

CALCIUM IN SMOOTH MUSCLE SARCOPLASMIC RETICULUM *IN SITU*

Conventional and X-Ray Analytical Electron Microscopy

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During the past few years it has become increasingly apparent that the sarcoplasmic reticulum (SR) of smooth muscle cells is sufficiently well developed and organized in its relationship to other subcellular components. Consequently, it has been suggested that the SR might play a role in smooth muscle similar to that in skeletal muscle in the storage and release of calcium, thereby controlling the contraction-relaxation cycle (4-6, 9, 10, 18, 19, 27, 31, 33). Indeed, there is circumstantial evidence in the literature to support this interpretation. The SR can accumulate strontium when the vascular smooth muscle is incubated for 1 h in a strontium-rich solution (10, 32, 33). Several groups (2, 7, 17, 22, 36) have found *in vitro* an energy-dependent calcium uptake by microsomal preparations isolated from a variety of smooth muscles, but such calcium uptake experiments with microsomes cannot be attributed exclusively to the SR (27). Although cytochemical studies have shown calcium localization in the smooth muscle SR *in situ* (27-29, 35), no direct evidence for calcium release from the SR on stimulation and/or calcium accumulation by the SR on return to the resting state has yet been provided.

We have recently demonstrated the fine structural localization of calcium in skeletal (11, 12) and cardiac (13-15) muscle, using a precipitation method with potassium oxalate. The reliability of the oxalate to mark the cellular sites of calcium storage has been proved by chelation with EGTA (11-13, 15), electron probe microanalysis (11, 12, 25), and correlated X-ray and electron diffraction studies (15). As an extension of our previous works, the present investigation was undertaken to demonstrate calcium accumulation inside the SR

of the smooth muscle cells under relaxation by using the same oxalate method. The principle of this method consists in the immobilization of calcium ions *in situ*, by precipitation with oxalate, as electron-opaque deposits of calcium oxalate. However, the polarized resting membrane of muscle is virtually impermeable to the oxalate anion. Therefore, muscle strips were incubated in a high-K medium containing oxalate to induce depolarization which increases membrane permeability. X-ray analytical electron microscopy was performed in order to determine by a separate method whether the precipitates found inside the SR contain calcium after the incubation of smooth muscle with high-K oxalate medium.

MATERIAL AND METHODS

Guinea pigs weighing 250-300 g were killed by a blow on the head, and pieces of *Taenia coli* (about 25 mm long) were dissected and, immediately after removal from the animal, were suspended isotonicly in a muscle bath.

The following solutions were employed: (a) Krebs' solution (mM): NaCl, 127.0; KCl, 4.7; CaCl₂, 2.6; MgCl₂, 1.2; NaHCO₃, 25.0; KH₂PO₄, 1.3; glucose, 5.6; pH adjusted to 7.4 with NaOH; 310 mosM; (b) high-K (depolarizing) Krebs' solution: 174.0 mM KCl was added to normal Krebs (solution a); pH adjusted to 7.4 with NaOH; 580 mosM; (c) high-K (depolarizing) oxalate medium (mM): K oxalate, 40.0; KCl, 140.0; adjusted to pH 7.4 with KOH; 360 mosM (27); K oxalate cannot be added to Krebs' solution since a precipitation of divalent cations occurs; (d) high-K (depolarizing) oxalate medium containing sucrose: 1.4% sucrose was added to solution c in order to prevent the swelling of smooth muscle cells produced in a high-KCl solution (23); pH adjusted to 7.4 with KOH; 450 mosM; (e) K-free (control) oxalate medium: 40 mM NH₄ oxalate; pH adjusted to 7.4 with NaOH and osmolarity to 310 mosM with sucrose.

