Cell Tiss. Res. 157, 125—140 (1975)

Effects of Hypoxia on the Distribution of Calcium in Arterial Smooth Muscle Cells of Rats and Swine*

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Received November 5, 1974

Summary. Exposure to hypoxia caused an increase in the hematocrit and right heart weight of experimental rats, but did not affect calcium-45 uptake by pulmonary arterial smooth muscle cells. However, autoradiographic studies showed that hypoxia apparently caused a shift of 45-Ca from primarily extracellular sites in arteries of control rats to intracellular sites in tissues of hypertensive rats. Cytochemical studies of calcium distributions in pulmonary arterial smooth muscle cells support the autoradiographic data and show that in both rats and swine the majority of pyroantimonate granules occur extracellularly in control tissues. In contrast, hypoxic tissues displayed a greatly reduced number of granules in extracellular sites and an increase in the amount of precipitate in intracellular sites. In pulmonary arterial smooth muscle cells from hypoxic rats most of the precipitate was associated with the caveolae intracellulares, while in corresponding cells from hypoxic swine the majority of the pyroantimonate granules were localized to the sarcoplasmic reticulum. Hypoxia may produce pulmonary hypertension by interfering with the ability of the arterial smooth muscle cells to maintain transmembrane ionic gradients, thus producing an effective increase in cytoplasmic calcium levels. The increased calcium may then activate the contractile apparatus to produce a sustained vasoconstriction.

Key words: Smooth muscle cells — Pulmonary artery — Calcium distribution — Hypoxia — Rat, swine.

Introduction

Calcium controls smooth muscle tone by acting as a membrane stabilizing agent (Axelsson *et al.*, 1967), a transmembrane charge carrier (Reuter, 1973), a coupling or triggering agent between excitation and contraction (Hurwitz and Joiner, 1969), an activator of the contractile apparatus (Sparrow *et al.*, 1970; Rüegg, 1971), and a regulator of smooth muscle metabolism (Anderson, 1972). Therefore, regulation of the intracellular calcium concentration is an important mechanism for controlling smooth muscle contractility. The plasmalemma, sarcoplasmic reticulum and mitochondria have each been implicated in this function (Fitzpatrick *et al.*, 1972; Somlyo and Somlyo, 1971; Hurwitz *et al.*, 1973; Somlyo *et al.*, 1974); however, the relative importance of each is unknown and may in fact vary in smooth muscle cells from different sources.

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* From a thesis submitted to the Graduate Faculty of Colorado State University in partial fulfillment of the requirements for the Doctor of Philosophy degree. Supported in part by Research Grant HE 13972 from the National Institutes of Health.

The mechanisms by which hypoxia produces pulmonary hypertension are presently obscure. There is, however, evidence for an effect of hypoxia on the ability of the vascular smooth muscle cell to regulate its cytoplasmic calcium levels. First, a primary response to hypoxia is a sustained contraction of the arterial smooth muscle (Alexander and Jensen, 1963; Arias-Stella and Saldana, 1963; Naeye, 1965; Hauge, 1970; Abraham *et al.*, 1971; Heath *et al.*, 1973). Second, in hypoxic smooth muscle cells, anaerobic glycolysis, which is stimulated by hypoxia, apparently can provide enough energy to sustain contraction but not transmembrane ionic gradients (Detar and Gellai, 1971; Detar and Bohr, 1972) and consequently cytoplasmic calcium levels increase (Casteels *et al.*, 1973). Third, hypoxia causes disruption of mitochondria in pulmonary arterial smooth muscle cells (Jaenke and Alexander, 1973) which presumably interferes with their ability to produce ATP. Fourth, increased cytoplasmic calcium levels increase the activity of actomyosin ATPase (Ebashi and Endo, 1968; Weber and Murray, 1973) which increases vascular tone (Rüegg, 1971). Finally, ATP is required not only for contraction, but also for dissociation of the actomyosin complex and calcium sequestration, both of which are required for relaxation (Ebashi and Endo, 1968; Weber and Murray, 1973). Therefore, it appears that hypoxia may produce pulmonary hypertension by interfering with the smooth muscle cell's ability to regulate its cytoplasmic calcium levels. There is, however, no direct evidence for this hypothesis.

Because calcium is intimately involved in smooth muscle function, a comparison of calcium localizations in pulmonary arterial smooth muscle cells from control and hypoxic animals might help to demonstrate the mechanism of action of hypoxia in inducing pulmonary hypertension. Also, by studying calcium localizations in smooth muscle cells in altered functional states, the relative roles of the sarcolemma, mitochondria and sarcoplasmic reticulum in calcium sequestration might be further elucidated. Therefore, the purpose of this study was to determine the effects of alveolar hypoxia on calcium distributions in pulmonary arterial smooth muscle cells by examining, cytochemically, calcium localizations in tissues from control and hypertensive rats and miniature swine. The data obtained in this study were then used to test the hypothesis that hypoxia produces pulmonary hypertension through an alteration of the arterial smooth muscle cell's ability to regulate its cytoplasmic calcium levels.

Materials and Methods

Six male albino rats and six miniature swine were placed in the hypo-hyperbaric chamber of the Department of Physiology and Biophysics, Colorado State University, and exposed to a simulated altitude of 18000 feet (5500 m) for four weeks. A second group of six rats and six pigs were maintained as ambient controls at an altitude of 5000 feet (1500 m).

