

The Sarcoplasmic Reticulum of Smooth Muscle Fibers

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Dedicated to Professor Wilhelm Hasselbach on the Occasion of his 60th Birthday

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The ability of the sarcoplasmic (endoplasmic) reticulum (SR, ER) of smooth muscle cells to accumulate Ca was demonstrated by measuring the uptake of ^{45}Ca in fibers which were chemically skinned with saponin, and by electron cytochemistry of the accumulated Ca. The Ca uptake was dependent on ATP and it was stimulated by oxalate, as it is the case in SR of striated muscle. Electron microscopy of the skinned smooth muscle preparations revealed the presence of calcium oxalate deposits in the reticulum.

The SR vesicles were isolated from several smooth muscles. The purification was carried out by taking advantage of the density increase of the SR vesicles after loading with calcium in the presence of oxalate. Among the muscles investigated the smooth muscle of the pig stomach was found to be the most suitable and it was selected for further biochemical and morphological characterization of the SR vesicles. These vesicles, which contain calcium oxalate crystals, were able to accumulate an additional amount of Ca. The Ca uptake was supported by several energy yielding substrates. Their order of potency was $\text{ATP} > \text{dATP} \approx \text{UTP} > \text{ITP} > \text{GTP} \approx \text{CTP}$. The rate of Ca uptake was two orders of magnitude slower than that in SR of skeletal muscle. The measurement of the level of phosphorylated Ca transport intermediate showed that this difference is due to smaller number of calcium transport sites per vesicle. The distribution of intramembrane particles in freeze-fractured specimens is in agreement with this conclusion.

Introduction

Although smooth muscle cells differ from skeletal muscle fibers in the structural organization of the contractile apparatus and in the chemistry of their activation mechanism, the contraction of smooth muscle is regulated as in skeletal muscle by the cytoplasmic Ca^{2+} concentration in the range 10^{-7} M to 10^{-5} M [1, 2]. Since skeletal muscle cells have a large diameter and because they are able to contract very fast, the diffusion of Ca from the extracellular space into the cells would not be sufficiently fast to account for the speed of contraction. Therefore, skeletal muscle cells require releasable stores of Ca which are distributed over the whole cell volume, and a system for the conduction of the electrical activation signal towards the Ca stores in the deeper parts of the cell. The activator Ca is stored in the sarcoplasmic reticulum (SR), a membrane system specialized in the rapid uptake and release of Ca.

In smooth muscle, sacs of internal membranes are present which have been designated as sarcoplasmic reticulum in analogy with striated muscle (for its morphological description see [3–5]). As compared

with skeletal muscle, the SR of smooth muscle is poorly developed. This is probably related to the fact that the diameter of smooth muscle cells is very small and to the fact that they contract and relax very slowly. It can be calculated that the influx of Ca alone would be sufficient to explain the speed of contraction. It has indeed been observed that the contraction of smooth muscle cells is dependent on the presence of Ca in the bathing solution, indicating that extracellular Ca is an important source for activation of the contractile proteins [6–10]. However, it has also been demonstrated that internal releasable Ca stores are present and that they too play an important role in the excitation-contraction coupling process [4, 6–10]. During incubation in Ca-free solution, one transient contraction can be induced by agonists such as noradrenaline in most vascular muscles and by acetylcholine in intestinal muscle. Depolarization with high K^{+} is ineffective. If the first stimulus is a maximal dose of the agonist, a second addition of the agonist has no contractile effect, indicating that the activator Ca after being released from the internal store is not reaccumulated in the store. Experiments using ^{45}Ca have shown that this Ca is extruded from the cells and that readmission of external Ca is required to refill

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the store [7–10]. The initial phase of the contraction induced by an agonist in Ca-containing solution is probably elicited by the release of intracellular Ca, while the tonic component is maintained by the continuous influx of Ca.