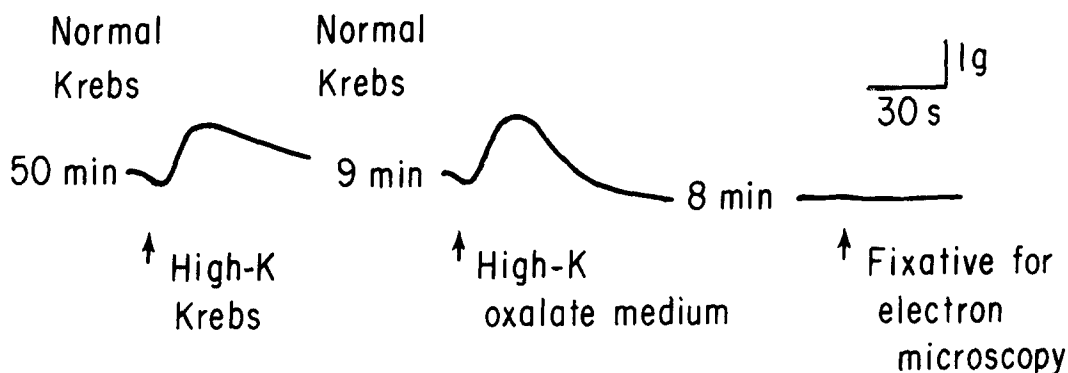


FIGURE 1 Isotonic contractile responses of the guinea pig *Taenia coli* under the conditions used for the ultrastructural detection of calcium in smooth muscle cells. The fluctuations in the base-line record before the contractures are artifacts caused by exchanging the solutions in the muscle bath. Muscle was equilibrated for 50 min, at 37°C, in aerated normal Krebs (solution *a*). A control K contracture was induced by the addition of high-K Krebs (solution *b*), and then normal Krebs (solution *a*) was added again allowing the muscle to relax. After 10 min the normal Krebs' solution was replaced with high-K oxalate medium (solution *c*) to precipitate calcium ions *in situ*. This medium produced a shorter K contracture followed by gradual relaxation maintained 10 min later when the fixative for electron microscopy (2.5% buffered glutaraldehyde) was introduced into the muscle bath. As the record shows, muscle was fixed under relaxation.

Procedure for Calcium Precipitation In Situ

Muscle strips were equilibrated in aerated Krebs (solution *a*) at 37°C for 50 min. At the end of that time, Krebs' solution was replaced with high-K Krebs (solution *b*), to test the response of muscle to depolarizing medium before the oxalate treatment, and then normal Krebs' solution was added again. Ten min later, Krebs' solution was replaced with high-K (depolarizing) oxalate medium (solution *c* or *d*) in order to introduce the oxalate into living muscle cells for precipitation of calcium ions *in situ*. After 10 min, the fixative for electron microscopy was added. Changes in muscle length were continuously recorded on a Tönies Electronic Equipment for Electrophysiology (Freiburg-Baden, West Germany) until the primary fixation. In control experiments, the muscle strips, initially equilibrated in aerated Krebs (solution *a*) for 50 min at 37°C, were incubated for 10 min with K-free oxalate medium (solution *e*) and then fixed for electron microscopy.