At the end of the treatment period the animals were removed from the chamber and processed as follows: The rats were weighed and anesthetized with ether, and the right jugular vein was exposed. Each rat was then given 150 μCi of calcium-45 (New England Nuclear, 1 mCi/mM) in 0.25 ml of physiological saline via the jugular vein. After five minutes the animals were sacrificed, the thorax was opened, and blood for hematocrit determinations was obtained from the left ventricle. The heart and lungs were then removed and slices of the middle lobe of the right lung were prepared for fixation as described below. After dissecting away the atria and major vessels the heart was rinsed, blotted and weighed. Then the right ventricle was removed and weighed for the determination of the ratio

of right ventricular weight to total ventricular weight (RV/TV ratios). Three portions of each intercostal muscle and lung were obtained, placed in glass liquid scintillation counting vials and frozen for processing at a later time. The procedures for collecting tissue and physiological data from the miniature swine have been presented elsewhere (McMurtry *et al.*, 1973).

The tissues from both rats and pigs to be processed for electron microscopy were immediately placed into a solution of 1% osmium tetroxide and 1% potassium pyroantimonate in 0.01 N acetic acid adjusted to pH 7.4 with 0.1 M potassium hydroxide. The tissues were carefully dissected into 1 mm cubes under a dissecting microscope and placed into small vials of fresh fixative. Fixation was carried out for 2 to 4 hours at room temperature with gentle agitation. After fixation the tissues were rinsed in distilled water, dehydrated with a graded series of alcohols and propylene oxide, and embedded in Epon 812. Silver sections were cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome and examined with a Siemens Elmiskop 1A. The sections were unstained, stained with uranyl acetate, or stained with uranyl acetate and lead citrate.

Tissues to be processed for liquid scintillation counting were thawed and weighed on a precision microbalance (Federal Pacific Electric Company). The tissues were returned to the counting vials and digestion was carried out in 1 ml of Protosol (New England Nuclear) overnight at 60° C. After cooling, 1 µl of glacial acetic acid and 10 ml of Aquasol universal liquid scintillation counting cocktail (New England Nuclear) were added to each vial. The samples were then counted in an Isocap 300 liquid scintillation counter (Nuclear Chicago).

The changes in precipitate distribution within the arterial smooth muscle cells were quantitated by determining the total number of granules in each of 20 different cells in each treatment group. The percentage of the granules associated with each of the following areas was calculated: the extracellular surface of the cell, inside the plasma membrane, caveolae, the contractile elements, mitochondria and sarcoplasmic reticulum. The relative distributions of granules over a given area in the control and hypoxic smooth muscle cells were compared and the Student's *t*-test was used to determine the significance of observed differences.

For autoradiography, one-micron thick sections were cut and mounted on glass microscope slides. The glass slides were then dipped in Kodak NTB-3 liquid emulsion, dried in a vertical position, and stored at 4° C in the presence of a desiccant in black plastic slide boxes which were sealed with black plastic tape. Test slides were developed at intervals to determine the optimal exposure time. The slides were developed in Kodak D-19 developer at 18° C for 2 minutes, stopped in distilled water for 1 minute and fixed in Kodak fixer for 5 minutes. After rinsing in running tap water for 15 minutes the sections were lightly stained with Toluidine blue, cover-slipped with glycerin jelly and examined by phase contrast microscopy.

Results

There was no significant difference between the body weights of the control and experimental rats (Table 1). The hypoxia, however, did produce significant increases in the weights of the right ventricles (12%, $p < 0.05$) and the hematocrits (9.5%, $p < 0.01$) of the experimental rats as compared to those of the control animals (Table 1). Thus, the degree of hypoxic stress, although not great enough to affect the body weights, did induce hypertrophy in the heart, and apparently increased both the production of red blood cells and their concentration in the blood. The physiological data for the effects of hypoxia on swine have been published elsewhere (McMurtry *et al.*, 1973).

Liquid scintillation counting of solubilized lung and skeletal muscle from rats injected with calcium-45 showed that although the tissues from hypoxic rats incorporated slightly more label than did tissues from control rats, the differences were not significant (Table 2). The skeletal muscle from both normal and hypertensive rats, however, contained about 70% fewer counts than did the lung tissue. This difference could be a reflection of a different degree of uptake in the

