ELECTRON MICROSCOPY AND ELECTRON PROBE ANALYSIS OF MITOCHONDRIAL CATION ACCUMULATION IN SMOOTH MUSCLE

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ABSTRACT

The contractile responses to barium and the ultrastructure and ionic composition of mitochondria were studied in vascular smooth muscle. In normal rabbit portal anterior mesenteric vein (PAMV) and main pulmonary artery (MPA) smooth muscle mitochondria were frequently associated with the surface vesicles. The average distance between the outer mitochondrial and inner surface vesicle membrane was 4–5 nm. Ba contractures of MPA were tonic and of PAMV were phasic. Incubation of MPA and PAMV with Ba resulted in the accumulation of mitochondrial granules, followed in the MPA by massive mitochondrial swelling. Oligomycin and anoxia inhibited the appearance of mitochondrial electron-opaque granules and prevented the Ba-induced mitochondrial swelling in the MPA. Electron probe analysis of mitochondria in PAMV incubated with Ba and containing granules showed characteristic Ba signals over the mitochondria. Electron probe X-ray microanalysis also showed a highly significant ($P < 0.001$) correlation of P with mitochondrial Ba, in an estimated elemental ratio of approximately 3 Ba/4 P. Mitochondrial granules were still prominent after block staining of the osmium-fixed, Ba-loaded PAMV, but electron probe microanalysis showed no Ba, but only U, emissions. Tissues incubated with strontium had electron-opaque mitochondrial granules and deposits in the sarcoplasmic reticulum. X-ray microanalysis of mitochondria containing granules showed the presence of characteristic Sr and Ca emissions. The presence of Sr was similarly verified in the sarcoplasmic reticulum. These findings indicate the energy dependent uptake of divalent cations, in association with phosphate, by mitochondria in vascular smooth muscle *in situ* and the possibility that mitochondria may contribute to the regulation of intracellular divalent cation levels in smooth muscle.

INTRODUCTION

The presence of a sarcoplasmic reticulum (SR) in smooth muscle (Somlyo et al., 1971; Somlyo and Somlyo, 1971; Devine et al., 1972; Gabella, 1973), and the isolation, from smooth muscle, of a

microsomal fraction that can accumulate Ca^{++} in the presence of ATP (Carsten, 1969; Batra and Daniel, 1971; Hurwitz et al., 1973; Baudouin-Legros and Meyer, 1973) have led to the suggestion that the SR functions similarly, in excitation-contraction (EC) coupling, in smooth and in striated muscles (for review see Somlyo, 1972). The volume of the SR, however, varies in different smooth muscles, and may be as little as approximately 2% of the cell volume (Somlyo et al., 1971; Devine et al., 1972). Therefore, the possibility that, in addition to the SR, other sites in smooth muscle also may sequester and release Ca^{++} has been considered (Somlyo and Somlyo, 1970).

The energy dependent accumulation of Ca^{++} by mitochondria (for review see Chance, 1965; Lehninger, 1970) has led to the suggestion from several laboratories (Chance, 1965; Patriarca and Carafoli, 1968; Harigaya and Schwartz, 1969; Sulakhe and Dhalla, 1970; Horn et al., 1971) that mitochondria may contribute to the regulation of intracellular free Ca^{++} levels in cardiac muscle. Our long-term aim is to explore this question (Somlyo and Somlyo, 1970, 1971; Somlyo, 1972; Devine et al., 1973) in smooth muscle. The immediate aim of the present work was to document the relationship of mitochondria to the surface vesicles, observed in the course of our studies on SR, and to establish the feasibility of using electron-opaque divalent cations for electron microscope studies of mitochondrial cation movements in situ. We also wanted to verify, with X-ray microanalysis, the nature of the deposits found in smooth muscles incubated with barium and strontium, and to determine the anion associated with some of these deposits.

Strontium and barium are particularly suitable elements for electron microscope studies of divalent cation accumulation, as they have sufficiently high atomic numbers for the deposits to be electron opaque (Peachey, 1964). Furthermore, the accumulation of strontium by fragmented skeletal muscle SR preparations (Nagai et al., 1965; Van der Kloot and Glovsky, 1965; Weber et al., 1966) and by mitochondria isolated from liver has been studied in detail (Carafoli, 1965 a, b; Carafoli et al., 1965; Greenawalt and Carafoli, 1966), and is rather similar to the accumulation of Ca^{++}.

Intramitochondrial electron-opaque granules are produced in smooth muscles through incubation in either strontium- or barium-containing solutions (Peachey, 1964; Somlyo and Somlyo, 1971). Electron-opaque deposits are also formed in the lumen of the SR of smooth muscles incubated in solutions containing strontium (Somlyo and Somlyo, 1971; Somlyo et al., 1972; Devine et al., 1973). The preferential compartmentalization of Ba into mitochondria, rather than SR, may give specific information about the action of physiological stimuli and of drugs on mitochondrial cation movements through observations on transmembrane Ba^{++} fluxes, and our present electron microscope studies are presented to provide an ultrastructural basis for flux experiments. Preliminary reports of some of our observations have been previously published (Somlyo and Somlyo, 1971; Somlyo et al., 1972).

MATERIALS AND METHODS

Physiological Studies

White male rabbits 2.0–2.7 kg were killed with a postcephalic blow. Circumferential strips of main pulmonary artery (MPA) and longitudinal strips of the portal anterior mesenteric vein (PAMV) were cut and stretched to 1.5 and 1.7 times, respectively, their excised length. The composition of the Krebs' solution and the methods of recording tension have been reported previously (Somlyo et al., 1971; Jones et al., 1973).

Tissues were loaded with barium or strontium by incubation for 60 min in one of the following solutions: (a) Ba or Sr Krebs' solution (mM): NaCl, 111.3; KCl, 4.7; KH_2PO_4, 1.2; $NaHCO_3$, 18.7; dextrose, 5.6; $MgCl_2$, 1.2; $SrCl_2$ or Ba acetate, 10.0. (b) Ba or Sr HEPES-buffered solution (mM): NaCl, 128.5; KCl, 5.9; $MgCl_2$, 1.2; dextrose, 5.6; HEPES, 2.0; $SrCl_2$ or Ba acetate, 10; pH adjusted to 7.4. (c) 1.2 mM KH_2PO_4 added to solution b. Some MPA strips were also examined after shorter exposure to Ba^{+-} (see Results).

In some experiments the tissues, initially loaded for 1 h in 10 mM Ba- or Sr-containing solution, were washed out over a 60-min period in four changes of Krebs' solution or a high KCl solution with 1.7% sucrose added to maintain normal cell volume (Jones et al., 1973). Since there was no detectable difference in the elemental ratios observed between spectra obtained over relatively faint granules analyzed in unloaded strips or in strips that were fixed immediately after barium loading, the electron probe results of both types of material are tabulated together.

The effects of metabolic inhibition were examined by the addition of 10 μg/ml oligomycin (15% oligomycin A, 85% oligomycin B, Sigma Chemical Co., St. Louis, Mo.) to incubation solution b, without added dextrose, gassed with 100% N_2. Oligomycin (4 mg/0.08 ml solvent) was dissolved in dimethyl sulfoxide (DMSO). Control experiments showed that the final concentration of DMSO delivered to the muscle bath did not block mitochondrial Ba^{++} uptake. Tissue Ba contents were determined by

atomic absorption spectroscopy (Perkin-Elmer model 303, Perkin-Elmer Corp., Boller & Chivens Div., South Pasadena, Calif.

Electron Microscopy

Tissues were fixed in: (a) 2% glutaraldehyde containing 4.5% sucrose in 0.075 M cacodylate buffer (pH 7.4) for 2 h and postfixed in osmium as reported previously (Devine et al. 1972). (b) 2% osmium tetroxide in 0.05 M cacodylate buffer (pH 7.4) or Krebs' bicarbonate buffer for 2 h. (c) 2% glutaraldehyde containing 4.5% sucrose in 0.075 M cacodylate buffer (pH 7.5) for 2 h. These tissues were not postfixed in osmium. (d) Very small (100–150 μm in diameter) strips in osmium tetroxide vapor for 15 min followed by a brief dehydration of 15 min in graded ethanols.

Osmium-fixed tissues were divided into two pieces, one of which was block stained with saturated aqueous uranyl acetate for 60 min. All tissues were embedded in Spurr's resin (Spurr, 1969). Unless indicated in the figure legends, tissues were examined unstained.

MITOCHONDRIAL SURFACE VESICLE RELA-TIONSHIPS VIEWED WITH SPECIMEN TILT-ING: The Hitachi HU11E electron microscope was fitted with a HK3A wide-range tilting stage ±30° with full 360° azimuth control and a large bore pole piece (Devine et al., 1972).