The amplitude of the transient contraction in Ca-free solution, which is an indication of the amount of intracellular Ca which is released, is dependent on many factors. When the cells are loaded with Ca in a Ca-containing K^+ -depolarizing solution immediately preceding the superfusion with Ca-free solution, and when this period in Ca-free solution is kept very short, the amplitude of the transient contraction is nearly maximal, indicating that the amount of Ca which can be accumulated in the store is sufficient for at least a near-maximal activation of the contractile proteins [9]. It has also been observed that β -agonists may increase the amount of Ca in the store [9–10]. The stimulation by β -agonists of the Ca uptake in the store may contribute to the relaxing effect of these agonists on smooth muscle.

The available evidence favours the SR as the most likely candidate for being the site of the internal Ca store in smooth muscle. This function of the SR is suggested by the analogy with striated muscle and by the finding that it contains a high concentration of Ca [11]. An alternative possibility, the reversible Ca binding to the inner surface of the plasma membrane has been proposed. Histochemical evidence has been obtained for this hypothesis, but more experimental evidence is not available [12, 13]. It is also possible that both Ca storage systems exist together in the same cell.

In this paper more evidence will be presented for similarities between the SR of striated and smooth muscle, suggesting that in smooth muscle, as in striated muscle, this subcellular structure may represent the internal Ca store. However, a direct demonstration of the release and uptake of Ca in the SR during contraction and relaxation of smooth muscle is still lacking.

Some of the results have been previously communicated [14].

Methods

Chemical skinning of smooth muscle fibers with saponin

Helical strips of the distal part of rabbit ear arteries were incubated in normal physiological

solution, then shortly washed in Ca-free solution to remove extracellular Ca, and incubated for 3 min in the "relaxing solution" containing 120 mM KCl, 6 mM $MgCl_2$, 5 mM ATP, 5 mM Na-azide, 2 mM EGTA, 25 mM imidazole pH 6.9. This solution was replaced by the skinning solution of similar composition but containing 100 μ g/ml saponin. After 25 min the saponin was washed out with relaxing solution, which was then replaced by "contracting solution" in which the Ca concentration was buffered at 10^{-5} M Ca with EGTA. This solution induced a tonic tension the amplitude of which was 60 to 80% of the tension evoked by K^+ -depolarization (80 mM K^+) before skinning, indicating that most cells had been rendered leaky by the treatment with saponin.

The Ca uptake in the chemically skinned fibers was measured by incubation for the indicated period of time in the "contracting solution" containing trace amounts of ^{45}Ca . They were then washed for 5 min in relaxing solution, blotted, weighed and dissolved for the determination of the ^{45}Ca content.

For the ultrastructural localization of the site of Ca uptake, 5 mM oxalate was always included in the contracting solution. After incubation for 1 h, the tissues were fixed at 0 °C in 1% OsO_4 in cacodylate buffer pH 7.4 containing 1 mM oxalate, dehydrated in oxalate-containing ethanol-water mixtures and embedded in araldite. Sections were observed in the electron microscope without additional staining in order to minimize the dissolution of the deposits.

Isolation of sarcoplasmic reticulum

The following smooth muscles were used: the antrum region of the pig stomach, bovine main pulmonary arteries, rabbit uterus obtained four days after injection of the rabbits with 1 mg estradiurine (Leo, Halsingborg, Sweden), and the media-intima layer of pig coronary arteries. Crude microsomal fractions were isolated as described previously for the pig stomach [15].

For the comparative study of the microsomal fraction of these muscles, the crude microsomal pellets were carefully resuspended in 0.25 M sucrose containing 10 mM imidazole pH 6.9. The suspensions were centrifuged at low speed ($27000 \times g$ for 15 min) in order to sediment large particles and aggregated vesicles. To the supernatant the ingredients required for Ca uptake were added: ATP 5 mM, $MgCl_2$ 5 mM, creatinephosphate 5 mM, creatine