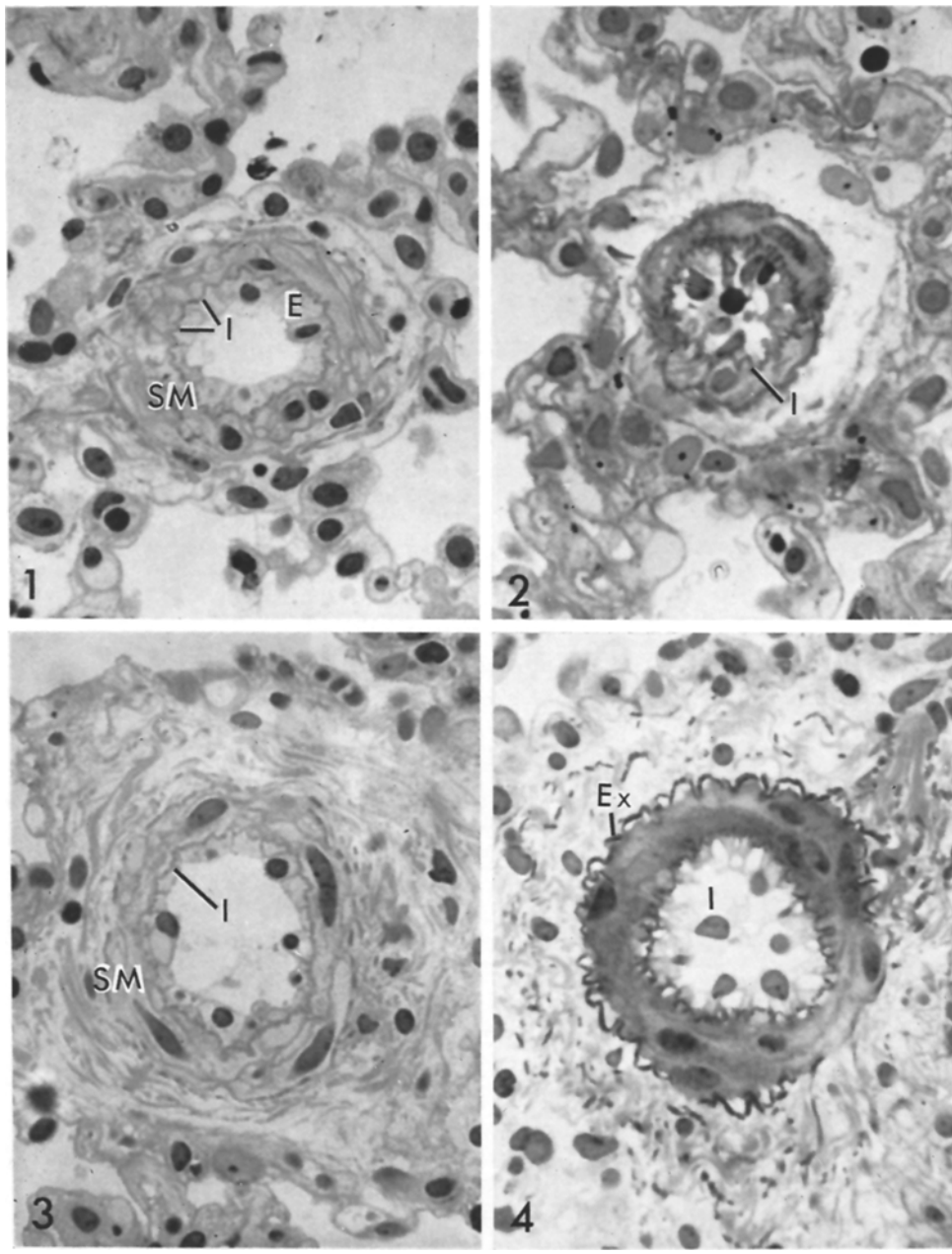

Fig. 1. A pulmonary artery from a control rat. The endothelial cells (*E*) are cuboidal in shape, possess rounded nuclei and lie on a mildly undulating internal elastic lamina (*I*). Some of the smooth muscle cells (*SM*) have an irregular outline while others are quite uniform. These cells do not appear to be contracted. 1 micron-thick section, Epon, Toluidine blue, × 900

Fig. 2. A pulmonary artery from a hypoxic rat .The endothelial cells are quite compressed and their nuclei extend into the lumen of the vessel. The internal elastic lamina (*I*) is highly folded.

Table 1. Systemic effects of hypoxia on rats

Control	Experimental	Significance
A. Body weights (mean)		
306 gm SE = ± 15	302 gm SE = ± 12	N.S.
B. Right ventricle wt./total ventricle wt. (mean)		
0.2342 gm SE = ± 0.0121	0.2710 gm SE = ± 0.0198	$p < 0.05$
C. Hematocrit (mean)		
51 SE = ± 1.47	56 SE = ± 1.44	$p < 0.01$

Table 2. Calcium-45 uptake rat

Tissue	Treatment mean gpm/mg	Significance
Lung	Control 247.76 SE = ± 23.47 Hypoxic 266.12 SE = ± 21.59	N.S.
Skeletal	Control 67.93 SE = ± 7.39 Hypoxic 81.22 SE = ± 6.27	N.S.

two tissues, but more likely it represents the dilution of label from its site of injection.

Autoradiography revealed that in the pulmonary arterial smooth muscle cells from control rats the silver grains were concentrated in the stroma and appeared to be most heavily localized around the perimeter of the smooth muscle cells. In contrast, the hypoxic smooth muscle cells exhibited a larger number of silver grains localized over the cell body. Background over the rest of the tissue, however, was too high to quantify this difference. In addition, wide variations in the number of silver grains in autoradiographs from different rats within a treatment group obscured the differences between the two groups. Thus, the results of the radio-

The smooth muscle cells have very irregular outlines and appear to be contracted. 1 micron-thick section, Epon, Toluidine blue, × 900

Fig. 3. A small artery from control pig lung. The endothelial cells lie close to the internal elastic lamina which is slightly folded (*I*). The smooth muscle cells (*SM*) have a regular outline and spindle shaped nuclei. 1 micron-thick section, Epon, Toluidine blue, × 900

Fig. 4. A pulmonary artery from a hypertensive pig. The endothelial cells possess ovoid nuclei which protrude into the lumen. Both the internal (*I*) and external (*Ex*) elastic laminae are highly folded. The outlines of the smooth muscle cells are indistinct but their nuclei are shorter and thicker than those in control vessels. 1 micron-thick section, Epon, Toluidine blue, × 900