Electron Probe Analysis

Tissues used for electron probe analysis included smooth muscle incubated in Ba- or Sr-containing solutions (a, b, or c, Physiological Studies) for 60 min and fixed (b, c, and d, preceding section) for 2 h in 2% glutaraldehyde (without postosmication), or in 2% osmium in buffered aqueous solution (without prefixation with an aldehyde), or with osmium vapor. Glutaraldehyde-fixed material was used for the simultaneous analysis of barium and phosphorus because the closely adjacent osmium M_α (1.91 keV) and phosphorus K_α (2.01 keV) lines cannot be resolved with energy dispersive detectors without further computer processing. Portal vein preparations that had been loaded with barium and contained large electron-opaque intramitochondrial granules were also analyzed after block staining with uranyl acetate (see Results).

An EMMA 4 instrument was used for electron probe analysis. The instrument, a transmission electron microscope fitted with two diffractive and one energy dispersive spectrometer, has been described in detail (Weavers, 1973). The X-ray microanalyses reported here were performed with a Kevex-retractable lithium-drifted silicon detector (resolution of 165 eV, active surface 40 mm^2) placed 10.5 cm from the specimen, and connected to a Kevex 5000A and 6000 multichannel analyzer-data processor operated at 10 (for Ba^{++}) or 20 eV (for Ca^{++} and Sr^{++}) per channel (Kevex Corp., Burlingame, Calif.). The memory of the analyzer permits the storage of

spectra for comparison with or subtraction from analytical data. In a few experiments, the spectrum of an Sn standard was stored and subtracted from the spectrum obtained over mitochondrial granules, to remove interference by the Sn, in the specimen rod, with the Ca K_α peak: the presence of calcium was verified by diffractive analysis of the same spot with a pentaerythritol (PET) crystal. Subsequently, a specimen rod with aluminum inserts and free of Sn interference, was used.

The approximately 100-nm sections were placed on large mesh copper grids, carbon coated, and analyzed at 40 kV. The mini lens of the EMMA 4 permitted adequate orientation for analysis, but, for obvious reasons, did not yield image resolution comparable to that obtained with thinner sections and higher accelerating voltages. Grading of the electron density of the granules (light, medium electron dense, very electron dense) was feasible and was done by the same operators who did the electron microscopy of barium accumulation.

The size of the focused spot used as the electron probe was from 200 to 300 nm. Unless noted otherwise, analyses were performed with 20 nA probe current over 300-s integrated detecting time. In addition to the aforementioned Sn lines generated by the copper bronze specimen rod, the Si K_α emission (1.74 keV), which may be generated by the inactive layer of the Si (Li) detector and the Cu lines due to the rod and specimen grids, there was also an extraneous S K_α line that may have been due to contamination from the oil in the vacuum pumps. This contamination could be eliminated with the liquid nitrogen anticontamination device, but, since we did not use it in all of our analyses, we do not identify sulfur in our material. The osmium M_α and chlorine K_α lines could be ascribed to the preparatory techniques and will be discussed below (see Results).

RESULTS

Electron Microscopy and Contractile Behavior

GENERAL RELATIONSHIP OF MITO-CHONDRIA: The general arrangement of the mitochondria in the rabbit PAMV is shown at low magnification in Fig. 1. Mitochondria are most frequently at the periphery (within 100 nm from the cell membrane) or in centrally located clusters at the nuclear poles. In longitudinal sections mitochondria are cigar-shaped, elongated structures up to 7 μm in length. In the rabbit PAMV, 39% (measurements in 251 cell profiles from five animals) of the mitochondria are peripheral (i.e., less than 100 nm from the cell membrane), and the majority of these (34% of the *total*) are associated with surface vesicles rather than the nonvesicular surface membrane. In rabbit MPA
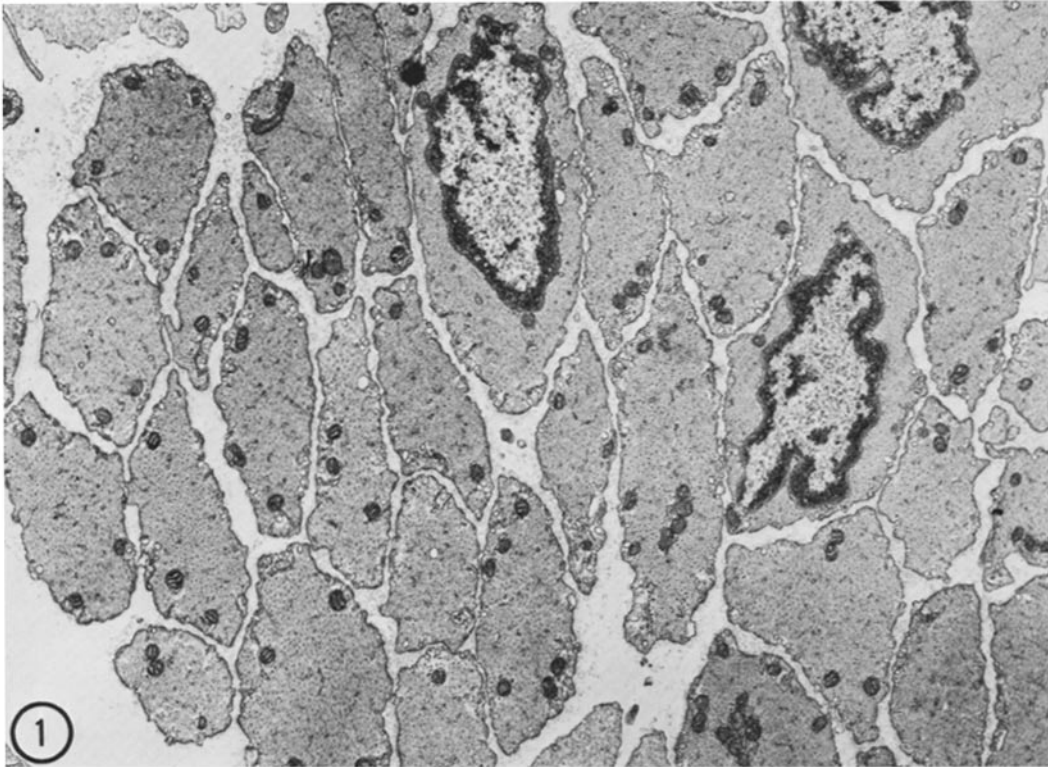
FIGURE 1 Transversely sectioned bundle of smooth muscle cells (outer, longitudinal muscle layer). The majority of the mitochondria are peripheral. In this micrograph approximately 50% are at a distance less than 50 nm from the surface vesicles or the nonvesicular cell membrane. Rabbit PAMV, incubated in Krebs' solution for 30 min, 2% glutaraldehyde fixative containing 4.5% sucrose, postfixation in 2% osmium, block stained with uranyl acetate, poststained with Pb citrate. × 11,100.

FIGURE 2 Longitudinally sectioned mitochondrion and adjacent surface vesicles (V). The distance between the outer mitochondrial membrane and the surface vesicles is 3–4 nm. Rabbit PAMV, incubated in Krebs' solution for 30 min, 2% glutaraldehyde fixative containing 4.5% sucrose, postfixation in 2% osmium, block stained with uranyl acetate, poststained with Pb citrate. × 216,000.

smooth muscle only 10% of the mitochondria were peripheral (50 cell profiles, three animals), the majority of the latter also being associated with the surface vesicles. Some of the difference between MPA and PAMV, however, is due to the larger total (including central) number of mitochondria in MPA smooth muscle (11 per cell profile in MPA vs. six per cell profile in PAMV).

Peripheral mitochondria formed close relationships with the surface vesicles: the average distance between the outer mitochondrial membrane and the inner surface vesicles membrane being 4-5 nm (Figs. 2, 3). To verify that these close relationships were not spurious, due to overlapping of two superimposed structures within the focal plane, longitudinal sections of PAMV were examined with specimen tilting. Tilting of the section from $+30°$ to $-30°$ (Fig. 3) were consistent with a close relationship between the two membrane systems: 3–5-nm gaps were abolished due to the superimposition of the two structures (mitochondrion and surface vesicles) by tilting, and previously superimposed structures became separated by a gap no greater than 5 nm. Further evidence of the close mitochondrial surface vesicle relationship was the indentation of some mitochondria by adjacent surface vesicles (Fig. 3), and the finding of similar close relationships in ultrathin (gray) sections.

A second type of peripheral relationship, found in approximately 15% of peripheral mitochondria of the MPA and PAMV, consisted of an element of SR interposed between the mitochondrial and the plasma membrane (Fig. 4).

Our preliminary observations on the close relationship between mitochondria and surface vesicles (Somlyo et al., 1972), illustrated here, suggested the possibility that mitochondrial-surface vesicle contacts may represent sites of transfer for cations, accumulated by mitochondria, into the extracellular space. The ensuing studies on the accumulation of barium and strontium, to be described below, were designed to explore the question of mitochondrial cation uptake in smooth muscle.

BARIUM-CONTRACTURE AND ELECTRON-OPAQUE MITOCHONDRIAL DEPOSITS IN SMOOTH MUSCLES EXPOSED TO BARIUM: Barium is a well-known smooth muscle stimulant (see Discussion), but the differences in the contractile response of different smooth muscles to Ba^{++} had not been previously appreciated.