Conventional Electron Microscopy

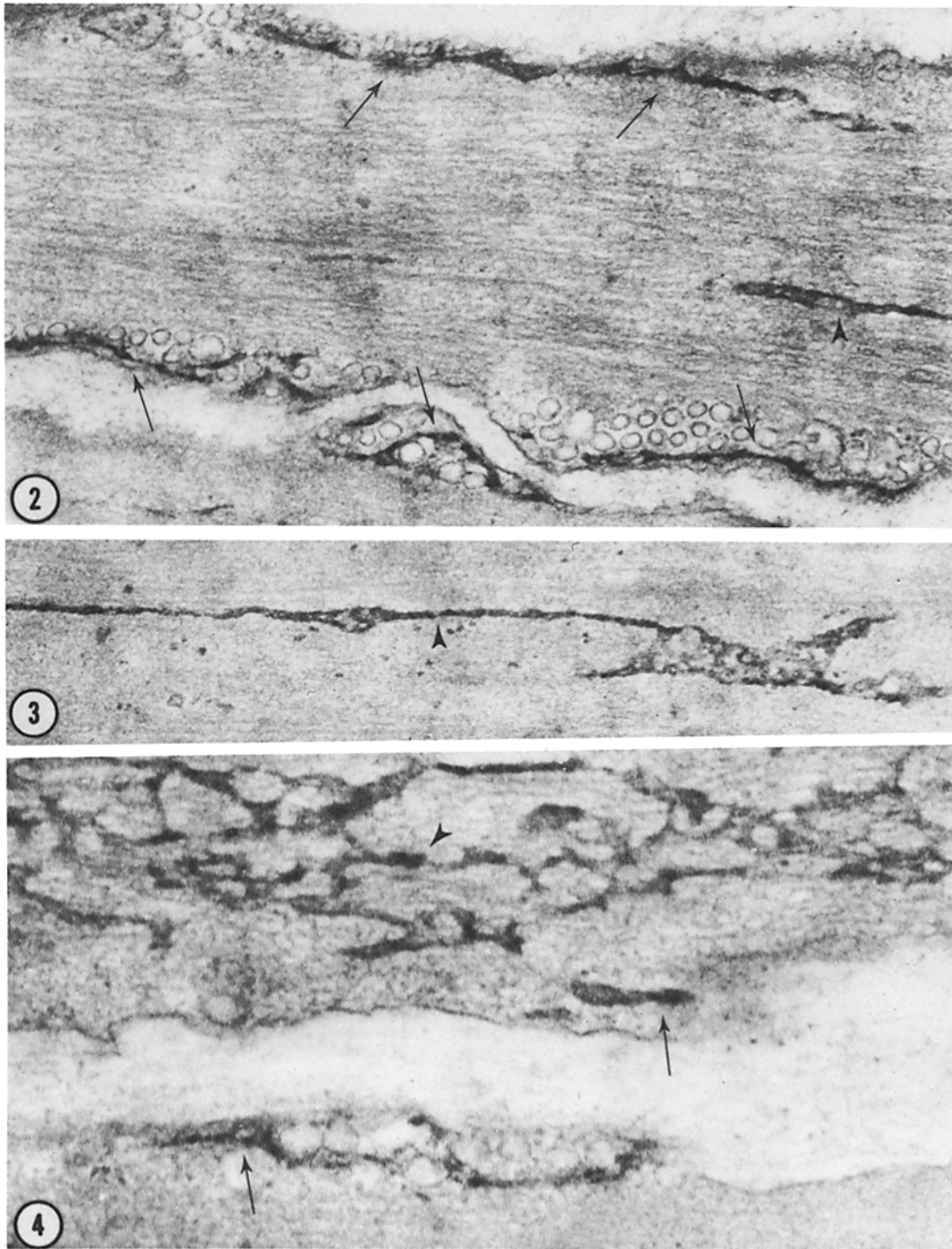
Muscle strips were prefixed for 2 h at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, rinsed in the same buffer and postfixed for 1 h, at 4°C, in 2% osmium tetroxide in 0.05 M cacodylate buffer. Each of these solutions also contained 40 mM potassium oxalate to minimize the loss of calcium during fixation and to obtain a satisfactory preservation of calcium oxalate deposits during washing (27). After being rapidly dehydrated through graded ethanols, the specimens were cleared in propylene oxide and embedded in Epon 812. Thin sections were cut with glass knives on a Porter-

Blum MT-1 ultramicrotome (Du Pont Instruments, Sorvall Operations, Newtown, Conn.), mounted on copper grids with Formvar-support membrane (Belden Mfg. Co., Chicago, Ill.) and examined at 40 or 60 kV with a Philips 301 electron microscope (Philips Electronic Instruments, Mount Vernon, N.Y.). Contrast staining of sections was omitted since uranyl acetate removes calcium oxalate deposits (1, 25), and an exchange of cations in the electron-opaque deposits could take place during staining (32).

