-gizzard or anti-brain tropomyosin and then washed several times over 18 h. The sections were then incubated for 18–24 h in 12.5 μ g/ml horseradish peroxidase-conjugated sheep anti-rabbit IgG Fab (Pasteur

Institute, Paris) and washed as above. Control sections were quenched and permeabilized in exactly the same manner but were then handled in one of three ways: (a) Some were incubated in nonimmune IgG and then in secondary antibody; (b) others were incubated in secondary antibody alone; and (c) still others were reacted as described below with no prior antibody incubation as a control for endogenous peroxidase activity.

PEROXIDASE REACTION: All sections were preincubated on ice for at least 15 min in 0.5 mg/ml 3,3'-diaminobenzidine (DAB)¹ in 0.1 M sodium monobasic phosphate, titrated to pH 7.0 with ammonium hydroxide. Reaction was initiated by the addition of hydrogen peroxide to 0.01% final concentration and was terminated by transfer of individual tissue sections to PBS. Tissue was reacted for 15, 30, and 60 min; 30-min reaction times appeared to be optimal, giving the best signal-to-background ratio.

SUBSEQUENT PROCESSING: After a PBS wash, the tissue was postfixed for 1 h on ice in 1% osmium tetroxide in veronal-acetate buffer, pH 7.4, then dehydrated and embedded in Poly/Bed 812 Embedding Medium (Polysciences Inc., Warrington, PA). Sections, \sim 1 μ m thick, were cut for light microscopy on a Sorvall Porter-Blum MT2-B ultramicrotome (DuPont Instruments-Sorvall Biomedical Div., Wilmington, DE) and photographed with a Zeiss Photomicroscope II on Kodak Technical Pan film. Thin sections, stained with Reynold's lead citrate and uranyl acetate, were examined and micrographed on a Philips 301 electron microscope.

RESULTS

Control Experiments

In tissue reacted without prior antibody incubation, the presence of dark reaction product (oxidized and polymerized DAB), indicating endogenous peroxidase activity, was consistently seen only in (a) granulocytes, especially eosinophils, in the lamina propria of the intestine as in Fig. 2*b*; (b) a rare erythrocyte within capillary lumina; and (c) occasional mast cells associated with blood vessels in all the organs examined. In addition, light brown staining, apparently due to nonspecific adsorption of DAB, was seen in fat cells occasionally present within the muscle connective tissue and lipid droplets within muscle fibers (micrographs not shown). A discontinuous light-staining band was also evident in the subendothelial layer of large arterioles and small veins (Fig. 2*c*). The stained areas correspond to the position of the internal elastica, which is discontinuous in these vessels. We observed no additional staining in tissues incubated with secondary antibody alone or in heart, diaphragm, and intestine incubated with nonimmune IgG (Fig. 2, *a-c*). Nonspecific staining was observed in pancreas incubated with nonimmune IgG on the apical plasma membrane of the acinar cells and on the luminal aspect of the ducts. Centroacinar cells were also stained. We never observed, however, nonspecific staining on pericytes, endothelial cells, vascular smooth muscle cells, or fibroblasts in this tissue. The companion paper (18) illustrates additional controls and nonspecific staining in the pancreas (Fig. 1*c*).

Antibody Penetration

In preliminary experiments, antibody penetration into the muscularis and mucosa of the intestine and into vascular smooth muscle cells was tested under a variety of experimental conditions. Antibody penetration deep within the muscularis and into the smooth muscle cells surrounding small arteries and small veins was consistently poor. Dense reaction product was usually visible only within the outermost cell layers, which suggests that significant antibody penetration beyond the first cell layer does not occur (micrographs not shown). Preincubating the sections in glycerol and extending the permeabilization step, the antibody incubation, or the

¹ Abbreviation used in this paper: DAB, 3,3'-diaminobenzidine.

FIGURE 1 Equal amounts of protein (75 μ g) from rat brain (lanes 1, 3, 5, and 7) and isolated intestinal smooth muscle extracts (lanes 2, 4, 6, and 8) were electrophoresed on 10% Laemmli polyacrylamide gels. Lanes 1 and 2 illustrate the Coomassie Blue-staining patterns obtained. After electrophoretic transfer of the separated polypeptides to nitrocellulose, individual nitrocellulose strips were incubated in nonimmune IgG (lanes 3 and 4), anti-brain tropomyosin (lanes 5 and 6), or anti-gizzard tropomyosin (lanes 7 and 8) and then incubated in 125 I-Protein A. Autoradiographic results show no binding of nonimmune IgG to peptides of either extract (lanes 3 and 4). Anti-brain tropomyosin recognized both nonmuscle tropomyosin (lane 5) and smooth muscle tropomyosin (lane 6). Anti-gizzard tropomyosin bound smooth muscle tropomyosin in the smooth muscle extract (lane 8) and recognized a peptide, present in apparently low concentrations, in the brain extract that corresponded in electrophoretic mobility to that of smooth muscle tropomyosin (lane 7). This antibody did not recognize nonmuscle tropomyosin in the brain extract. Small arrowhead, position of smooth muscle tropomyosin subunits (M_r 35,000); large arrowhead, position of nonmuscle tropomyosin subunits (M_r 30,000). *B*, brain; *I*, intestine; *NI*, nonimmune IgG; *a-B*, anti-brain tropomyosin; *a-G*, anti-gizzard tropomyosin; *TM*, tropomyosin.