Characteristic responses of rabbit MPA and PAMV smooth muscle to 10 mM Ba^{++} are shown in Fig. 5. While the Ba^{++} contracture in the MPA is tonic (maintained throughout the observation for 1 h), the response of the PAMV smooth muscle is relatively phasic, and relaxes to nearly base line by 1 h. Maintained Ba contractures were observed in 12 out of 13 MPA strips, and phasic contractures in 26 out of 34 PAMV strips. The remaining PAMV strips did not relax fully by the end of the 1 h observation period.

Electron micrographs of PAMV and MPA strips incubated for 1 h in 10 mM Ba^{++} containing Krebs' solution showed marked differences between the mitochondria in the two types of smooth muscle. In PAMV the mitochondria contained opaque deposits (Figs. 6, 7), but were otherwise normal. Only occasionally were some swollen and/or damaged mitochondria seen in these tissues. The illustrations shown in this section include material that has been block stained with uranyl acetate to visualize the membranes. Casual observation showed no major difference between the electron-opaque granules in unstained (osmium fixed) and in uranyl acetate-stained smooth muscles, although, as will be shown below, the nature of the granules turned out to be rather different when subjected to electron probe analysis.

In contrast to the relatively normal appearance of PAMV smooth muscle exposed for 1 h to Ba^{++}, many of the mitochondria in MPA smooth muscle similarly exposed to Ba^{++} for 1 h were massively swollen and their cristae obliterated (Figs. 11, 14). Since it was very likely, on the basis of previous studies of isolated mitochondria (Peachey, 1964; Caplan and Carafoli, 1965; Hackenbrock and Caplan, 1969), that the appearance of the mitochondria in MPA represented the end state of massive Ba^{++} accumulation and osmotic swelling, we have examined the appearance of MPA at various stages of exposure to Ba^{++}. As shown in Figs. 8–11, the mitochondria of MPA after 5-min exposure to Ba^{++} contain only a few, faint electron-opaque granules, while their number and electron opacity greatly increase after 13-min exposure to Ba^{++}, and resemble the appearance of PAMV smooth muscle after exposure to Ba^{++} for 1 h. The last micrograph taken in this sequence (Fig. 11) shows the final stage of massive mitochondrial swelling in the same MPA, after 1-h exposure to Ba^{++}.

INHIBITION OF BARIUM UPTAKE WITH METABOLIC INHIBITORS: If, as suggested from the properties of isolated mitochondria, Ba
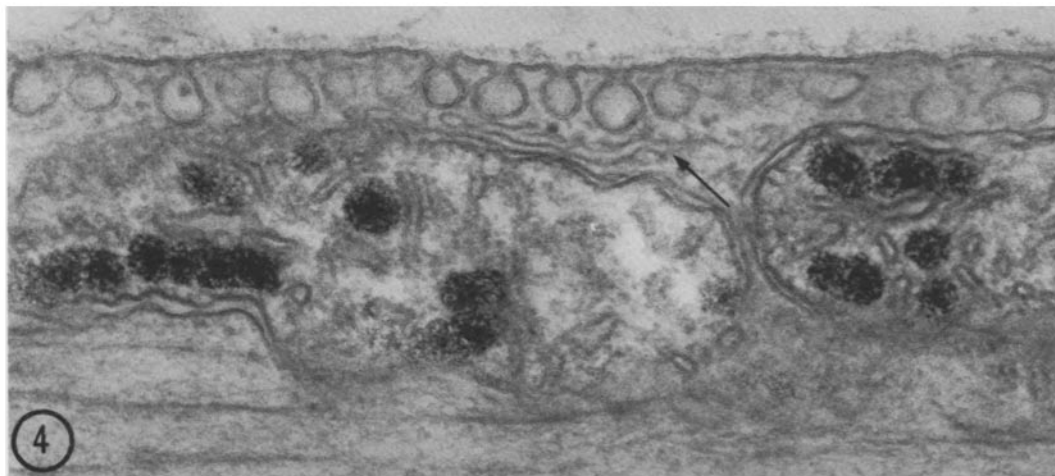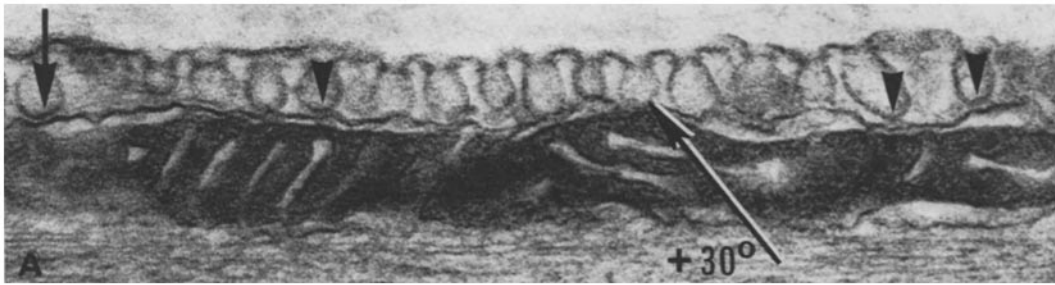
accumulation by mitochondria in smooth muscle is an active process, it should be blocked by metabolic inhibitors. The respiration-supported uptake of calcium or strontium by isolated mitochondria is blocked by respiratory inhibitors (e.g. cyanide or anoxia), while ATP-supported uptake is blocked by oligomycin (e.g. Brierley, 1963; Carafoli et al., 1965; Bielawski and Lehninger, 1966). We therefore made observations on smooth muscle exposed to both 10 $\mu g/ml$ oligomycin and 100% N_2 for 15 min before and during the 1-h incubation with Ba^{++}-containing solutions.

Oligomycin and anoxia prevented the appear-

ance of electron-opaque granules in PAMV smooth muscle exposed for 1 h to Ba^{++} (Figs. 12, 13). The massive swelling of the mitochondria, normally found after a 1 h Ba^{++} contracture in MPA smooth muscle, was also inhibited by oligomycin and anoxia, although small and irregular intramitochondrial granules were present (Figs. 14, 15) in some of the fibers. These may have been due to incomplete block of ATP-supported uptake or to passive entry of Ba^{++} (energy-independent binding).

Electron Probe X-Ray Microanalysis

IDENTIFICATION OF BARIUM IN MITOCHONDRIA AND ITS REPLACEMENT DURING BLOCK STAINING WITH URANYL ACETATE: The majority of the mitochondria analyzed contained, within the area covered by the probe, from one to four electron-opaque granules; while relatively large and electron-opaque single granules did generate a small Ba^{++} peak, the large peaks of the type illustrated in Fig. 16 were generated when four to five granules were excited. For a given counting interval, the size of the Ba^{++} X-ray peak (relative to background) increased with the number and electron opacity of intramitochondrial granules covered by the exciting beam.

The X-ray spectrum in the upper panel of Fig. 16 was obtained with the probe placed on a mitochondrion containing electron-opaque granules:
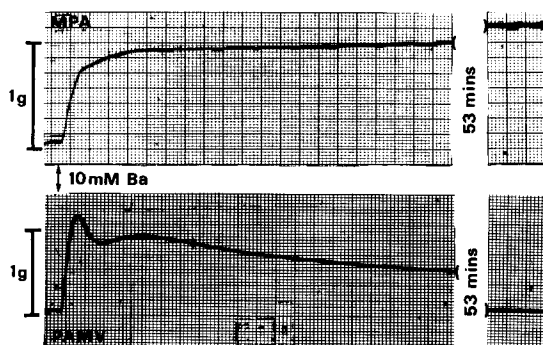

FIGURE 5 Isometric contractile responses of PAMV and MPA to 10 mM Ba^{++}-Krebs' solution. MPA tension is still maintained by 60 min while the phasic PAMV has relaxed.

FIGURE 3 a-c A section of a portion of a vascular smooth muscle cell containing a longitudinally running mitochondrion in close relationship to surface vesicles. The distance between the outer mitochondrial membrane and the surface vesicles is 3–4 nm. This section has been photographed at 0° tilt and +30° and −30° tilt in the Y axis (the direction of tilt is indicated by the long arrows). The images indicating the relationship between the surface vesicles and the outer mitochondrial membrane change. At 0° tilt (Fig. 3 b) several vesicles and mitochondrial membrane profiles (arrowheads) are clearly resolved, but in the +30° and −30° tilt directions (Fig. 3 a and 3 c, respectively) the vesicle and mitochondrial membrane profiles overlap or are not clearly resolved, indicating that their images at 0° tilt (Fig. 3 b) probably represent the correct relationship. In other cases, the membranes are more easily resolved in the image resulting from +30° tilt (Fig. 3 a, short arrow), indicating that this vesicle and mitochondrion (obliquely sectioned at one point) appear to overlap in the 0° tilt (Fig. 3 b and −30° tilt (Fig. 3 c). In the +30° tilt (Fig. 3 a) and 0° tilt (Fig. 3 b) some vesicles appear to have a "diaphragm" at the opening, but in the images from the −30° tilt (Fig. 3 c) the same vesicles have openings, indicating that the diaphragm is due to super-imposed images. Rabbit PAMV incubated in Krebs' solution for 30 min, 2% glutaraldehyde fixative containing 4.5% sucrose, postfixation in 2% osmium, block stained with uranyl acetate, poststained with Pb citrate. All micrographs × 100,000.