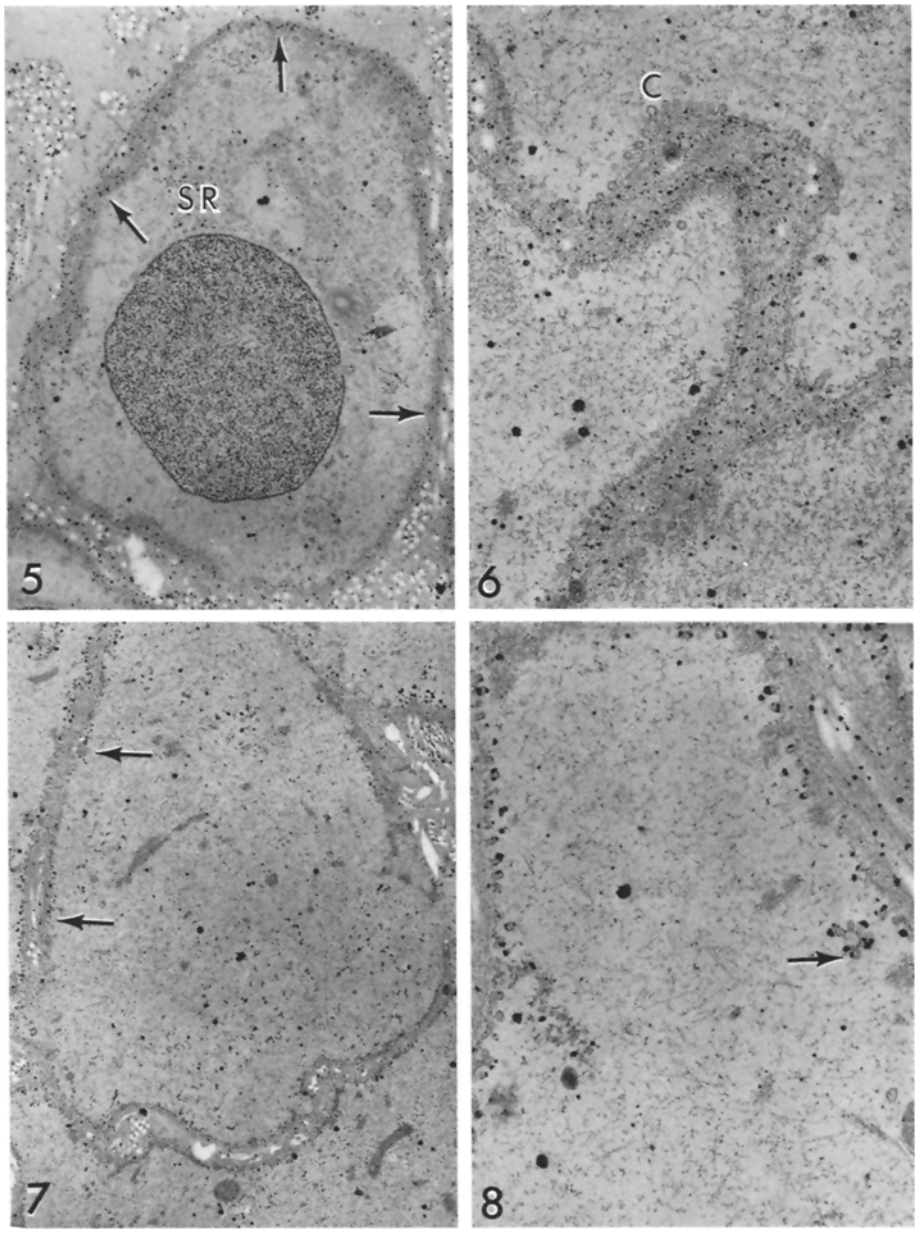

Fig. 5. A section of a pulmonary arterial smooth muscle cell in a control rat. The predominant distribution of pyroantimonate granules (*arrows*) along the extracellular surface of the cell is readily apparent. Some granules also occur in the sarcoplasmic reticulum (*SR*). Uranyl acetate, lead citrate, × 13500

Fig. 6. A portion of the extracellular space between two smooth muscle cells from a control rat. Pyroantimonate granules are abundant in this space but few appear in the cytoplasm of the cells. The caveolae (*C*) are empty or contain a few very small granules. Uranyl acetate, lead citrate, × 24000

active calcium studies are equivocal and, while they do indicate a trend, do not by themselves provide definitive evidence for hypoxia-induced calcium shifts in hypertensive animals.

Histologically, striking differences were noted between the pulmonary arteries from control animals (Fig. 1 and 3) and hypertensive animals (Figs. 2 and 4). The changes were similar in both the rat and the pig. The smooth muscle cells in the pulmonary arteries from the hypertensive animals (Figs. 2 and 4) were shorter, thicker and more pleomorphic than the corresponding cells from control animals (Figs. 1 and 3). The endothelial cells lining the arteries of hypertensive animals (Figs. 2 and 4) were more compressed and their nuclei protruded into the lumens of the vessels, while the endothelial cells in control arteries were more elongated and their nuclei tended to lie closer to the walls of the vessels. Thus, the pulmonary arteries from the hypertensive animals of both species, while not demonstrating any obvious pathological alterations, did appear to be more highly contracted than the corresponding arteries from control animals.