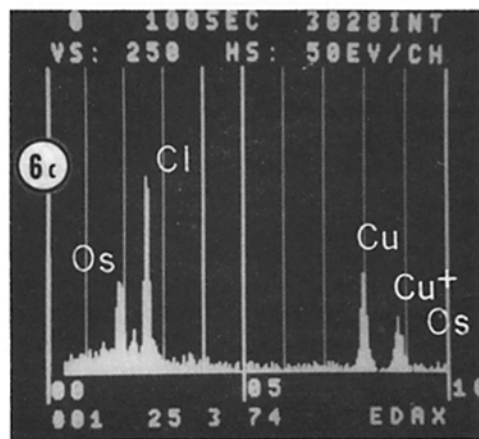
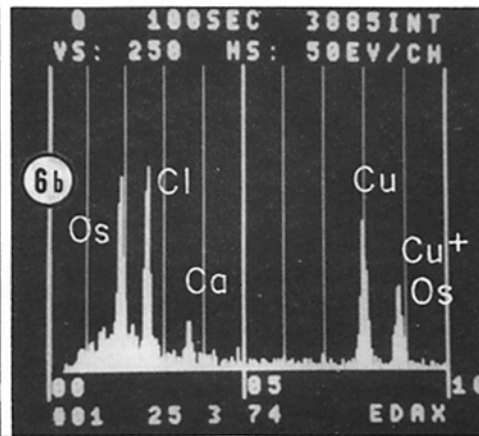
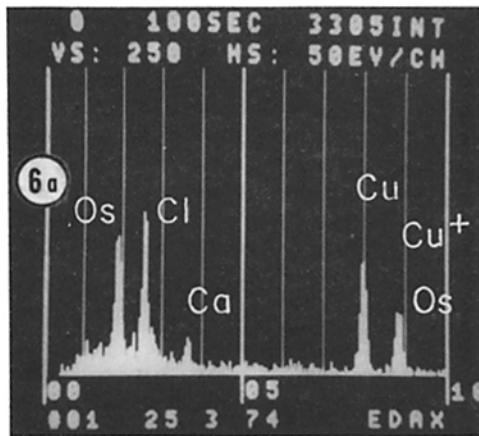
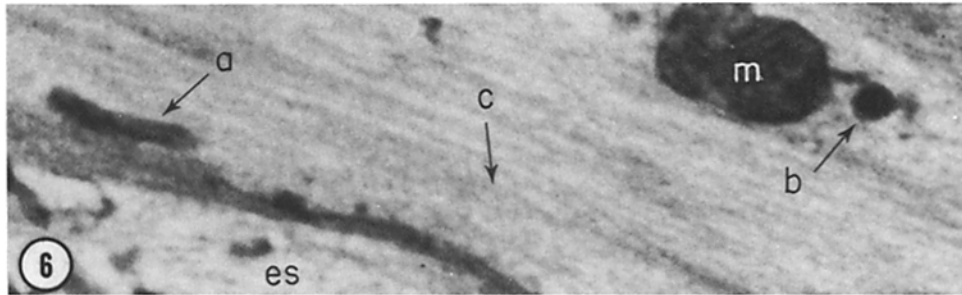
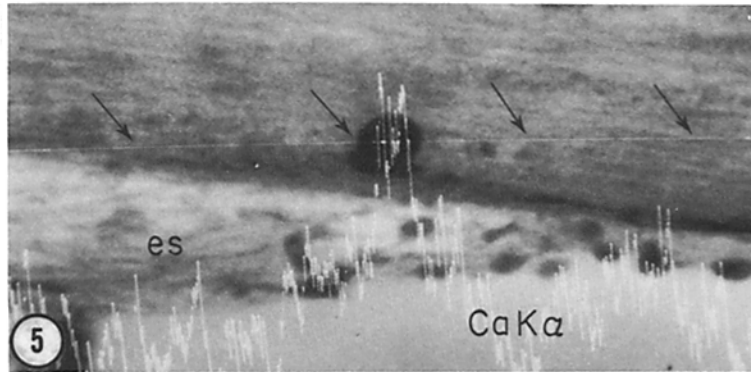
X-Ray Analytical Electron Microscopy