kinase 100 µg/ml, oxalate 5 mM, Na-azide 5 mM, Ca^{2+} buffered at 10^{-5} M with EGTA and trace amounts of ^{45}Ca . The microsomal suspension was then incubated at 37 °C for 1 h. The uptake of Ca in the presence of oxalate induces an appreciable density increase of the sarcoplasmic reticulum vesicles because they are permeable to oxalate and intravesicular calcium oxalate deposits will be formed during Ca uptake [15]. Instead of 5 mM oxalate, 40 mM phosphate can be used [16]. The Ca uptake was stopped by cooling at 0 °C and the oxalate loaded vesicles were sedimented by centrifugation at $27000 \times g$ for 15 min. The protein content and the intravesicular ^{45}Ca content of the pellet was measured in order to determine the enrichment of the isolated material.

For further study of the isolated SR of the pig gastric smooth muscle, the low speed centrifugation preceding the loading with Ca was carried out three times instead of once. The calcium oxalate loading was stopped after 30 min instead of after 60 min to avoid complete filling of the vesicles.

For the observation of thin sections of the SR vesicles, fixation and embedding was carried out as described for the chemically skinned muscle fibers. Replicas of freeze-etched specimens were prepared as described previously [16].

The Ca uptake in the isolated vesicles was measured by Millipore filtration. The nucleoside 5'-triphosphates, creatine phosphate and creatine kinase were obtained from Boehringer, Mannheim.

Results

Ca uptake in the SR of chemically skinned smooth muscle fibers

Chemical skinning of smooth muscle cells with saponin preserves the SR membranes functionally intact [17]. Chemically skinned rabbit ear arteries were incubated at 37 °C in the presence of ATP, magnesium, oxalate and 10^{-5} M Ca^{2+} buffered with EGTA. Azide was included to inhibit the Ca uptake by mitochondria. Fig. 1 shows the time course of the Ca uptake in these preparations. The Ca uptake is stimulated by oxalate, suggesting that it occurs by the SR membranes. In the presence of oxalate more than 3 mmol Ca per kg wet weight are accumulated in 60 min. During the first 20 min the rate of uptake is about $70 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. This value is an

underestimation of the capability of the SR *in vivo*, because some cells may resist the treatment with saponin, while in others the SR may have been damaged. Surprisingly, the Ca uptake in the absence of oxalate does not reach a plateau value, as it is the case in isolated vesicles (see Fig. 1 and 5). ATP is required for the Ca uptake. The addition of a Ca-ionophore to the ATP-containing medium has the same effect as the omission of ATP. The treatment with saponin also leaves the mitochondria intact. The Ca uptake in these organelles, which was determined by omission of azide, reaches more than $2 \text{ mmol} \cdot \text{kg}^{-1}$ in 20 min. A value of about $10 \text{ mmol} \cdot \text{kg}^{-1}$ was found when the same solution was supplemented with 10 mM succinate (data not shown).

The site of the ATP-dependent and oxalate-stimulated Ca uptake was determined by electron microscopy of saponin-treated fibers which had been incubated in a solution for Ca uptake. As shown in Fig. 2, deposits of calcium oxalate are