The general cytology of the smooth muscle cells from the pulmonary arterial tree was similar to that published elsewhere for other vascular smooth muscle cells (Rhodin, 1962, 1967; Fernando and Movat, 1964; Movat and Fernando, 1963; Gabella, 1973). The ultrastructure of the smooth muscle cell, however, was not as well preserved by the osmium-pyroantimonate fixative as by more conventional fixatives. Osmium-pyroantimonate produces dark granules 20 to 45 nm in diameter which have been shown through electron elemental analysis (Spicer and Swanson, 1972) to contain principally calcium pyroantimonate, and to be representative of the subcellular distribution of calcium in tissues. In smooth muscle cells from control rats, the highest density of pyroantimonate granules was localized extracellularly along the cell surface, over the basement membrane, and intercellular connective tissue (Figs. 5 and 6). Granules, though fewer in number, also appeared scattered over areas of the cytoplasm containing the contractile elements and the sarcoplasmic reticulum. Quantitation of the relative granule distributions in these cells (Table 3) revealed the following distributions: 54.3% of the granules were located in the extracellular space; 6.7% were adjacent to the cytoplasmic surface of the plasma membrane; 4.4% were associated with the caveolae; 24.3% occurred in the contractile elements; 28.6% were located in the sarcoplasmic reticulum, the mitochondria contained 3.3% of the granules. The smooth muscle cells from the control pigs (Figs. 9 and 10) revealed a basically similar distribution of granules. That is, the majority of the granules appeared in association with the extracellular sites, while a smaller number of granules were distributed more diffusely over intracellular structures. Determination of the relative distribution of pyro-

Fig. 7. A smooth muscle cell from a hypertensive rat. The pyroantimonate granules appear along the intracellular surfaces of the sarcolemma (*arrows*) rather than in the extracellular spaces. Granules are also apparent in the cytoplasm. Uranyl acetate, lead citrate, × 9000

Fig. 8. A small portion of a smooth muscle cell from a hypoxic rat. The majority of the pyroantimonate granules present are associated with the caveolae (*C*). The granules, especially the smaller ones (*arrow*), appear to be localized to the cytoplasmic surfaces but some of the larger granules extend into the lumina. Compare with Fig. 10. Uranyl acetate, lead citrate, × 24500

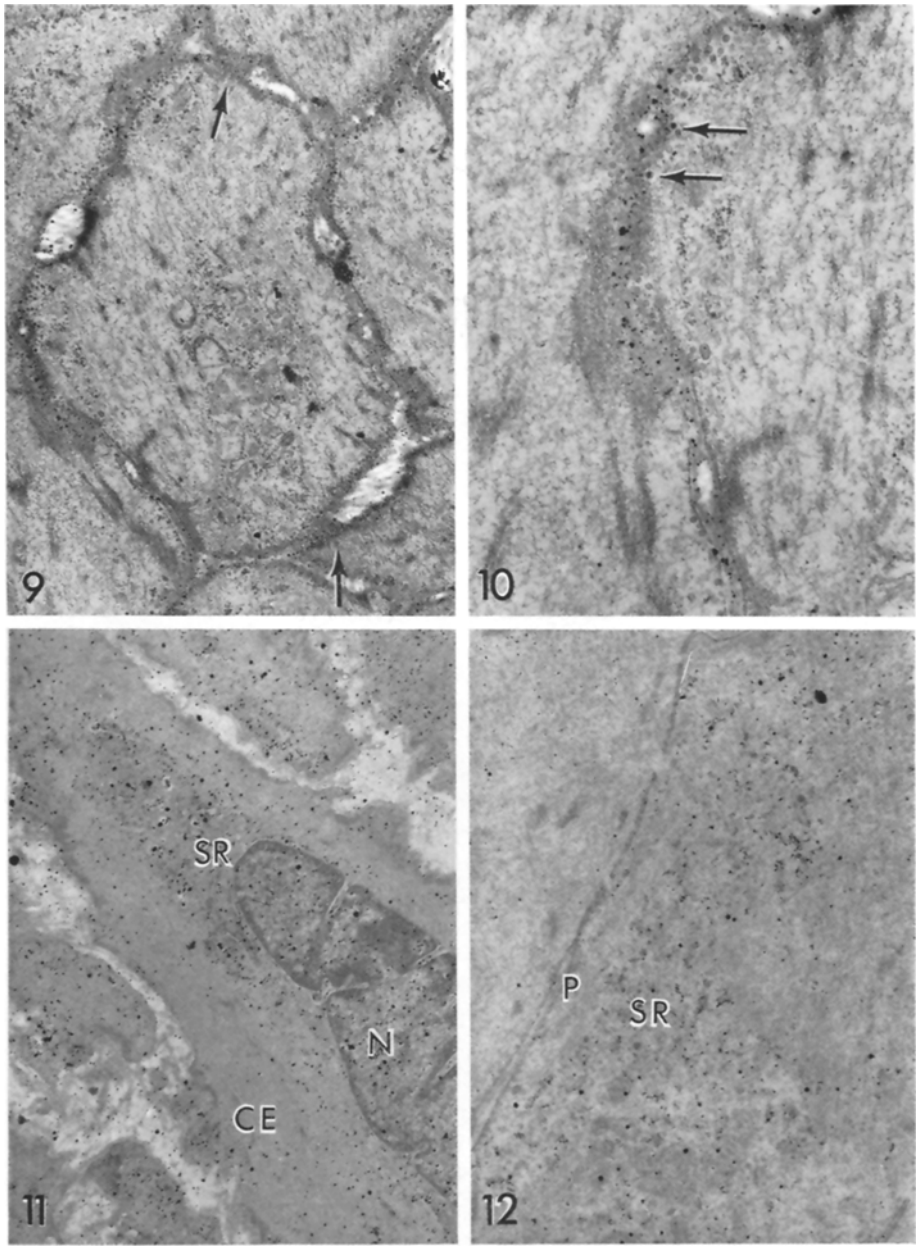

Fig. 9. A smooth muscle cell from a pulmonary artery of a control pig. The pyroantimonate granules (*arrows*) are localized primarily on the extracellular surfaces of the cell. Uranyl acetate, lead citrate, × 9500