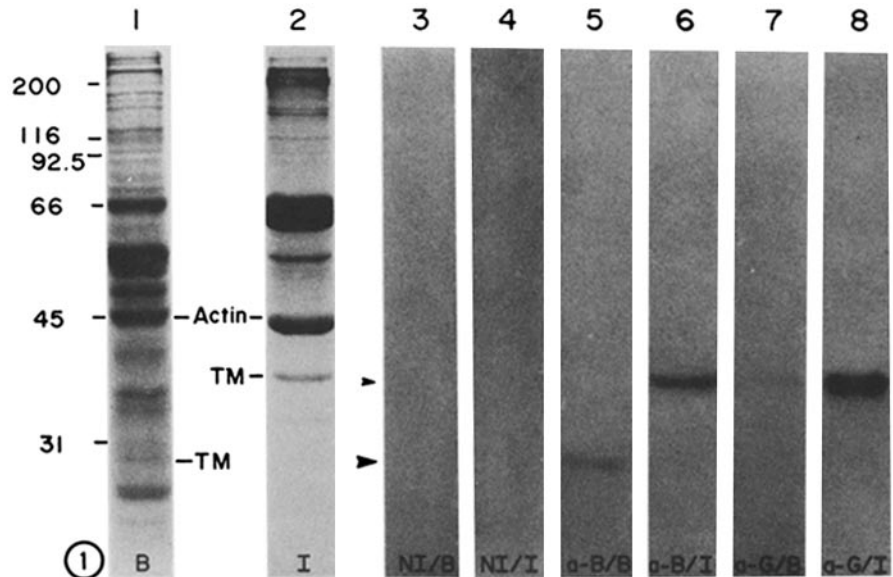
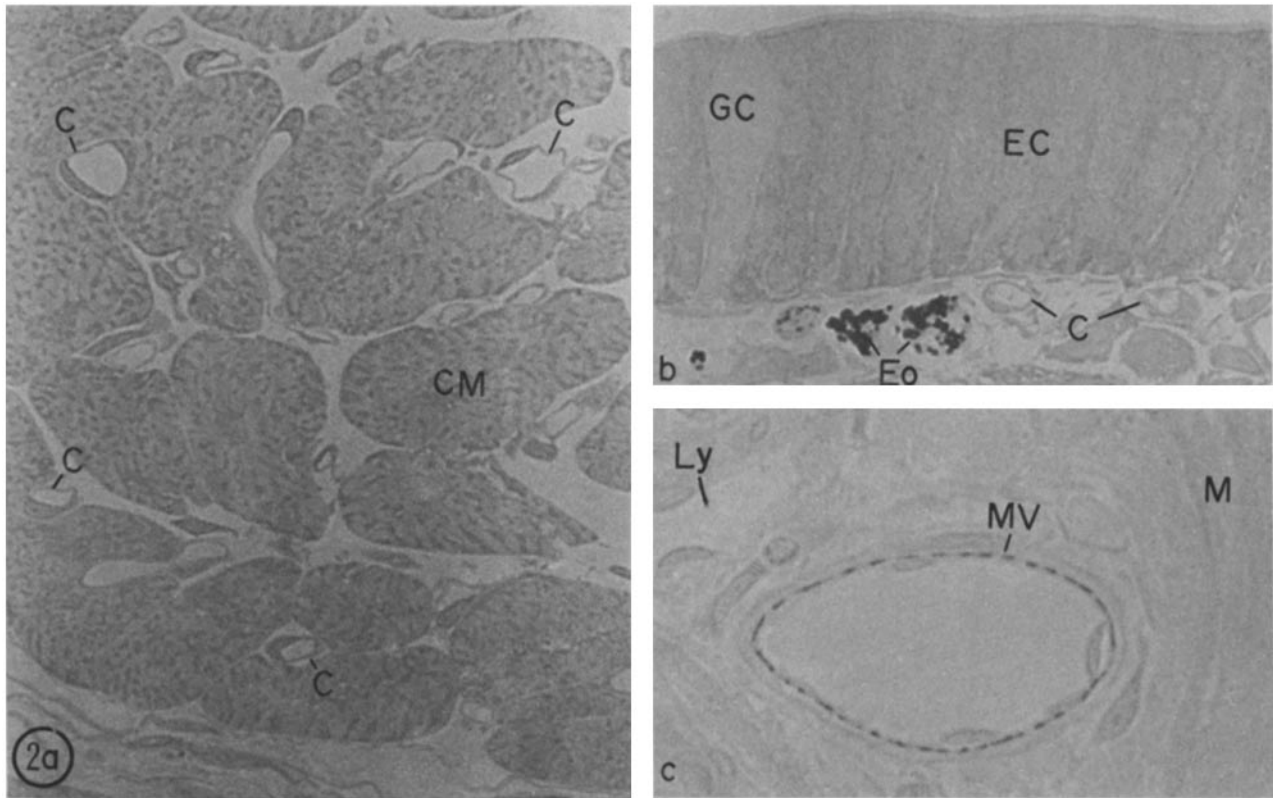


FIGURE 2 Sections of rat heart (a) and intestine (b and c) were incubated in secondary antibody alone and reacted with DAB and hydrogen peroxide. The lack of reactivity observed in the cardiac muscle tissue (a) is typical for all the organs studied under control conditions. No staining is visible except that due to endogenous peroxidase activity, illustrated by the eosinophils (*Eo*) in the intestinal lamina propria (b), or apparent nonspecific DAB adsorption, such as that seen in the periodic staining of the discontinuous elastica in the muscular venule (*MV*) within the intestinal submucosa (c). *C*, capillary; *CM*, cardiac muscle cell; *GC*, goblet cell; *EC*, epithelial cell; *Ly*, lymphatic vessel; *M*, muscularis. \times 1,000.



DAB reaction beyond the times given in Materials and Methods led to no significant improvement.