FIGURE 4 High magnification view of mitochondria containing electron-opaque granules, close to the surface vesicles. The granules appear to lie in the mitochondrial matrix space. There is an element of the SR (arrow) lying between one of the mitochondria and the surface vesicles. Rabbit PAMV incubated for 60 min in 10 mM Ba^{++}-Krebs' solution. Fixed in 2% osmium tetroxide. Stained en bloc with uranyl acetate. × 105,000.
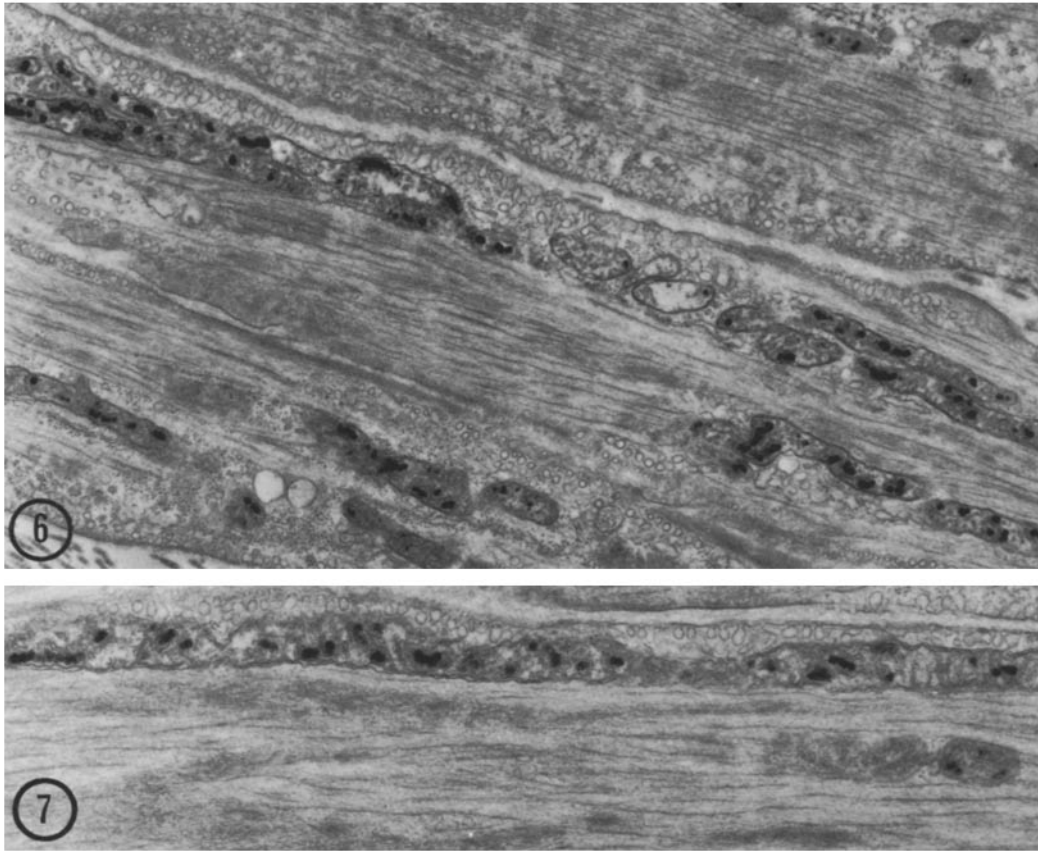
FIGURE 6 Longitudinal section through smooth muscle cells with mitochondria containing electron-opaque granules. Rabbit PAMV incubated for 60 min in 10 mM Ba^{++}-Krebs' solution. Fixed in 2% osmium tetroxide, block stained with uranyl acetate. × 17,000.

FIGURE 7 A longitudinally running mitochondrion (6.0 μm long within the section) adjacent to the surface vesicles and the cell membrane, containing electron-opaque granules. Rabbit PAMV incubated for 60 min in 10 mM Ba^{++}-Krebs' solution. Fixed in 2% osmium tetroxide, block stained with uranyl acetate. × 23,000.

peaks are present at the energy levels corresponding to the Ba^{++} L$_\alpha$, L$_\beta$, and L$_\gamma$ lines. When the probe was moved off the mitochondrion to the adjacent cytoplasm, the characteristic lines generated by Ba^{++} were no longer detectable, as shown in the lower panel of Fig. 16. This preparation also showed, as all osmium-fixed tissues, a large peak corresponding to the osmium M$_\alpha$ line and an overlapping phosphorus K$_\alpha$ line. In this, as in the other tissues analyzed, the osmium peaks were larger over the mitochondria than over the cytoplasm, presumably reflecting the greater amount of osmiophilic material in the mitochondrial membranes and, possibly, incorporation of Os into the granules. The chlorine K$_\alpha$ line detectable in all of our material could be ascribed to the Cl content of

the embedding medium, and these counts showed no significant variation over a given section.

A dramatic change in the X-ray spectrum was produced by block staining the Ba^{++}-loaded tissues with aqueous uranyl acetate although, as noted earlier, the electron-optical image of the intramitochondrial granules was not noticeably changed by this procedure. The upper and lower panels of Fig. 17 show spectra from paired strips of the same portal vein loaded with Ba^{++} before fixation, respectively, with and without block staining of the fixed tissue with uranyl acetate. In both instances the probe was placed over mitochondria containing several electron-opaque granules. In the block-stained preparation (upper panel), a very prominent uranium M line (actually

a combination of the $M_{\alpha 1}$ and $M_{\beta 2}$ lines) was present without a significant Ba^{++} emission, while in the paired unstained preparation the characteristic Ba^{++} L lines were present (lower panel). These findings clearly indicate the replacement of Ba^{++} with uranium during block staining. The uranium peak was more prominent over the mitochondrial granules (14,312 counts, peak P minus background b) than over the cytoplasm (3,237 counts, P − b, spectrum not shown).

To verify independently the removal of Ba by block staining, the Ba content of fixed tissues was determined via atomic absorption spectroscopy. The tissue Ba contents of osmium-fixed tissues were 26.4 $\mu M/g$ wet weight ± 9.9 SE (n = 4) before and 1.8 $\mu M/g$ wet weight ± 0.7 SE (n = 4) after block staining with uranyl acetate (P = <0.001).

THE ASSOCIATION OF BARIUM WITH PHOSPHORUS IN INTRAMITOCHONDRIAL GRANULES: Since it seemed probable that Ba^{++} would be deposited as some form of phosphate in mitochondrial granules, we have determined the correlation of Ba with P through X-ray microanalysis of intramitochondrial granules in Ba-loaded PAMV fixed with glutaraldehyde, to eliminate interference with the P signal by osmium. A representative electron micrograph of such a preparation is shown in Fig. 18. Fig. 19 is an example of the spectrum over intramitochondrial granules, and shows both the characteristic Ba and P lines. The lower panel of this figure shows the same spectrum after the Sn lines, generated by the specimen rod, have been subtracted. There remains (lower panel) at 3.69 keV a small, but significant Ca K_{α} peak, also confirmed with diffractive analysis of the same spot with the PET crystal tuned to the Ca K_{α} wavelength.

In this, as in the osmium-fixed material, the number and electron opacity of the granules correlated with the X-ray peaks, and no Ba signals were detected over areas of cytoplasm.

The results of 18 analyses showing the peak and peak minus background counts in the Ba L_{α} and P K_{α} peaks, as well as the $(P - b)_{Ba}/(P - b)_{P}$ ratios, are shown in Table I. The average ratio of the respective peak counts was 1.1 : 1.

Regression analysis of Ba^{++} (P − b) counts vs. P (P − b) counts was performed on the 15 mitochondria subjected to 300-s counts tabulated in Table I. The regression line calculated was P_{counts} = 0.92 Ba^{++}_{counts} + 85. The Y intercept

(85 counts in 17 channels) is not significantly above background, and the line can be considered effectively to be passing through the origin. The correlation coefficient was 0.89, highly significant $P < 0.001$.

To obtain a correlation between the ratio of the X-ray peaks and the ratio of the elemental concentration of Ba^{++} vs. P, Ba orthophosphate $(BaHPO_4)$ standards were analyzed with the electron probe. The $Ba^{++}_{(P-b)}/P_{(P-b)}$ of this standard, containing an elemental ratio of 1:1, was 1.55 ± 0.045 SE (n = 12). Since elemental ratios are linearly proportional to the count ratios (Hall, 1971), it can be estimated that the peak count ratio (1.1:1) observed over intramitochondrial granules corresponds to an elemental ratio of approximately 3 $Ba^{++}/4$ P.