Several thick sections (purple sections of about 2,000 Å) were cut from the same blocks as the sections used for conventional electron microscopy and were subjected to X-ray analytical electron microscopy without any contrast staining. The analytical equipment consisted of a JEM 100 B transmission electron microscope (TEM) fitted with the following attachments: (a) side entry goniometer stage (SEG); (b) scanning device (ASID); (c) energy-dispersive X-ray spectrometer (solid-state detector). By operating the microscope in this mode, scanning transmission electron microscope (STEM) images of higher contrast than the TEM images were obtained. However, since the STEM does not afford the high spatial resolution of the TEM and the sections were thicker, the ultrastructural details cannot be so evident in the STEM images as in the TEM images.

X-ray microanalysis was carried out in two modes: (a) Spot analysis, by using the electron beam as a stationary probe (60 kV, 10^{-11} A) positioned over the area of



FIGURES 2, 3, and 4 Examples of TEM images of smooth muscle cells of the guinea pig *Taenia coli* fixed under relaxation, subsequent to the incubation in high-K (depolarizing) oxalate medium containing sucrose (solution *d*); no staining was used. Note electron-opaque deposits inside the peripheral (arrows) and deep SR (arrow-heads). Figs. 2 and 3, $\times 50,000$; Fig. 4, $\times 75,000$.



interest; minimum spot diameter of approximately 200 Å (21, 30). The emitted X rays were collected for 100 s. (b) Line-scanning analysis, by using the electron beam as a scanning probe. The X rays emitted at 3.69 keV (corresponding to the emission of calcium K_{α} shell) were monitored along a line trace. Thus, differences in calcium content were detected across a region of interest. The principles of the identification of chemical elements by X-ray analytical electron microscopy have been recently reviewed (8, 30). In the absence of computer processing, a signal was considered real, and the element present, if the corresponding peak rose to more than twice the background necessary to distinguish the signal from noise in X-ray spectra (24).

RESULTS AND DISCUSSION

As shown in Fig. 1, the exposure of muscle to the high-K oxalate medium produced a K contracture followed by gradual relaxation that was maintained 10 min later when the fixative for electron microscopy was introduced into the muscle bath. When the smooth muscle cells treated with high-K oxalate medium (solution *c* or *d*) were examined in the electron microscope, numerous electron-opaque deposits were found at the level of the SR irrespective of its intracellular topography.¹ More-

over, in the examination of unstained sections, the electron-opaque deposition appeared virtually as an electron-opaque marker of the SR. The electron-opaque deposits were either attached to the SR membrane or located inside the lumen of the SR. However, in a given element of the SR, the membrane could be clearly seen only if the electron-opaque deposits did not fill its lumen completely. As reported previously (27), in muscle strips treated with the high-K oxalate medium without the addition of sucrose (solution *c*), electron microscopy revealed light cells (reduced electron opacity of the cytoplasm and the nuclei) and dilated SR elements because of the swelling produced in a high-KCl solution (23). The addition of sucrose to the high-K oxalate medium (solution *d*) prevented the swelling of cells and preserved better the SR (Figs. 2-4). No electron-opaque deposits were found at the level of the SR when muscle strips were incubated with a K-free oxalate medium (solution *e*). This finding indicates that the depolarization is really a prerequisite for the penetration of the oxalate into living smooth muscle cells.