In contrast, positive reaction product was consistently present in the less tightly packed smooth muscle cells that composed the muscularis mucosae and the smooth muscle cells of arterioles and muscular venules. Apparently, antibody penetrated into these cells more effectively because it had access from all directions. A high reaction level in such cells thus became the criterion for effective antibody penetration in subsequent experiments.

Immunoperoxidase Localization of Tropomyosin in Capillary and Postcapillary Venule Pericytes and Vascular Smooth Muscle Cells

Pericytes of capillaries and postcapillary venules and vascular smooth muscle cells were consistently positive for tropomyosin in all the tissues examined regardless of the antitropomyosin used. By light microscopy, diffuse DAB reaction product was visible in pericytes and the staining patterns were

very similar to those previously described (17). Because pericytes, especially those on capillaries, are highly elongated cells (17, 24, 37), the staining pattern obtained depended on where along its length the cell was sectioned. For example, when a pericyte was sectioned through the cell body, as in Fig. 3, *a*, *b*, *d*, *f*-*i* and in Fig. 4, *a* and *c*, diffuse reaction product was visible in the perinuclear cytoplasm and proximal portions of cell processes but the nucleus was unstained. When it was sectioned through more distal segments of its processes, a positive reaction appeared as discrete "spots" on the outer aspect of the vessel wall, as illustrated in Figs. 3, *c* and *e* and 4*b*. The galleries of capillaries in Fig. 3 and postcapillary venules in Fig. 4 illustrate the similarity of positive tropomyosin staining patterns in pericytes on the two types of vessels. The positivity of pericytes was most clearly seen in specimens in which they had become separated (probably artifactually, during tissue preparation) from the underlying endothelium. Such a cell is illustrated in Fig. 3*b*, where a positive pericyte is contrasted against the negative endothelium. For comparison, a capillary with an accompanying pericyte from a control

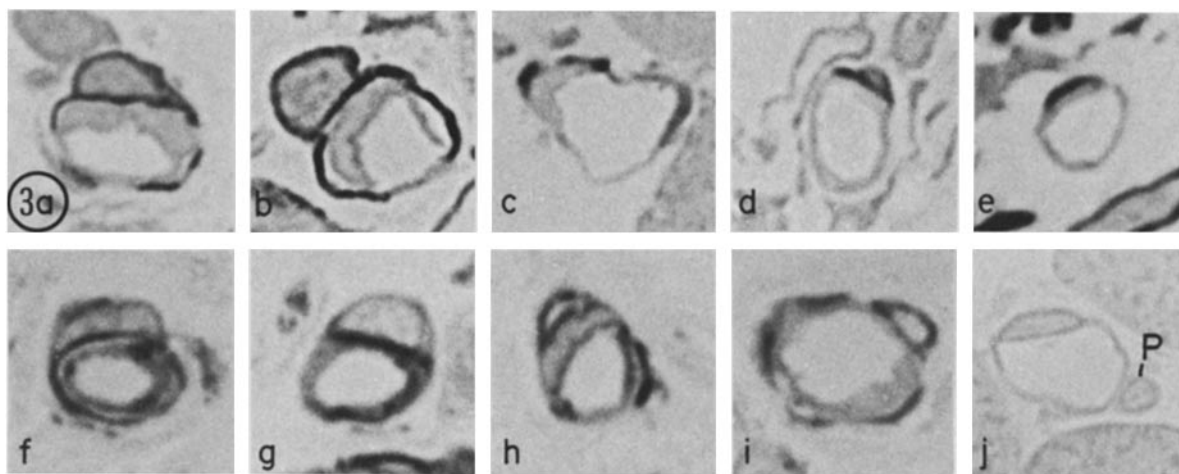


FIGURE 3 This gallery of capillaries from tissue sections of rat intestine (*a*, *b*, *d*-*i*) and heart (*c* and *j*) incubated in anti-gizzard tropomyosin (*a*-*e*), anti-brain tropomyosin (*f*-*i*) or nonimmune IgG (*j*) illustrates a variety of pericyte and endothelial cell staining patterns. Pericytes are positive and the overall staining patterns are similar regardless of the antitropomyosin IgG used. In sections incubated with anti-gizzard tropomyosin, no staining is evident in the endothelium, whereas discrete "spots" of positivity can often be seen in endothelia when sections are incubated with non-isoform specific anti-brain tropomyosin (*f*-*h*). Regions of positivity are apparently spaced far apart so the endothelium does not appear positive in all sections (*i*). The unstained capillary with an accompanying pericyte (*P*) in *j*, which was incubated in nonimmune IgG, is included for comparison. (*a*, *b*, and *g*) \times 2,200; (*c*) \times 2,400; (*d*, *e*, *h*, and *i*) \times 2,600; (*f*) \times 2,300; (*j*) \times 1,800.