ANALYSIS OF STRONTIUM-LOADED MITOCHONDRIA CONTAINING CALCIUM: Electron probe analysis of smooth muscle loaded with Sr before fixation revealed the characteristic Sr^{++} K_{α} line in the X-ray spectrum when the probe was placed over mitochondria containing electron-opaque granules, as shown in the upper panel of Fig. 20. The lower panel shows the absence of the Sr^{++} emission after the probe was moved from the mitochondrion to the cytoplasm of the same fiber. In addition to the Sr signal, there was a very prominent Ca^{++} K_{α} signal generated over the mitochondrion-containing granules; this signal was also absent when the adjacent cytoplasm was analyzed.

Although the exciting spot did not cover more than the diameter of a single mitochondrion in these studies, some of the mitochondria analyzed formed part of a cluster, and it may be argued that the association of strontium with calcium in the X-ray spectrum reflected signals generated from two adjacent mitochondria. To eliminate this possibility, X-ray counts were obtained over single mitochondrial profiles that were found without adjacent mitochondria. An example of such an analysis is shown in Fig. 21, that again clearly indicates the coexistence of the Sr^{++} and Ca^{++} emissions. It is clear that microanalytically detectable calcium and strontium stores are present within a single mitochondrion, although the X-ray spatial resolution of the present studies would not permit us to determine whether the two elements form part of a single, mixed granule.

The results of analyses similar to those shown in Figs. 20 and 21 are tabulated in Table II, and show
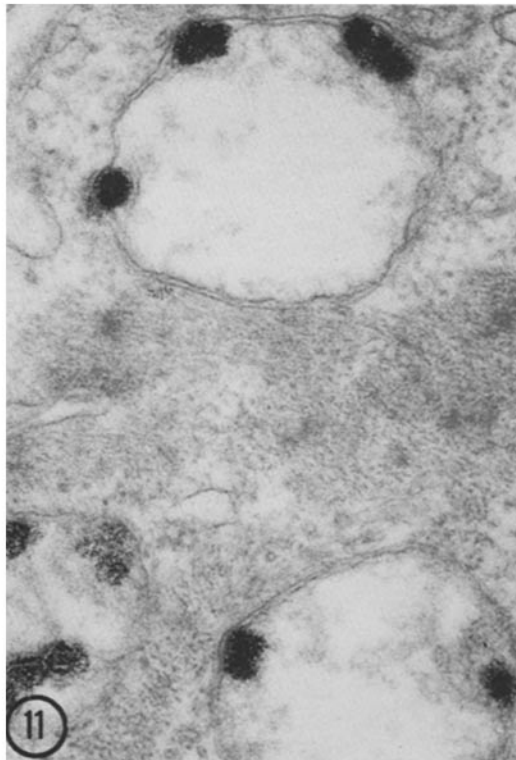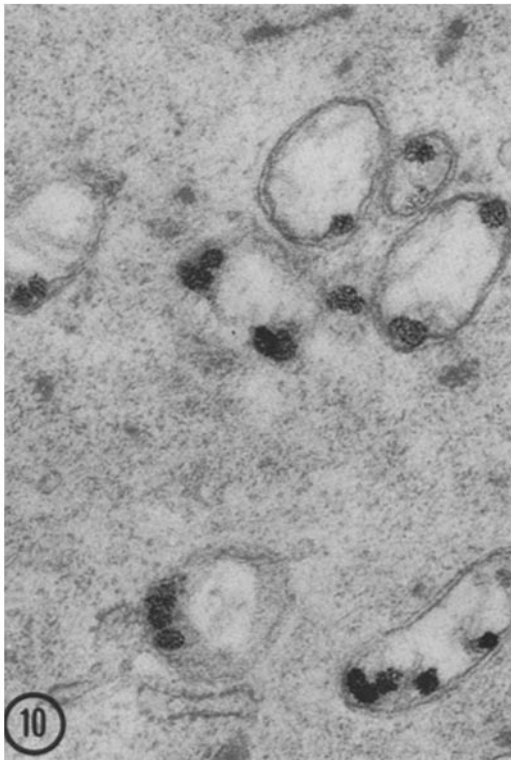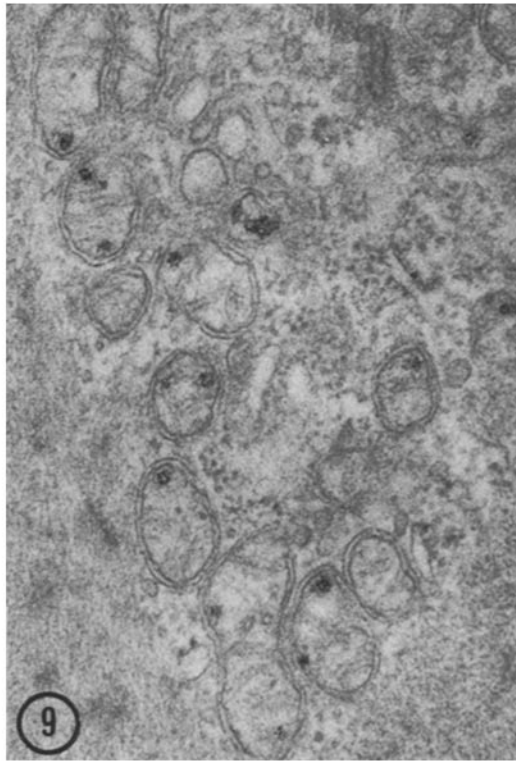
the coexistence of Sr^{++} and Ca^{++} signals originating from the mitochondria-containing granules.

Finally a few electron-opaque deposits were also analyzed that were not in mitochondria, but in peripheral elements of the SR. An example of such an analysis (Fig. 22) shows a peak in the Sr^{++} K_α region, the small size of which is consistent with the small size and electron opacity of the deposit analyzed.

DISCUSSION

Ultrastructure, Mitochondrial Cation Uptake, and Ba Contractures

The close (4–5 nm) proximity of mitochondria to the surface vesicles of vascular smooth muscle, verified through specimen tilting, is compatible with a functional relationship, such as the extracellular transport of ions accumulated by mitochondria (Somlyo et al., 1971; Goodford and Wolowyk, 1972). The distance between the mitochondrial and the surface vesicle membranes is less than that (10–12 nm) between the SR and the nonvesicular surface membrane at the couplings (Somlyo et al., 1971; Devine et al., 1972, 1973). The absence of apparent connections between the mitochondrial and surface vesicular membranes, except for occasional and possibly random electron opacities, does not necessarily rule out a specialized function of these sites. Mitochondria, by discharging accumulated ions in the vicinity of the surface membrane, may generate local variations of transmembrane ionic gradients and thereby facilitate ion extrusion. Localization of transport enzymes to the surface vesicles would further contribute to the transport function of the mitochondrial-surface vesicle complex. There is some evidence, subject to the uncertainty of the histochemical techniques involved, that ATPase is localized at the surface vesicles (Santos-Buch, 1966; Hoff, 1968).

Statistical proof of the significance of the mito-chondrial-surface vesicle relationship is difficult to obtain, as it is possible that mechanical limitations (e.g., insertion of filaments on and around dense bodies, Somlyo et al., 1973) may prevent the mitochondria from forming close relationships with some regions of the plasma membrane not occupied by surface vesicles. Nor should one infer a fixed relationship of the mitochondria to the surface vesicles, since as in other cells (Barasa et al., 1973) mitochondria in smooth muscle may also undergo slow intracellular movements. The morphological relationship described in the present study merely suggests that the possibility of the functional relationship between the two structures deserves further exploration.

The accumulation of electron-opaque intramitochondrial granules in smooth muscle exposed to Ba-containing solutions and its inhibition by oligomycin[1] and anoxia indicates, in conjunction with electron probe analysis (see below), the energy dependent mitochondrial uptake of divalent cations with phosphate in intact smooth muscle. This aspect of cation transport has been well characterized in mitochondria isolated from several systems (see Introduction). It has also been specifically shown that Ba accumulation by liver mitochondria is energy dependent (Vainio et al., 1970), and that mitochondria isolated from smooth muscle accumulate Ca (e.g., Batra, 1973). The accumulation of Ba by PAMV smooth muscle in the absence of significant mitochondrial damage, and the electron microscope detection of Ba deposits, will provide a convenient means for further study of factors affecting mitochondrial cation movements in situ. We have blocked Ba uptake by the combined use of oligomycin and anoxia to inhibit, respectively, ATP- and respiration-sup-

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[1] Our experimental method, unlike studies of isolated mitochondria, can not exclude the possibility that metabolic inhibitors may also reduce the influx of Ba^{++} through the plasma membrane.