Fig. 10. A portion of the extracellular space between two smooth muscle cells from a control pig. The pyroantimonate granules are abundant in the space but are sparse in the cytoplasm of the two cells. Most of the caveolae are empty but a few (*arrows*) contain granules in their lumina. Uranyl acetate, lead citrate, × 18000

Table 3. Distribution of calcium pyroantimonate granules over smooth muscle cells rat

Location	Treatment	Mean percent of granules	Significance
Extracellular	Control rat	54.9 SE = ±3.3	$p<0.01$
	Hypoxic rat	29.7 SE = ±3.3	
Cytoplasmic surface of membrane	Control rat	6.7 SE = ±1.6	N.S.
	Hypoxic rat	7.8 SE = ±0.6	
Caveolae	Control rat	4.4 SE = ±0.8	$p<0.01$
	Hypoxic rat	20.8 SE = ±2.7	
Contractile elements	Control rat	24.3 SE = ±2.9	N.S.
	Hypoxic rat	26.6 SE = ±2.4	
Mitochondria	Control rat	3.3 SE = ±1.2	N.S.
	Hypoxic rat	5.5 SE = ±1.4	
Sarcoplasmic reticulum	Control rat	28.6 SE = ±6.1	$p<0.05$
	Hypoxic rat	19.0 SE = ±1.4	

antimonate granules in the various subcellular compartments of the pig smooth muscle cells (Table 4) showed the following: extracellular sites 54.0%, cytoplasmic surfaces of plasmalemma 12.3%, caveolae 6.6%, contractile elements 20.1%, sarcoplasmic reticulum 11.7%, and mitochondria 4.5%. These data indicate that by far the largest amount of pyroantimonate precipitate was associated with extracellular sites on the arterial smooth muscle cells in control animals of both species.

In contrast, there was a striking reduction (45%) in the relative number of granules associated with the extracellular surfaces of smooth muscle cells from the pulmonary arteries of the hypertensive rats (Figs. 7 and 8). Only 29.7% of the granules were found to be associated with the extracellular spaces of cells from the hypertensive rats (Table 3) compared to 54.9% for the controls ($p<0.01$). In addition, there was also a dramatic shift in the relative number of granules over the different subcellular compartments. For the control and hypertensive animals, respectively, these were: cytoplasmic surface of the plasma membrane (6.7% vs 7.8%, N.S.), caveolae (4.4% vs 20.8%, p. <0.01), contractile elements (24.3% vs 26.6, N.S.), sarcoplasmic reticulum (28.6% vs 19.0%, $p<0.05$) and mitochondria (3.3% vs 5.5%, N.S.). Thus, in the arterial smooth muscles of the rat, the primary and most significant shift of the pyroantimonate granules was from the extracellular surface to the region of the caveolae that lines the sarco-

Fig. 11. A section of a pulmonary arterial smooth muscle cell from a hypoxic pig. The pyroantimonate granules appear to be primarily associated with the sarcoplasmic reticulum (*SR*). However, some granules are also associated with the nucleus (*N*) and the contractile elements (*CE*). Uranyl acetate, lead citrate, × 7500

Fig. 12. A portion of a smooth muscle cell from a hypoxic pig. The plasma membrane (*P*) is devoid of caveolae. Few pyroantimonate granules appear on either surface of the membrane. The majority of the granules present are scattered over the sarcoplasmic reticulum (*SR*). Uranyl acetate, lead citrate, × 15000

Table 4. Distribution of calcium pyroantimonate granules over smooth muscle cells pig

Location	Treatment	Mean percent of granules	Significance
Extracellular	Control pig	54.0 SE = ± 2.9	$p < 0.01$
	Hypoxic pig	21.9 SE = ± 2.7	
Cytoplasmic surface of membrane	Control pig	12.3 SE = ± 1.9	N.S.
	Hypoxic pig	9.4 SE = ± 1.2	
Caveolae	Control pig	6.6 SE = ± 0.7	$P < 0.05$
	Hypoxic pig	10.5 SE = ± 2.8	
Contractile elements	Control pig	20.1 SE = ± 2.2	N.S.
	Hypoxic pig	22.8 SE = ± 2.9	
Mitochondria	Control pig	5.4 SE = ± 2.5	N.S.
	Hypoxic pig	9.2 SE = ± 1.8	
Sarcoplasmic reticulum	Control pig	11.7 SE = ± 1.2	$p < 0.01$
	Hypoxic pig	38.0 SE = ± 2.9	

lemma. In addition, most of the granules appeared to be localized to the cytoplasmic surface of the caveolae. This, however, could not be clearly defined because many of the grains were so large that they extended across both sides of the membrane. There was also a less dramatic but significant ($p < 0.05$) reduction in the number of granules associated with the sarcoplasmic reticulum of hypoxic smooth muscle cells. Thus, the hypertensive state in the rat appeared to be associated with a reduction in the number of precipitate granules localized extracellularly and an increase in their intracellular concentration.