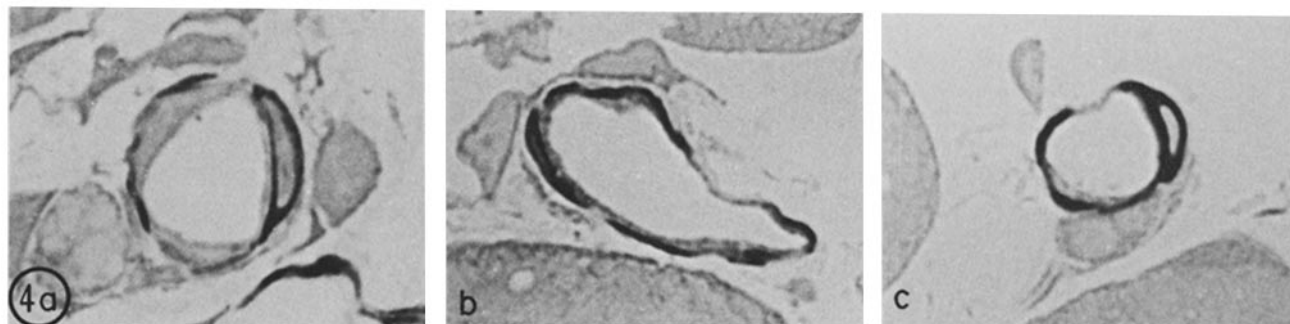


FIGURE 4 A selection of postcapillary venules from intestine (*a*) and diaphragm (*b* and *c*) illustrates positive pericyte staining patterns obtained in sections incubated in anti-gizzard tropomyosin. As with capillary pericytes, postcapillary venule pericytes contain diffuse DAB reaction product distributed throughout the perinuclear cytoplasm (*a* and *c*) and cell processes (*a*-*c*). Endothelial cells in these vessels are consistently negative with this smooth muscle-specific antibody. Pericytic venules were identified by two criteria: vessel diameter and extent of pericytic coverage. \times 1,600.

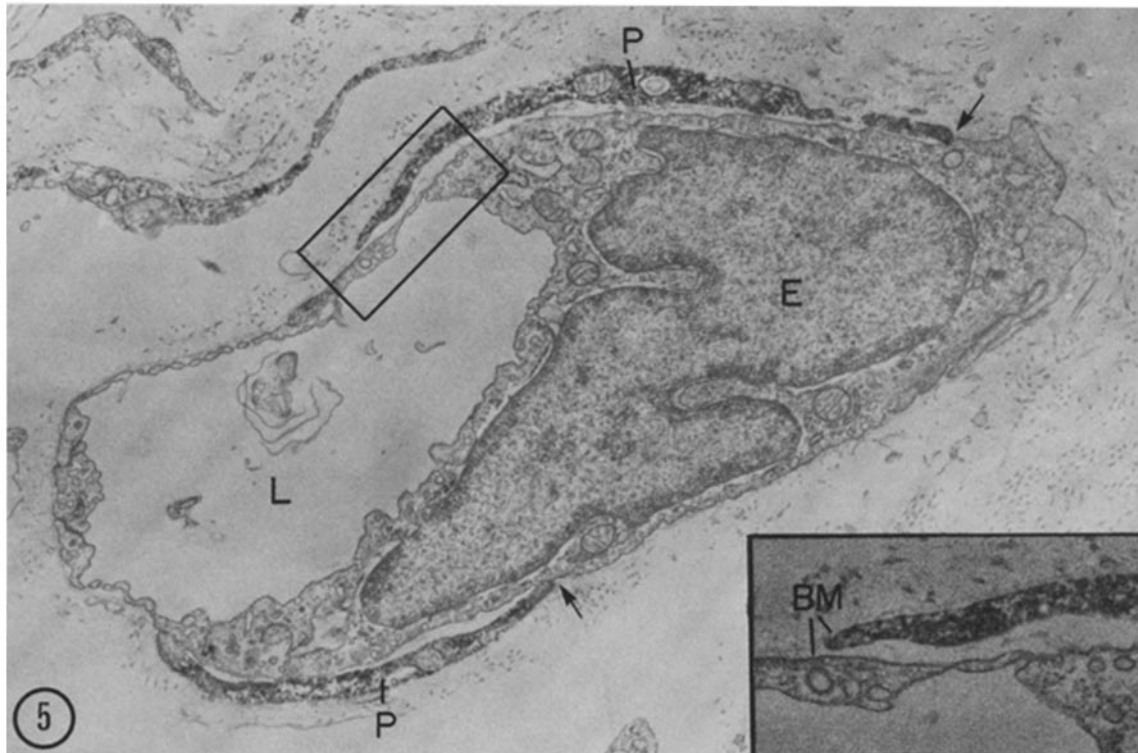


FIGURE 5 This electron micrograph from a section of intestine incubated with anti-gizzard tropomyosin shows a capillary with associated pericyte foot processes (P). Diffuse DAB reaction product is clearly visible in the cytoplasm of the processes, whereas the endothelial cell (E) appears unstained. The processes are identified as those belonging to a pericyte by two criteria: the close apposition of tips of the foot processes to the underlying endothelium, seen at the arrows; and enclosure of the processes within vessel basement membrane leaflets (BM). Staining of the basement membrane is poor in this micrograph and the inset has been included to show the leaflets at higher contrast. L, lumen. $\times 13,000$ (Inset) $\times 28,500$.

specimen is included (Fig. 3j). In this case neither the pericyte nor the endothelial cell is stained. The staining patterns of vascular smooth muscle cells can be seen on the larger vessels of the mucosa and submucosa of the intestine in Fig. 6, c and d.

By electron microscopy, reaction product was found diffusely distributed within the cytoplasm of both the perinuclear region and the processes of pericytes. Fig. 5 illustrates the immunoperoxidase localization of tropomyosin within the processes of an intestinal capillary pericyte. In this micrograph, the basement membrane that encloses the pericyte processes is poorly visualized. An inset showing a segment of the vessel in higher contrast is included to permit the membrane leaflets to be seen more easily.