FIGURE 8–11   The time-course of barium accumulation by mitochondria of the main pulmonary artery after incubation in 10 mM Ba^{++}-Krebs' solution. Fig. 8: 2-min incubation in Ba^{++}-Krebs'. Fig. 9: 5-min incubation in Ba^{++}-Krebs'. Small electron-opaque deposits are present in the mitochondria. Fig. 10: 13-min incubation in Ba^{++}-Krebs'. There is a greater accumulation of barium in the mitochondria and the mitochondria are swollen. Fig. 11: 60-min incubation in Ba^{++}-Krebs'. The mitochondria are grossly swollen and large deposits of barium are present. All tissues in Figs. 8–11 are from strips of the same rabbit MPA incubated for various times in 10 mM Ba^{++}-Krebs' solution. Fixed in 2% osmium tetroxide. All micrographs × 60,000.
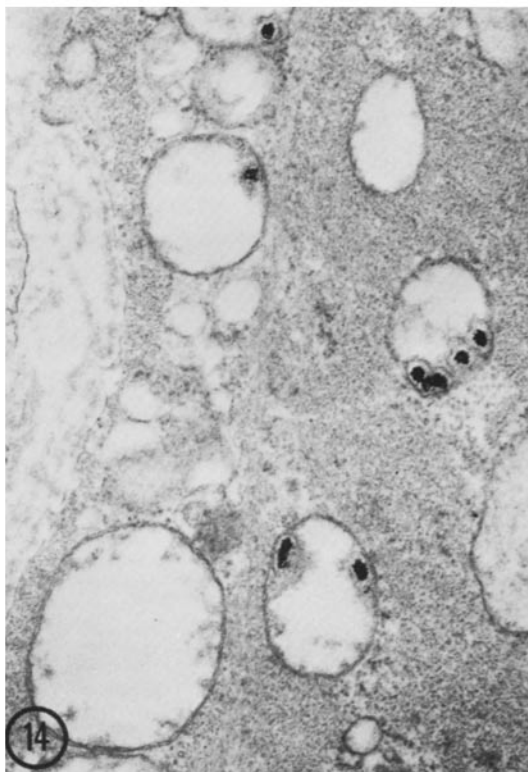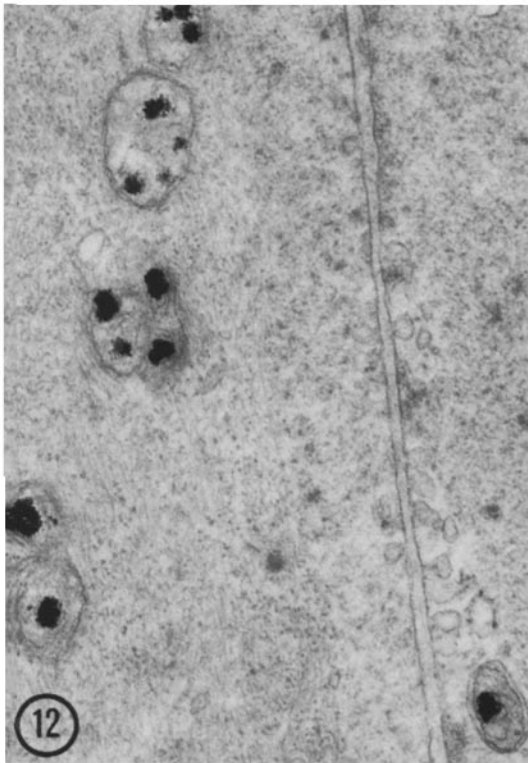
ported ion transfer. It will be of interest to determine which one of these transport mechanisms dominates in the intact muscle. Preliminary observations (A. V. Somlyo and A. P. Somlyo, unpublished) suggest that the inhibition of Ba uptake in the rabbit PAMV is largely due to oligomycin, rather than to anoxia.

The contractile responses elicited by Ba and the ultrastructure of the mitochondria after 1-h exposure to Ba were different in the two types of vascular smooth muscle. The tonic Ba contracture of the MPA smooth muscle and the more phasic response of PAMV smooth muscle resemble the contractile responses of these two preparations to high K solutions (Somlyo et al., 1969). The tonic contractures were accompanied by marked swelling of the mitochondria in MPA smooth muscle, while the more phasic Ba contractures in the PAMV smooth muscle were associated with good preservation of the mitochondrial ultrastructure. The inhibition of mitochondrial swelling in MPA smooth muscle by metabolic inhibitors is consistent with the explanation that the swelling is an osmotic effect due to massive accumulation of Ba.[2] The most economical interpretation is that during the tonic contracture there is a well-maintained Ba influx exceeding the uptake capacity of MPA mitochondria, while during the phasic contracture of PAMV there is a gradual reduction in Ba influx and mitochondrial uptake capacity is not exceeded. In cardiac muscle, the effects of Ba on the membrane conductance are transient (Hermsmeyer and Sperelakis, 1970) as is the calcium influx stimulated by Ba in taenia coli smooth muscle (Karaki et al., 1967). The mitochondrial damage caused by Ba in MPA smooth muscle is clearly

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[2] Increased mitochondrial membrane permeability to other ions, produced by Ba^{++} (Drahota et al., 1969), may also contribute to swelling.
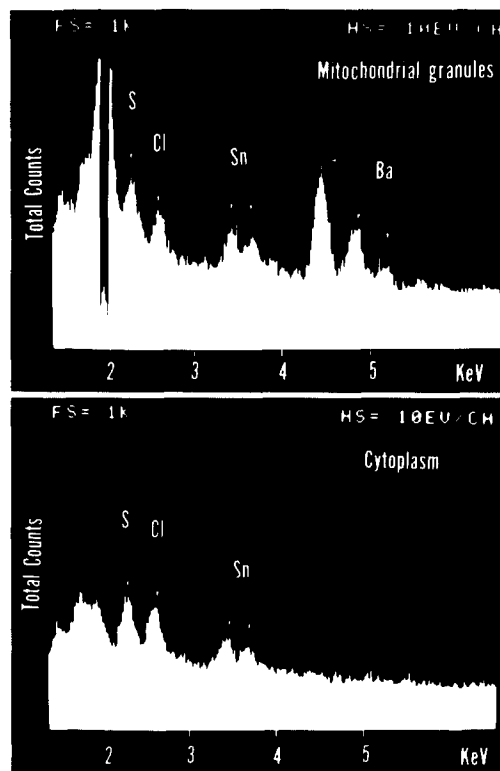

FIGURE 16   X-ray emission from rabbit PAMV smooth muscle incubated in 10 mM Ba^{++}-Krebs' and fixed in 2% osmium tetroxide. Upper panel: spectrum over mitochondrion containing four to five electron-opaque granules. Lower panel: spectrum obtained over cytoplasm of the same fiber. Note the prominent Ba L lines over the mitochondrial granules, and the absence of these peaks over the cytoplasm. The break in the Os-P peak (upper panel) reflects the accumulation of X-ray counts in excess of full scale.

suggestive of markedly impaired oxidative phosphorylation, and may explain the dependence of Ba contractures on glycolytic substrates (Altura and Altura, 1970).

FIGURES 12-15   The effect of oligomycin and anoxia on barium accumulation by mitochondria in PAMV and in MPA. Paired strips from the same PAMV (Figs. 12 and 13) and MPA (Figs. 14 and 15) were incubated for 60 min in Ba^{++}-Krebs' solution. Fig. 12: Opaque deposits of barium in mitochondria in PAMV. Fig 13: No granules are found in the mitochondria of PAMV smooth muscle treated with oligomycin and 100% N$_2$, and the small "flakes" of electron opacity were seen in only some of the fibers. Fig. 14: Massive swelling of mitochondria containing large electron-dense bodies in Ba^{++}-loaded MPA. Fig. 15: Mitochondria, without massive swelling, containing only small electron-opaque deposits, in MPA smooth muscles treated with oligomycin and 100% N$_2$. Many other fibers in this block contained no detectable Ba^{++} deposits. Rabbit PAMV and MPA fixed in 2% osmium tetroxide. × 50,000
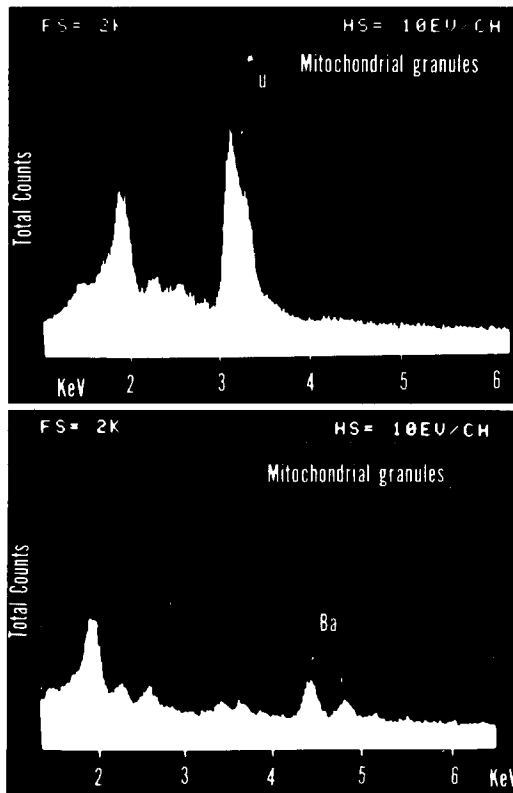
FIGURE 17 The effect of block staining with uranyl acetate on the X-ray emission spectra of electron-opaque intramitochondrial granules in Ba-loaded smooth muscle. Upper panel: spectrum over mitochondrion containing four very electron-dense granules; there is a large uranium peak, but no evidence of Ba. Lower panel: spectrum obtained over mitochondrion containing five electron-opaque granules in a strip of smooth muscle from the same PAMV as the upper panel, but analyzed without block staining. The characteristic Ba peaks are present, while the U peak is absent. Rabbit PAMV incubated in 10 mM Ba-Krebs', 2% osmium fixation.