As in the cells from hypertensive rats, the smooth muscle cells from the arteries of the hypertensive pigs showed a reduction in the number of granules associated with the extracellular sites (Figs. 11 and 12). The shifts in granule distributions in cells from control and hypertensive respectively, were found to be: extracellular (54.0% vs 21.9=, $p < 0.01$), cytoplasmic surface of membrane (12.3% vs 9.4%, N.S.), caveolae (6.6% vs 10.5%, $p < 0.05$), contractile elements (20.1% vs 22.8%, N.S.), sarcoplasmic reticulum (11.7% vs 38.0%, $p < 0.01$) and mitochondria (5.4% vs 9.2%, N.S.) (Table 4). This data indicated that as in the rat, hypoxia in the pig caused a significant decrease in the percentage of granules associated with the extracellular surfaces of smooth muscle cells. However, in contrast to the rat, there was only a slight increase in the distribution of granules over the caveolae of hypoxic smooth muscle cells in the pig, while there was a large increase in the number of granules associated with sarcoplasmic reticulum. One possible reason for this is that the sarcoplasmic reticulum appeared to be less abundant in the smooth muscle cells from rats as compared to pigs. The caveolae, which greatly increase the cell surface area, may perform the function of calcium sequestration in rat cells in place of the sarcoplasmic reticulum. Thus, the primary shift of pyroantimonate granules was from the extracellular surface in control animals to intracellular membrane surface in hypertensive animals.

In summary, granule distributions in control smooth muscle cells from rats and pigs differed significantly from those in cells from hypertensive animals in

three locations: the extracellular surface, caveolae and sarcoplasmic reticulum; and from each other in two locations: the caveolae and sarcoplasmic reticulum. The differences in precipitate localizations between control and hypertensive rats appeared as a decrease in the number of granules associated with the extracellular surface and the sarcoplasmic reticulum and an increase in the number associated with the caveolae of the hypertensive animals. In the tissues from the pig, the differences appeared as a decrease in the distribution of granules over extracellular sites, a slight increase in the region of the caveolae and a large increase in the number of granules associated with the sarcoplasmic reticulum.

Discussion

These data indicate that there is a major redistribution of pyroantimonate granules in arterial smooth muscle cells from hypertensive animals as compared to those from control animals. This has been interpreted to mean that hypoxia produces a significant shift of calcium from extracellular to intracellular sites. The validity of this assumption rests on the sensitivity and specificity of the pyroantimonate anion for calcium ions.

The potassium pyroantimonate-osmium tetroxide fixative was initially developed by Komnick (1962, 1963) in an attempt to localize sodium in the herring gull salt gland. Komnick, however, pointed out that this technique could also be adapted for the subcellular localization of calcium. In fact, the affinity of pyroantimonate for calcium has been shown to be ten times higher than for magnesium and 10000 times higher than for sodium (Klein *et al.*, 1972). Electron microprobe, X-ray microprobe and elemental analyses of pyroantimonate precipitates have demonstrated that the primary components of these granules were calcium and antimony (Spicer and Swanson, 1972; Herman *et al.*, 1973; Yarom and Meri, 1973) and that the amounts of K^+, Na^+ and Mg^{2+} present in the granules were too small to analyze statistically (Yarom and Chandler, 1974). In cytochemical studies the formation of pyroantimonate granules in myocardial tissue was unaffected, or even slightly increased, by sodium removal, but was prevented by the chelation of calcium with EGTA even when an excess of magnesium was added (Legato and Langer, 1969). Finally, the greatest distribution of pyroantimonate granules in relaxed skeletal muscle occurs in the sarcoplasmic reticulum, while in contracted muscle the granules are more numerous at the site of actin and myosin interaction (Yarom and Meri, 1972; McCallister and Hadek, 1973; Davis *et al.*, 1974). This observation corresponds well with the proposed role of calcium in muscle contraction (Podolsky and Constantin, 1964; Ebashi and Endo, 1968), the localization of calcium demonstrated by other cytochemical techniques (Constantin *et al.*, 1965; Diculescu *et al.*, 1971), and the movements of calcium-45 observed by means of autoradiography (Winegrad, 1965a, b, 1968, 1970). Therefore, it appears that the pyroantimonate technique is relatively specific for calcium in biological tissues and that the resultant granules give a good indication of where high concentrations of calcium occur within a cell. The technique, however, does not show whether the calcium is bound and/or free.

The distribution of calcium pyroantimonate granules in control smooth muscle cells differs significantly in three areas from those in cells from hypertensive