The relative staining intensities of the immunoperoxidase reaction provide a semiquantitative estimate of the amount of antigen present in different cell types. For instance, pericyte staining on both capillaries and postcapillary venules is similar, which suggests that the amount of tropomyosin present in these cells is comparable regardless of their location. Pericytes appear to be somewhat less intensely stained than the smooth muscle cells of arterioles and muscular venules. This difference becomes more evident when tissues are reacted with DAB and hydrogen peroxide for different periods. Staining is quite intense in vascular smooth muscle cells after 10 to 15 min, whereas a similar intensity is not consistently seen in pericytes until after 30 min of reaction time. This difference may reflect a lower tropomyosin concentration in pericytes, smaller volumes (especially in foot processes), or a somewhat different reactivity of the polyclonal antibody for smooth muscle and pericyte tropomyosins.

Differences in Tropomyosin Localization by Anti-Gizzard or Anti-Brain Tropomyosin

Besides pericytes and vascular smooth muscle cells, there are other cells in which tropomyosin was detected by the immunoperoxidase technique. The cell types containing positive reaction product differed, however, depending on the antitropomyosin antibody used. The small intestine contained the largest number of differentially reactive cells. Fig. 6, a-d illustrates the staining patterns obtained with either anti-gizzard (Fig. 6, a and c) or anti-brain tropomyosin (Fig. 6, b and d). Within the wall of the small intestine, several cell types reacted positively with both antibodies. This was the case for the cells that compose the muscularis mucosae, and the smooth muscle cells that extend from the latter into the villus, parallel to the lacteal. Cells identified as forming the pericryptal (26) and subepithelial fibroblastic sheaths (23) were positively stained by both antibodies to approximately the same extent as pericytes but to a somewhat lesser extent than smooth muscle cells. In addition, a stellate cell located within the lamina propria of the villus stained positively. This cell has a large, oval nucleus, scanty cytoplasm, and several thin processes that extend from the cell body. Reaction product was present within its thin perinuclear cytoplasm and individual foot processes. Although this cell was stained with both antitropomyosins, it appeared to stain more intensely with the gizzard tropomyosin antibody, but to a lesser extent than pericytes.

The most striking difference in reactivity was seen in intestinal epithelial cells of the villus, the less differentiated cells of the crypts of Lieberkuhn and in endothelia. No reactivity