The mechanisms of the contractile effect of Ba^{++} on smooth muscle have not been clearly established, but it is unlikely that Ba directly replaces calcium in the contractile process. The contractile effect of Ba on taenia coli (Karaki et al., 1967), arterial (Northover, 1968), and PAMV (A. V. Somlyo and A. P. Somlyo, unpublished observations) smooth muscle is markedly reduced in calcium-free solutions, and the affinity of Ba for troponin is very low (Ebashi, 1971).

It is probable that Ba contractures of smooth muscle are, at least partly, mediated by an influx of extracellular Ca^{++}. The persistence of some contractile effect on uterine (Daniel, 1963), MPA, and PAMV (A. V. Somlyo and A. P. Somlyo, unpublished observation) smooth muscle in the absence of extracellular Ca^{++} is more readily explained by intracellular translocation of calcium by Ba from the SR and/or the mitochondria. Under normal conditions, depolarization of smooth muscle by Ba^{++} (Bülbring and Tomita, 1969; Sakamoto and Kuriyama, 1970) may trigger Ca^{++} release from the SR. In comparison with Ba, strontium behaves more like Ca^{++} as it is taken up by SR (see Introduction), activates striated muscle actomyosin (Edwards et al., 1966), and can also support contractions of PAMV in the absence of extracellular calcium (A. V. Somlyo and A. P. Somlyo, unpublished observation).

Electron Probe X-Ray Microanalysis

The principles of energy dispersive electron probe X-ray microanalysis, the identification of elements ($Z \geq 11$) by the characteristic X-ray energy spectra emitted by them in response to bombardment by electrons, have been reviewed elsewhere (Hall, 1971; Hall et al., 1972; Beaman and Isasi, 1972). Biological X-ray microanalysis, both wave diffractive and energy dispersive, has been particularly useful for the chemical identification of localized intracellular deposits such as calcium oxalate crystals in striated muscle (Podolsky et al., 1970) and Ca^{++}, Mg^{++}, K, and P in refractive bodies of Amoeba and Tetrahymena (Coleman et al., 1972, 1973). The high sensitivity of the energy dispersive detectors permits the use of the relatively low levels of exciting currents available in scanning and transmission electron microscopes for microanalysis (e.g., Russ, 1972). This technique has been used to identify Ca^{++} and P within single intramitochondrial granules in chondrocytes (Sutfin et al., 1971).

Energy spectra clearly diagnostic of Ba^{++} were recorded over mitochondria containing electron-opaque granules in vascular smooth muscle exposed to Ba^{++}. The presence of electron-opaque granules in such tissue after glutaraldehyde fixation is, in itself, indicative of their Ba^{++} content.[3] After osmium fixation, however, electron probe analysis is required for conclusive distinction between osmiophilic structures and other deposits.

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[3] The possible presence of other components, in particular Ca^{++}, can only be established through electron probe analysis.
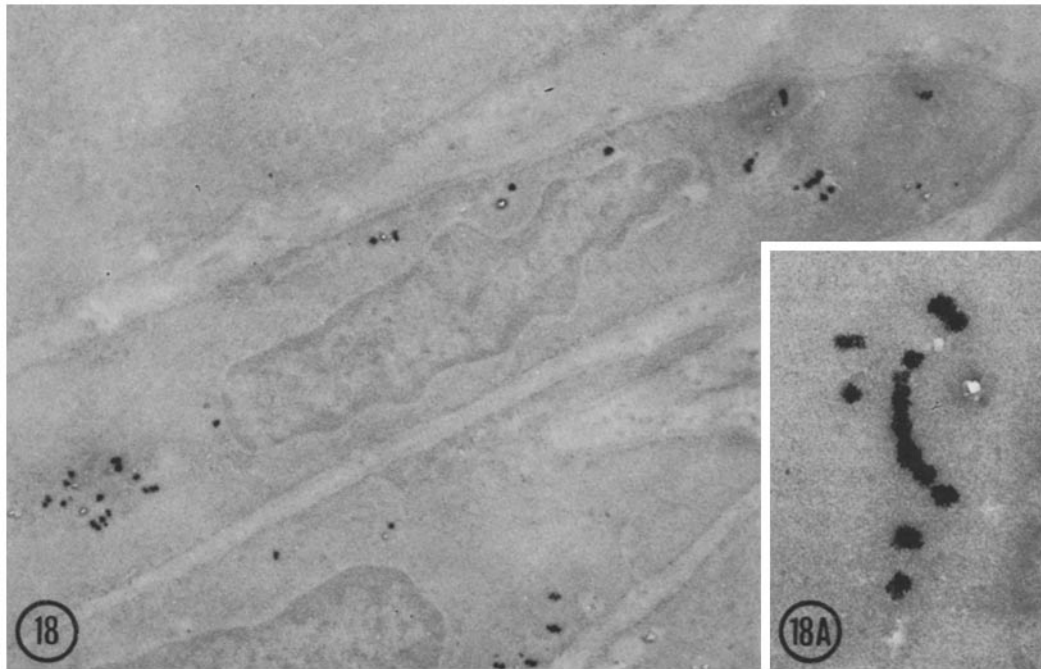
FIGURE 18 Appearance of Ba-loaded smooth muscle after glutaraldehyde fixation: oblique transverse section showing electron-opaque granules in PAMV smooth muscle incubated in Ba-HEPES solution containing PO_4. Such granules were not present in control tissues, not incubated with Ba. The small holes in the section were frequently seen in Ba-loaded smooth muscle after glutaraldehyde fixation, and presumably represent sites from which granules were removed during sectioning. (Similar holes have been observed in glutaraldehyde-fixed, Sr-loaded mitochondria by Greenawalt and Carafoli, 1966.) × 19,800.

FIGURE 18 a Higher magnification view of electron-opaque deposits in the same tissue. PAMV fixed in 2% glutaraldehyde. × 60,000.

Our results with electron probe X-ray microanalysis of Ba^{++}- and Sr^{++}-loaded smooth muscle indicate that observations on electron-opaque granules and deposits in mitochondria and SR, in fixed tissue, provide a valid measure of Ba and Sr localization.

The potential effect of preparative procedures on the ion content of intramitochondrial granules was dramatically demonstrated through the analysis of Ba^{++}-loaded smooth muscles block stained, *after fixation*, with uranyl acetate. In spite of the marked similarity of the electron images of granules in stained and in unstained tissues, the X-ray spectra clearly showed that the Ba content of the granules was replaced by uranium during staining. It is very likely that such exchange was facilitated by the acid pH of the saturated aqueous uranyl acetate solution, as barium orthophosphates would be soluble in acid media.

The association of Ba with P, clearly demon-

strated through X-ray microanalysis of glutaraldehyde-fixed material, is consistent with the accumulation of divalent cations in the form of phosphates in isolated mitochondria (Greenawalt et al., 1964; Brierley and Slautterback, 1964; Carafoli et al., 1965). The Ba/P atomic ratio of 3:4 is not characteristic of any of the natural barium orthophosphates, and may represent a mixture of Ba orthophates and Ba salts of adenine nucleotides. The Sr/P ratio in isolated mitochondria accumulating Sr is also not that of any of the natural strontium phosphates (Carafoli et al., 1965).

The intercept of the regression analysis of the correlation of Ba with P suggests that all the phosphorus detected by X-ray microanalysis in mitochondria is associated with Ba. This could reflect either a low sensitivity of our methods for determining phosphorus in the concentrations normally present in mitochondria and not associated with Ba, or the extraction of intrinsic mitochondrial P
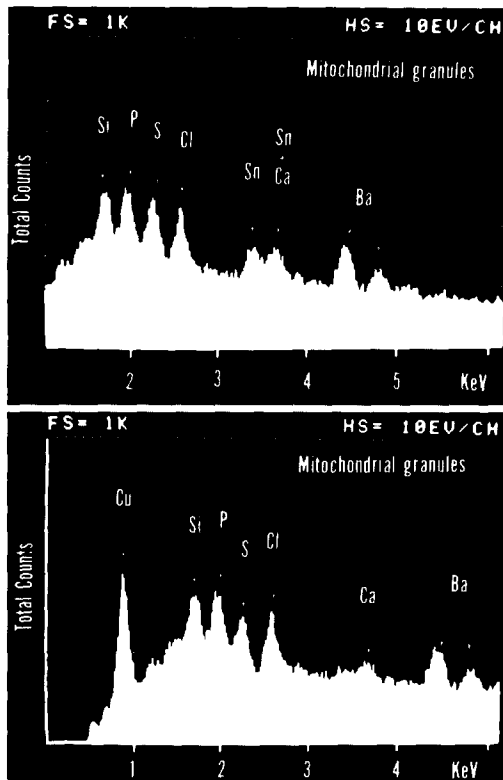
FIGURE 19 X-ray emission spectrum showing the association of Ba and P in mitochondrial granules. Upper panel: before subtraction. Lower panel: after subtraction of the interfering Sn peak. Note the prominent Ba and P signals, and the small but significant Ca peak found after subtraction of the interfering Sn peak. The spectrum was obtained over a mitochondrion containing approximately four electron-dense granules. The presence of Ca was confirmed by diffractive analysis of the same spot with PET crystal. Rabbit PAMV incubated in 10 mM Ba-Krebs', 2% glutaraldehyde fixation.