Since the electron-opaque deposits appeared only after the incubation with high-K oxalate medium, it seems reasonable to believe that the precipitates consisted primarily of calcium oxalate. Indeed, direct evidence for the presence of calcium in the electron-opaque deposits found inside the SR was provided by X-ray analytical

¹The following SR subclassifications were used: (a) *peripheral* SR, adjacent to the surface microvesicles and to nonvesicular cell membrane; (b) *deep* SR, surrounded by myofilaments; (c) *central* SR, located in the central sarcoplasmic axis.

FIGURE 5 Smooth muscle cell of the guinea pig *Taenia coli* incubated with high-K (depolarizing) oxalate medium (solution *c*). STEM image of an *unstained* section of about 2,000 Å showing the line-scanning analysis for calcium along the tracing marked by arrows. The distribution of characteristic X-ray pulses of calcium along the scanning line reveals a very prominent signal for calcium over the electron-opaque deposit, but no signals for calcium appear when the adjacent cytoplasm was analyzed. *es*, extracellular space. $\times 90,000$.

FIGURE 6 Smooth muscle cell of the guinea pig *Taenia coli* incubated with high-K (depolarizing) oxalate medium (solution *c*). STEM image of an *unstained* section of about 2,000 Å. Note electron-opaque deposits inside the peripheral (*a*) and deep (*b*) SR. The areas marked *a*, *b*, *c* were submitted to X-ray spot analysis. *m*, mitochondrion; *es*, extracellular space. $\times 72,000$.

FIGURES 6*a*, 6*b*, and 6*c* Emission spectra of X rays generated by the electron bombardment of the points *a*, *b*, and *c*, marked by arrows in the STEM image illustrated in Fig. 6. The energy dispersive spectrograms in Figs. 6*a* and *b* reveal distinct peaks corresponding to the emission line of calcium K_{α} shell (3.69 keV). They clearly indicate that calcium is present in a significant amount in the electron-opaque deposits which appeared inside the SR after the incubation of muscle with oxalate. When the SR-free area was analyzed (Fig. 6*c*) no signals for calcium were recorded. On the other hand, X-ray spectra show strong peaks for osmium (M_{α} line 1.91 keV; L_{α} line 8.91 keV), chlorine (K_{α} line 2.62 keV), and copper (K_{α} line 8.04 keV; K_{β} line 8.90 keV). They can be ascribed to the preparatory techniques: osmium, due to the fixative, chlorine, from the embedding medium, and copper, from the grid. The Os L_{α} and Cu K_{β} lines cannot be resolved and are responsible for the single peak in the spectra at about 8.9 keV.

electron microscopy,² which offers the ability to perform a chemical analysis of very small and well-defined areas from specimens while they are being observed microscopically. Line-scanning analysis across the SR containing electron-opaque deposits and the adjacent regions of the cytoplasm showed that the amount of calcium contained in the SR was significantly greater (Fig. 5). The emission spectra of X rays generated by the spot analyses of the electron-opaque deposits located in the peripheral and deep SR (Figs. 6 *a* and *b*) exhibited well-defined signals at the 3.69-keV energy level characteristic of calcium K_{α} shell. Although magnesium and zinc form oxalate precipitates *in vitro* and could possibly give rise to oxalate deposits *in situ*, particularly if these ions are concentrated in SR (25), no peaks corresponding to magnesium (K_{α} line 1.25 keV) or zinc (L_{α} line 1.01 keV; K_{α} line 8.63 keV; K_{β} line 9.57 keV) were detectable. In addition, the presumptive existence of potassium in the electron-opaque deposits (because of the incubation in high-K oxalate medium before fixation) was ruled out, due to the absence of an unambiguous peak centered at 3.31 keV (potassium K_{α} line). As a control, the analyzer spot was placed on a SR-free area of the cytoplasm. The corresponding X-ray emission spectrum did not display characteristic calcium signals (Fig. 6 *c*). Most prominent in all spectra (Fig. 6 *a-c*), irrespective of the analyzed areas, were peaks for osmium (M_{α} line 1.91 keV; L_{α} line 8.91 keV), chlorine (K_{α} line 2.62 keV), and copper (K_{α} line 8.04 keV; K_{β} line 8.90 keV). They are related to the fixative (osmium) and supportive materials; chlorine from the embedding medium, since Epon contains a lot of chlorine (34), and copper from the grid. The peaks for chlorine and copper were similar in all spectra, but the peak for osmium was higher over SR containing electron-opaque deposits (compare Fig. 6 *a, b* with *c*), suggesting that osmium, besides calcium, was also present in the electron-opaque deposits. This might be explained by a "tricomplex flocculation" of calcium, osmium, and phospholipids (16). However, the ratio of osmium peak to calcium peak is not directly a good measure of concentration since, for instance, the light and