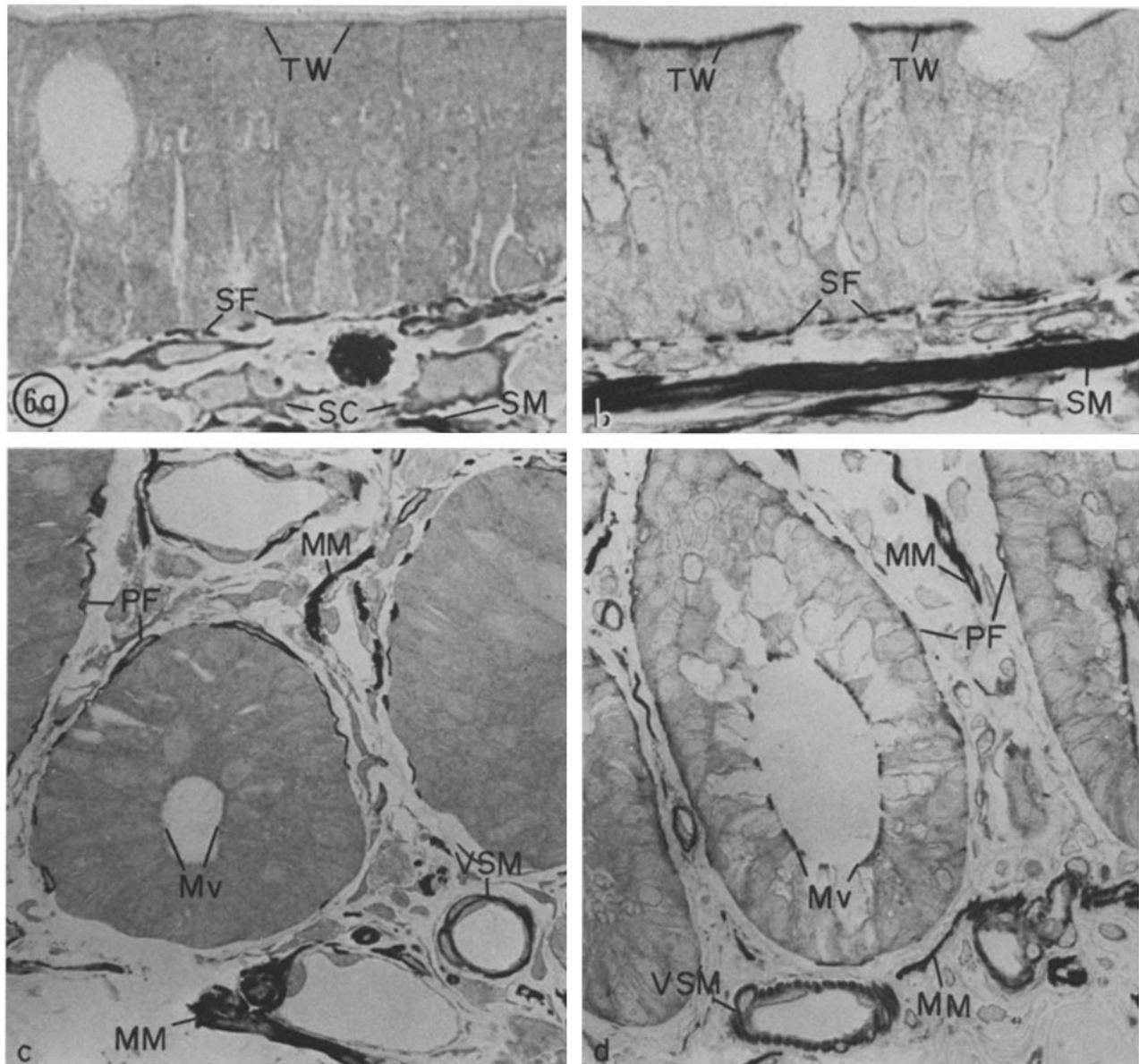


FIGURE 6 Lower magnification micrographs of sections from rat intestinal villi (a and b) and mucosal/submucosal regions (c and d), incubated in either anti-gizzard (a and c) or anti-brain tropomyosin (b and d), illustrate some differences in positive staining patterns obtained with the two antibodies. Anti-gizzard tropomyosin stains only smooth muscle and smooth muscle-like cells. Within the villus, these include the smooth muscle cells (SM), that extend from the muscularis mucosae, subepithelial fibroblasts (SF), and stellate cells (SC). In the mucosa and submucosa, this antibody stains the muscularis mucosae (MM), vascular smooth muscle cells (VSM), and pericryptal fibroblasts (PF). Anti-brain tropomyosin stains all the cells mentioned above and also portions of nonmuscle cells such as the terminal web (TW) of intestinal epithelial cells and the microvilli (Mv) of crypt cells. Endothelia and connective tissue fibroblasts are also stained by anti-brain tropomyosin but these cells, as well as pericytes, are not easily distinguishable at this magnification. (a) $\times 1,400$; (b) $\times 1100$; (c and d) $\times 700$.

was detectable in these cells when anti-gizzard tropomyosin was used. However, when tissue was incubated in anti-brain tropomyosin, the terminal web of the intestinal epithelial cells was positive, as were the microvilli of the cells forming the crypts (compare Fig. 6, a with b and c with d). The endothelial cells of capillaries also reacted positively with this antibody; the reaction product was unevenly distributed and appeared in small darkened areas within the endothelial cytoplasm (compare the negative endothelium in Fig. 3, a-e with the darkened regions in the endothelium of Fig. 3, f-h). The small areas of positivity are spaced (apparently) far apart, so that in some vascular profiles (as in Fig. 3i) the endothelium appears

unstained.

Connective tissue fibroblasts, adventitial fibroblasts, and skeletal and cardiac muscle cells all appeared unreactive when tissue was incubated in anti-gizzard tropomyosin. Fibroblasts were stained to a lesser extent than endothelia or stellate cells when sections were incubated in anti-brain tropomyosin. In skeletal muscle cells incubated with this antibody, positive reaction product was periodically distributed and appeared to correspond to the I-band regions of the sarcomere, a localization reported previously (27). In pancreatic acinar cells, no specific reactivity was observed with either antibody (micrographs not shown).

DISCUSSION

The resolution and signal intensity provided by the immunoperoxidase procedure applied in this study allows a clear distinction between adjacent positive and negative cells, and an estimate of the relative intensity of the reaction, presumed to reflect cell-to-cell variations in antigen concentration. On both accounts, the results are an improvement over immunofluorescence procedures. We attribute the gain to the amplification of the signal by the peroxidase tag and to the use of thick sections ($\sim 1 \mu\text{m}$) of plastic-embedded tissues. At the light microscope level the procedure appears to average the signal within each cell: It gives a relatively homogeneous silhouette and thereby facilitates the recognition of positive cells. At the electron-microscopic level, however, the same procedure does not provide additional information about antigen localization beyond the whole cell level. Structural preservation is unsatisfactory and the diffusion of DAB reaction product together with its preferential partition in membranes precludes a reliable localization of tropomyosins. These proteins are known to be closely associated with actin filaments in other cells, but better controlled localization procedures must be used for both antigens to confirm this association in pericytes.

Previous studies (17) have shown that the shapes of pericytes differ greatly depending on their location. On capillaries they are highly elongated with long, slender processes, whereas on postcapillary venules they are less extended and have thicker, more radial processes. Because of their polymorphism, it is important to determine whether all pericytes have similar biochemical features, regardless of their shape and location, or whether there are biochemical differences which may correspond to functional differences, connected to differences in location. The results obtained in these studies indicate that, in the organs examined, pericytes on both types of vessels are positive for tropomyosin and that antigen concentration in all pericytes throughout the microvasculature is comparable, to the extent quantitative estimates are possible.

Pericytes and vascular smooth muscle cells were compared in these studies, since morphological and biochemical findings suggest that the two cell types are related. In fact, gradual shape changes have been observed in the transition from vascular smooth muscle cells to pericytes at both the arteriolar and venular end of the microvascular bed (17, 19, 37), suggesting that there is no clear demarcation between these cell types within the microvasculature. In all the organs examined, the vascular smooth muscle cells of arterioles and muscular venules were well penetrated by antibody and highly reactive for tropomyosin. The results suggest that although both types of cells are positive for tropomyosin, pericytes may have somewhat less tropomyosin than do vascular smooth muscle cells. This interpretation is based, however, on the relative staining intensity in any single cross-sectional area of tissue. As mentioned previously, the staining patterns of pericytes differ depending on where the cell is sectioned. On any given tissue section, only a small fraction of the total volume of a pericyte may be examined, whereas a much greater part of an individual vascular smooth muscle cell may be visible because of characteristic differences in cell shape. Thus, what appears to be a quantitative difference in tropomyosin concentration in the two cell types may simply reflect a quantitative difference in total cross-sectional area and volume of the cell examined.