animals. The extracellular space in tissues from hypertensive rats contained 45% fewer granules than in tissues from control rats, and there were 33% fewer grains associated with the sarcoplasmic reticulum of smooth muscle cells from hypertensive rats. The caveolae in cells from hypertensive rats, however, contained 78% more granules than caveolae in control cells. In tissues from hypertensive pigs, the granule distributions were 60% lower in the extracellular spaces, 37% higher in the caveolae, but 70% higher in the sarcoplasmic reticulum than the distributions in corresponding areas of control cells. There is, therefore, a definite shift in the distribution of calcium pyroantimonate from extracellular sites in the control animals to intracellular sites in the hypoxic animals. The results of autoradiographic studies of calcium-45 distributions in arterial smooth muscle cells, although not as definitive, also provide evidence for an hypoxia-induced movement of calcium from extracellular to intracellular sites. These data are consistent with: 1) the observation that agents which cause smooth muscle contraction also increase the amount of intracellular calcium (Briggs and Melvin, 1961; Briggs, 1962; Hinke, 1965; Waugh, 1965) while relaxing agents decrease intracellular calcium levels (Seidel and Bohr, 1971), and 2) the fact that the degree of smooth muscle contraction is directly related to cytoplasmic calcium levels (Rüegg, 1971). Furthermore, these data correlate well with the vasoconstriction observed in the pulmonary arteries from hypertensive animals (Alexander and Will, 1963; Hauge, 1970) and provide a basis for the hypothesis that pulmonary hypertension results from long-term interference with the regulation of cytoplasmic levels of calcium.

Biochemical and cytochemical studies have implicated both the sarcolemma and the sarcoplasmic reticulum of smooth muscle cells in the regulation of cytoplasmic calcium concentrations; however, the relative importance in each of this function has not yet been ascertained (Carsten, 1969; Batra and Daniel, 1971; Devine *et al.*, 1971, 1972, 1973; Somlyo and Somlyo, 1971; Fitzpatrick *et al.*, 1972; Hurwitz *et al.*, 1973). The present study has shown that the greatest proportion of intracellular calcium in arterial smooth muscle cells from hypertensive rats is associated with the caveolae which line the sarcolemma, while in cells from hypertensive pigs, most of the intracellular calcium is associated with the sarcoplasmic reticulum. The lack of a significant increase in the distribution of calcium pyroantimonate granules over the myocontractile elements is surprising in view of the intimate role of calcium in smooth muscle contraction. However, the technique may not be able to demonstrate calcium increases in this region because half-maximal activation of the contractile apparatus occurs at a calcium concentration of approximately 10^{-6} M (Sparrow *et al.*, 1970). which represents the lower limit for the sensitivity of the pyroantimonate anion for calcium (Klein *et al.*, 1972). The differences in the sites of intracellular calcium localizations between the two species may be explained by the observation that smooth muscle cells from the pulmonary arteries of pigs seem to contain much more sarcoplasmic reticulum than do the cells of rats. In contrast, there appear to be slightly fewer caveolae lining the plasmalemma of porcine smooth muscle cells than in rat smooth muscle cells. Thus, the apparent differences in the amount of calcium associated with the two systems of organelles between pigs and rats, and the differences in the amount of sarcoplasmic reticulum or caveolae indicate that in porcine arterial smooth muscle cells, where it is abundant, the sarcoplasmic

reticulum probably plays the predominant role in intracellular sequestration. The sarcoplasmic reticulum, however, is less apparent in the rat arterial smooth muscle cells. Therefore, in these cells the caveolae, which amplify both sarcolemmal surface area and function, may play the predominant role in calcium sequestration.

Sustained alveolar hypoxia elicits pulmonary hypertension which involves an initial vasoconstriction (Alexander and Will, 1963; Hauge, 1970) followed, according to some authors, by hypertrophy and proliferation of the arterial smooth muscle cells (Alexander and Jensen, 1963; Abraham *et al.*, 1971; Heath *et al.*, 1973). There is, however, no direct evidence for hypertrophy of these cells. Recent ultrastructural studies have revealed that hypoxia of a degree sufficient to induce pulmonary hypertension rapidly produced degenerative lesions in the intimal and medial cells of the pulmonary arteries (Jaenke and Alexander, 1973). Thus, it seems possible that some mechanism other than hypertrophy of the medial smooth muscle may be primarily responsible for the production of pulmonary hypertension. The sustained reduction in the luminal diameter of the arterial tree, which is a consistent finding in studies of pulmonary hypertension (Alexander and Jensen, 1963; Naeye, 1965; Abraham *et al.*, 1971; Heath *et al.*, 1973) may indicate that this disorder results from prolonged contraction of the arterial smooth muscle cells. Studies with hypoxic smooth muscle have shown that while anaerobic glycolysis in these cells is able to produce enough ATP to support contraction, it is not capable of maintaining the transmembrane ionic gradients and the cells gain calcium (Detar and Gellai, 1971; Detar and Bohr, 1972; Casteels *et al.*, 1973). Thus, it seems that this apparent increase in membrane permeability produced by hypoxia might allow calcium to continually leak into the cell. Since calcium is known to activate the smooth muscle contractile apparatus (Sparrow *et al.*, 1970) the continued presence of calcium in the cytoplasm could elicit a sustained vasoconstriction.

In conclusion, hypoxia causes a shift in calcium pyroantimonate localizations in pulmonary arterial smooth muscles from extracellular to intracellular sites. Inside the cell, the precipitate granules accumulate predominantely on the caveolae in cells from rats, but in cells from pigs the doposits are primarily localized to the sarcoplasmic reticulum. Thus, it appears that in rat arterial smooth muscle cells, the caveolae play a predominant role in calcium regulation, but in porcine arterial smooth muscle the sarcoplasmic reticulum is the primary calcium sequestering agent. Hypoxia, therefore, may produce hypertension by interfering with the ability of these cells to maintain transmembrane ionic gradients, thus producing an effective increase in the cytoplasmic calcium levels. The resulting apparent increase in cytoplasmic calcium may then activate the contractile apparatus to produce a sustained vasoconstriction.

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