² X-ray spectra generated by the electron bombardment of the large electron-opaque deposits visible on the surface and outer mitochondrial membranes also revealed the existence of calcium (Diculescu, I., and L. M. Popescu. Manuscript in preparation).

heavy elements are not excited with the same efficiency (for details see ref. 24).

The correlational observations reported here provide conclusive evidence for calcium accumulation inside the SR of intact smooth muscle cells under relaxation, subsequent to a K-induced contraction. The most direct interpretation of this conclusion is that the SR of smooth muscle can take up, store, and (implicitly) release calcium ions, thereby participating in the regulation of intracellular free-calcium levels during the contraction-relaxation cycle, as in skeletal muscle. The finding of calcium accumulation by the peripheral SR on return to the resting state supports the assumption that, for the excitation-contraction coupling, the peripheral SR is the most probable intracellular site of calcium release by the action potential because of its close apposition with the surface membrane (4, 9, 10, 27, 31, 33). A rough estimate based on volumetric and microchemical data suggests that, under our experimental conditions, the amount of calcium sequestered in the peripheral SR would be enough for electromechanical coupling.³ However, the fact that there is sufficient calcium does not necessarily mean that calcium for contraction comes *only* from the superficial SR. Calcium influx across the cell membrane and/or calcium stored in the deep SR, which is connected with the peripheral SR (see Fig. 4), could participate also in triggering contraction.

Finally, it is notable that previous cytochemical studies (20, 27, 29, 35) showed that, in addition to the SR, other cell components (mitochondria, surface membrane, surface microvesicles, nucleus) can store calcium *in situ*. The possible role of mitochondria (3, 10, 27, 32, 33), surface microvesicles (9, 10, 26-28), surface membrane (20), or nucleus (27) in the control of myoplasmic calcium concentration has been considered. It might be expected that further physiological and pharmacological studies combined with the "ultrastructural

³ If we can extrapolate the concentration of calcium found in microsomes (0.022 M, after 1-h incubation of muscle in Krebs' solution) to the SR *in situ*, and if we consider previous morphometric data (27), it follows that the peripheral SR of *one* smooth muscle cell of the guinea pig *Taenia coli* would contain about 24.2×10^{-17} mol Ca. Since 4.7×10^{-17} mol Ca/cell (26) are needed to produce maximal contraction, it appears that the release of about 20% of the calcium stored in the peripheral SR would be sufficient for at least partial activation of the contractile machinery.

dissection" of smooth muscle cells by chemical methods will provide sufficient evidence to establish the relative contribution of various subcellular structures to calcium movements.

SUMMARY

Numerous electron-opaque deposits appear in the SR of the relaxed smooth muscle cells of the guinea pig *Taenia coli* that had been treated, before fixation, with a depolarizing medium containing oxalate to precipitate calcium ions *in situ*. X-ray spectra obtained by spot and line-scanning analyses of these deposits *in situ* show characteristic calcium signals, thus providing direct evidence for calcium accumulation inside the sarcoplasmic reticulum of smooth muscle.

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