The two antitropomyosins used in these studies were useful tools to probe further the relationship between pericytes and vascular smooth muscle cells. In these studies anti-pigeon gizzard tropomyosin IgG recognized only smooth muscle tropomyosin in both the immunoblots of rat tissue extracts and immunocytochemical localization experiments on different rat tissues. The fact that pericytes were positively stained with this antibody provides evidence that these cells contain tropomyosin antigenically similar to that of smooth muscle cells. Smooth muscle tropomyosin was also detected by the cognate antibody in the cells of the subepithelial and pericyptal fibroblast sheaths and in the stellate cells of the lamina propria within the intestinal villi, but the intensity of the reaction was less or considerably less than in pericytes.

Anti-bovine brain tropomyosin IgG recognized several tropomyosin isoforms and was therefore non-isoform specific. This antibody was a useful control, however, since it reacted with tropomyosin from nonmuscle as well as muscle cells, increasing the degree of confidence in the results obtained with the smooth muscle-specific anti-pigeon gizzard tropomyosin IgG. Results obtained with anti-brain tropomyosin indicate a wider distribution of tropomyosin (presumably of the nonmuscle type) in the organs examined than was suggested by the gizzard antibody. Due to the lack of a nonmuscle tropomyosin-specific antibody, we could not determine whether pericytes also have nonmuscle tropomyosin. Pericytes appear, therefore, more closely related to smooth muscle than to other cell types, with respect to both the type and concentration of the isotropomyosin they contain. These parameters define them as contractile elements differentiated above the general level of contractility of nonmuscle cells.

Although tropomyosin is a highly conserved protein, its isoforms vary in certain physical, biochemical, and functional characteristics. The isotropomyosins of skeletal, cardiac, and smooth muscle cells are similar in that they form paracrystals in the presence of divalent cations with an axial repeat of ~ 41 nm and in vitro bind with similar actin/tropomyosin ratios. They differ slightly from each other in subunit M_r (average M_r 35,000), pI (4.5–5.0), and amino acid composition. In contrast, tropomyosin forms detected in differentiated nonmuscle cells have a paracrystal axial repeat of ~ 34 nm and an average subunit M_r of $\sim 30,000$. Amino acid sequence analysis of equine platelet tropomyosin indicates a high degree of homology with muscle tropomyosins except at the amino and carboxyl termini, where the sequences differ significantly from those of muscle tropomyosins (22). The sequence differences in these two regions of the molecular appear to correlate with certain characteristics of nonmuscle tropomyosin such as a reduced ability for head-to-tail association (5), a weaker binding to F-actin (6), and decreased affinity for muscle troponin (4).

The mechanism by which tropomyosin helps regulate actomyosin ATPase activity is poorly understood in cells other than those of striated muscle. In the latter, tropomyosin, together with the troponin complex, inhibits Mg^{2+} -stimulated actomyosin ATPase activity in the absence of calcium. This inhibition is released upon binding of calcium to the Tn-C component of the troponin complex. Experimental evidence so far obtained suggests that tropomyosin in smooth muscle (2) and nonmuscle cells (25, 31) enhances actin-activated, Mg^{2+} -stimulated ATPase activity and that this enhancement is independent of calcium concentration.

Although both smooth muscle and nonmuscle tropomyo-

sins appear to function similarly, a number of physical and biochemical differences distinguish the two. The fact that pericytes have an isotropomyosin immunologically related to that of smooth muscle cells suggests that at least one pericyte isotropomyosin may have the physical and biochemical characteristics of smooth muscle cells. These results demonstrate that pericytes have tropomyosin in significant amounts and strongly suggest that they are indeed potentially contractile cells which, like vascular smooth muscle cells, may function in blood flow regulation in the microvasculature.

We thank Drs. Jan DeMey and Anthony Bretscher for their gifts of antitropomyosin IgG and Cindy Davis for her efficient preparation of the manuscript.

This work was supported by National Institutes of Health grant HL17080 to Dr. Palade and by Research Service Award S-T32-GM07223-09 to Dr. Joyce.

Received for publication 18 July 1984, and in revised form 22 January 1985.