PAMV suggests, if one can extrapolate from the relationship between ultrastructure and Sr accumulation (Greenawalt and Carafoli, 1966), an accumulation in excess of 475 ng-atoms Ba/mg mitochondrial protein. According to the electron probe results of a ratio of 3 Ba/4 P, this would imply an accumulation of P_i in excess of 632 nmol/mg protein. This is greatly in excess of an estimated endogenous P_i of 10–40 nmol/mg mitochondrial protein (Chappell et al., 1963; Carafoli, 1965 b). Therefore, the additional phosphate accumulated with Ba^{++} may have originated from the cytoplasm or have been liberated from ATP (Bielawski and Lehninger, 1966) during ATP-supported cation uptake *in situ*.

Electron probe X-ray microanalysis detected calcium in association with Sr and less frequently

during fixation and dehydration. Most of the intrinsic mitochondrial phosphorus is in the form of phospholipids (Vasington and Murphy, 1962; Chappell et al., 1963) susceptible to extraction during preparative procedures. Mitochondrial P is detectable by electron probe X-ray microanalysis of frozen sections (Weavers, 1972). Osmium fixation also removes 80% of P_i associated with Mg and 40–50% of that associated with Ca in isolated mitochondria (Brierley and Slautterback, 1964). It is probable, therefore, that the exclusive association of P and Ba in the present study was due to the extraction of intrinsic mitochondrial phosphorus during specimen preparation.

The number and size of granules in Ba-loaded

TABLE I

Electron Probe Analysis over Mitochondria Containing Electron-Opaque Granules

Barium counts		Phosphorus counts		$(P - b)_{Ba^{++}}$ / $(P - b)_P$
P	P – b	P	P – b	
1,345*	466	1,492	268	1.7
1,197*	367	1,525	580	0.6
9,467‡	2,758	11,279	2,526	1.1
10,919	3,836	12,002	3,545	1.1
9,023	1,825	10,503	1,455	1.3
10,657	1,808	10,647	1,290	1.4
3,146	410	4,401	334	1.2
3,254	904	4,856	919	1.0
2,186	1,131	2,032	592	1.9
4,143	1,202	5,697	1,002	1.2
8,053	1,181	8,711	1,269	0.9
5,895	2,084	7,686	2,384	0.9
5,755	1,751	7,541	1,535	1.1
8,841	1,769	9,736	2,670	0.7
8,128	1,693	9,294	1,264	1.3
5,737	675	6,477	559	1.2
6,110	403	7,426	847	0.5
8,457	1,396	9,057	1,836	0.8

Rabbit portal-anterior mesenteric veins incubated in 10 mM Ba-containing solutions before glutaraldehyde fixation. Sections from five blocks obtained from three animals were analyzed.

Probe size: 200–300 nm. Current: 20 nA. Accelerating voltage 40 kV. Live counting time: 300 s except * = 80 s, ‡ = 200 s.

P = peak, the number of counts under the elemental peak integrated over the counting interval; b = background counts integrated over the same interval.

with Ba, in mitochondria of vascular smooth muscle. This observation, while certainly not sufficient proof of a physiological role of mitochondria in regulating intracellular Ca^{++} in smooth muscle,
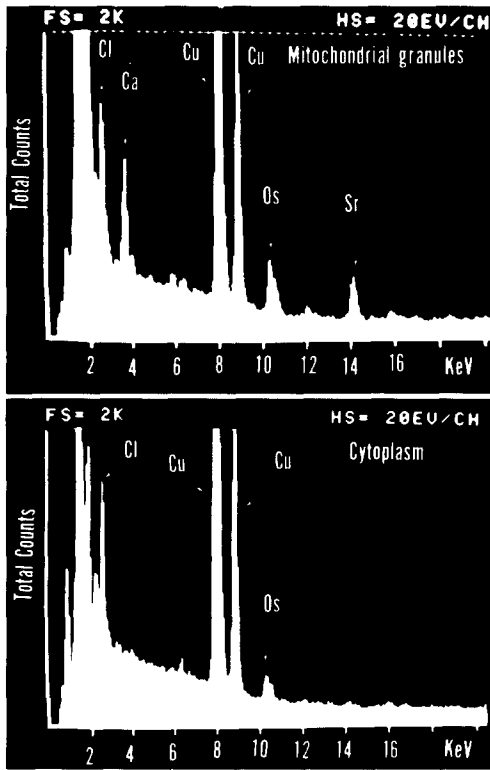

FIGURE 20 X-ray spectrum of smooth muscle incubated in strontium-containing solution before fixation. Upper panel: spectrum over mitochondrial electron-opaque granules, showing the prominent Sr and Ca peaks. Lower panel: the spectrum over the cytoplasm showing the absence of both Ca and Sr peaks. Rabbit PAMV, osmium vapor fixation.
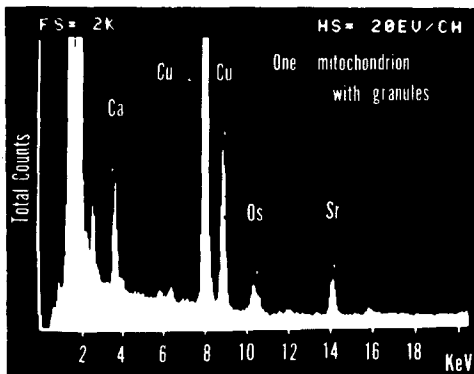

FIGURE 21 Coexistence of Ca and Sr peaks over a mitochondrion not forming part of a cluster. Same tissue block as Fig. 20.
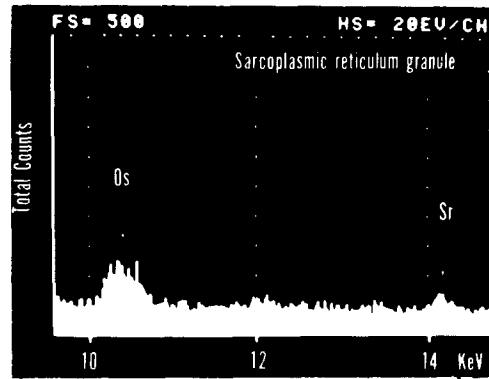

FIGURE 22 X-ray spectrum from SR, containing electron-opaque granule, in PAMV smooth muscle incubated in Sr-Krebs' solution; osmium vapor fixation.

TABLE II

Electron Probe Analysis over Mitochondria Containing Electron-Opaque Granules

Live counting time	Calcium counts		Strontium counts	
	P	P – b	P	P – b
s				
300	‡		9,917	3,717
300	‡		5,733	2,647
100	‡		3,162	1,926*
100	16,255	6,693	5,262	3,412*
50	6,686	1,383	2,179	1,114
50	6,561	2,043	2,440	1,507
50	7,703	3,128	2,557	1,486
50	13,854	6,083	4,691	3,130
50	12,388	5,235	4,169	2,633*

Rabbit portal-anterior mesenteric vein incubated in 10 mM Sr-containing solution before osmium vapor fixation.

Probe size: 200–300 nm. Current: 20 nA. Accelerating voltage 40 kV.

P and P – b as defined in Table I.

* In these experiments the electron probe was subsequently moved off the mitochondria onto the cytoplasm and no Sr signal was obtained.

‡ Calcium counts were not done due to Sn interference from the specimen holder. All other counts were obtained with aluminum inserts in the specimen holder, eliminating Sn emission.

is at least compatible with such a function. It is noteworthy that the prominent Ca emission lines originating from mitochondria in Sr-loaded muscles were due to intrinsic Ca, as no Ca was added to either the Sr-Krebs' incubation medium or to the fixative. The ability of the electron probe to

detect calcium and other cations in mitochondria suggests that this may be a useful technique for the study *in situ* of mitochondrial ion movements in smooth muscle.

Note Added in Proof: More recently we have been able to show by electron probe X-ray microanalysis (with a Philips EM 301, equipped with an energy dispersive detector), significant quantities of mitochondrial calcium associated with phosphorus in unfixed, unstained frozen-dried thin sections of normal mouse heart muscle (A. P. Somlyo, A. V. Somlyo, and J. Silcox, unpublished observation).

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