REFERENCES

- Bruns, R. R., and G. E. Palade. 1968. Studies on blood capillaries. I. General organization of blood capillaries in muscle. *J. Cell Biol.* 37:244-276.
- Chacko, S. 1981. Effects of phosphorylation, calcium ion, and tropomyosin on actin-activated adenosine 5'-triphosphatase activity of mammalian smooth muscle myosin. *Biochemistry.* 20:702-707.
- Cohen, D. M., and R. A. Murphy. 1978. Differences in cellular contractile protein contents among porcine smooth muscles. *J. Gen. Physiol.* 72:369-380.
- Cote, G. P., W. G. Lewis, M. D. Pato, and L. B. Smillie. 1978. Platelet tropomyosin: lack of binding to skeletal muscle troponin and correlation with sequence. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 94:131-135.
- Cote, G., W. G. Lewis, and L. B. Smillie. 1978. Non-polymerizability of platelet tropomyosin and its NH₂- and COOH-terminal sequences. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 91:237-241.
- Cote, G. P., and L. B. Smillie. 1981. The interaction of equine platelet tropomyosin with skeletal muscle actin. *J. Biol. Chem.* 256:7257-7261.
- Cummins, P., and S. V. Perry. 1973. The subunits and biological activity of polymorphic forms of tropomyosin. *Biochem. J.* 133:765-777.
- Cummins, P., and S. V. Perry. 1974. Chemical and immunochemical characteristics of tropomyosins from striated and smooth muscle. *Biochem. J.* 141:43-49.
- D'Amore, P. A., D. Edelman, D. Stark, and D. M. Form. 1983. Tissue culture and characterization of microvascular pericytes. *J. Cell Biol.* 97:334a. (Abstr.)
- Farquhar, M. G., and J. F. Hartman. 1956. Electron microscopy of cerebral capillaries. *Anat. Rec.* 124:288-289.
- Fine, R. E., and A. L. Blitz. 1975. A chemical comparison of tropomyosins from muscle and non-muscle tissues. *J. Mol. Biol.* 95:447-454.
- Forbes, M. S., M. L. Rennels, and E. Nelson. 1977. Ultrastructure of pericytes in mouse heart. *Am. J. Anat.* 149:47-70.
- Gruetter, C. A., P. Y. Gruetter, J. E. Lyon, P. J. Kadowitz, and L. J. Ignarro. 1981. Relationship between cyclic guanosine 3':5'-monophosphate formation and relaxation of coronary arterial smooth muscle by glyceryl trinitrate, nitroprusside, nitrite and nitric oxide: effects of methylene blue and methemoglobin. *J. Pharmacol. Exp. Ther.* 219:181-186.
- Herman, I. M., and P. A. D'Amore. 1983. Discrimination of vascular endothelium, pericytes and smooth muscle with affinity-fractionated antiactin IgGs. *J. Cell Biol.* 97:278a. (Abstr.)
- Hodges, R. S., and L. B. Smillie. 1970. Chemical evidence for chain heterogeneity in rabbit muscle tropomyosin. *Biochem. Biophys. Res. Commun.* 41:987-994.
- Izant, J. G., and E. Lazarides. 1977. Invariance and heterogeneity in the major structural and regulatory proteins of chick muscle cells revealed by two-dimensional gel electrophoresis. *Proc. Natl. Acad. Sci. USA.* 74:1450-1454.
- Joyce, N., P. DeCamilli, and J. Boyles. 1984. Pericytes, like vascular smooth muscle, contain high levels of cyclic GMP-dependent protein kinase. *Microvasc. Res.* 28:206-219.
- Joyce, N. C., M. F. Hairer, and G. E. Palade. 1985. Contractile proteins in pericytes. II. Immunocytochemical evidence of two isomyosins in graded concentrations. *J. Cell Biol.* 100:1387-1395.
- Krogh, A. 1924. *The Anatomy and Physiology of Capillaries.* Yale University Press, New Haven, CT. 1-276.
- Kuwabara, T., and D. G. Cogan. 1960. Studies of retinal vascular patterns. I. Normal architecture. *Arch. Ophthalmol.* 64:904-910.
- LeBeux, Y. J., and J. Willemot. 1978. Actin and myosin-like filaments in rat brain pericytes. *Anat. Rec.* 190:811-826.
- Lewis, W. G., G. P. Cote, A. S. Mak, and L. B. Smillie. 1983. Amino acid sequence of equine platelet tropomyosin. Correlation with interaction properties. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 156:269-273.
- Marsh, M. N., and J. S. Trier. 1974. Morphology and cell proliferation of subepithelial fibroblasts in adult mouse jejunum. I. Structural features. *Gastroenterology.* 67:622-635.
- Mazanet, R., and C. Franzini-Armstrong. 1982. Scanning electron microscopy of pericytes in rat red muscle. *Microvasc. Res.* 23:361-369.
- Onji, T., and N. Shibata. 1982. Tropomyosin enhances actomyosin ATPase activity in platelets. *Biochem. Biophys. Res. Commun.* 109:697-703.
- Pascal, R. R., G. I. Kaye, and N. Lane. 1968. Colonic pericyptal fibroblast sheath: replication, migration, and cytodifferentiation of a mesenchymal cell system in adult tissue. I. Autoradiographic studies of normal rabbit colon. *Gastroenterology.* 67:622-635.
- Pepe, F. A. 1966. Some aspects of the structural organization of the myofibril as revealed by antibody staining. *J. Cell Biol.* 28:505-525.
- Rapoport, R. M., M. B. Draznin, and F. Murad. 1982. Sodium nitroprusside-induced protein phosphorylation in intact rat aorta is mimicked by 8-bromo cyclic GMP. *Proc. Natl. Acad. Sci. USA.* 79:6470-6474.
- Rhodin, J. A. G. 1968. Ultrastructure of mammalian venous capillaries, venules and small collecting veins. *J. Ultrastruct. Res.* 25:452-500.
- Schultz, K. D., K. Schultz, and G. Schultz. 1977. Sodium nitroprusside and other smooth muscle-relaxants increase cyclic GMP levels in rat ductus deferens. *Nature (Lond.)* 265:750-751.
- Sobieszek, A. 1982. Steady-state kinetic studies on the actin activation of skeletal muscle heavy meromyosin subfragments. Effects of skeletal, smooth and nonmuscle tropomyosins. *J. Mol. Biol.* 157:275-286.
- Tilton, R. G., C. Kilo, and J. R. Williamson. 1979. Pericyte-endothelial relationships in cardiac and skeletal muscle capillaries. *Microvasc. Res.* 18:325-335.
- Tilton, R. G., E. J. Miller, and J. R. Williamson. 1983. Differences in pericyte distribution in rat retinal and uveal vasculatures. *Microvasc. Res.* 25:259. (Abstr.)
- Vegge, R. 1972. A study of the ultrastructure of the small iris vessels in the vervet monkey (*Cercopithecus aethiops*). *Z. Zellforsch. Mikrosk. Anat.* 123:195-208.
- Wallow, I. H., and B. Burnside. 1980. Actin filaments in retinal pericytes and endothelial cells. *Invest. Ophthalmol. & Visual Sci.* 19:1433-1441.
- Weibel, E. R. 1974. On pericytes, particularly their existence on lung capillaries. *Microvasc. Res.* 8:218-235.
- Zimmerman, K. W. 1923. Der feinere Bau der Blutcapillaren. *Z. Anat. Entwicklungs-gesch.* 68:3-109.