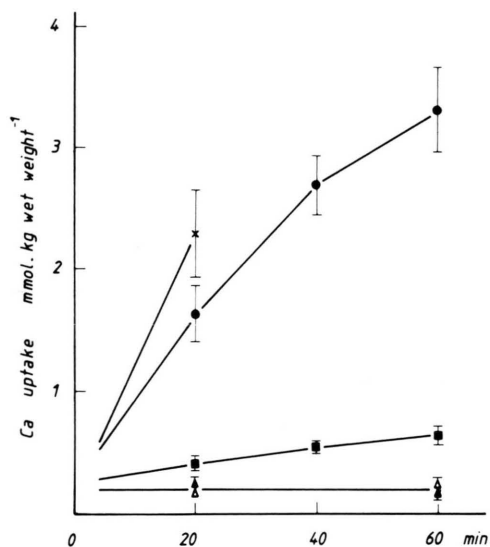


Fig. 1. Time course of the Ca uptake (mmol Ca/kg wet weight) in smooth muscle cells of the rabbit ear artery chemically skinned with saponin, with preservation of intact SR membranes. The complete medium contained ATP, oxalate and azide (●). The effect of omission of oxalate (■), ATP (▲) and of oxalate and azide (x) is shown. The Ca uptake in the absence of azide represents the Ca uptake mainly in mitochondria. The open triangles show the Ca uptake during incubation in the complete medium supplemented with $10 \mu\text{M}$ of the Ca ionophore A23187. The vertical bars represent the S.E. of the mean of 3 observations.

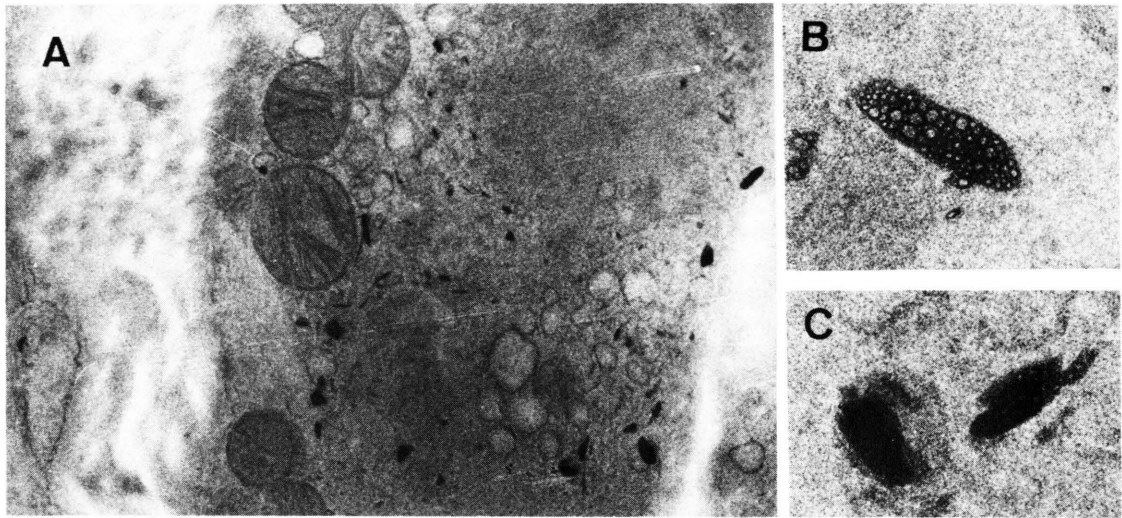


Fig. 2. Electron micrograph of a thin section of the smooth muscle of the rabbit ear artery chemically skinned with saponin and incubated in a solution containing oxalate in addition to the ingredients required for Ca uptake ("contracting solution", see text). Deposits of calcium oxalate are seen on areas occupied by smooth muscle cells. They are never seen on mitochondria. The crystals present a nodular morphology (Fig. B), similar to that of crystals in the isolated SR (see Fig. 3). In some cases deposits can be seen which are surrounded by a membrane (Fig. C). Fig. A $\times 24000$, B $\times 105000$, C $\times 140000$.

formed in these tissues. They occur only on areas occupied by muscle cells. They are never present on areas occupied by nuclei or mitochondria, indicating that they are formed inside the SR. Fine details are obscured in these sections because the observations were made on rather thick sections (to avoid the deposits to fall out of the section) which were weakly stained to diminish the dissolution of the deposits. However in some cases calcium oxalate crystals can be seen which are surrounded by a membrane (Fig. 2 C).

Isolation of sarcoplasmic reticulum of smooth muscle

In most types of smooth muscle, the total area of the internal membranes is smaller than that of the plasmalemma [5]. Therefore, purification of sarcoplasmic reticulum requires its separation from plasma membrane fragments. Several difficulties are encountered in the separation of these subcellular organelles. The most important ones are probably the aggregation of different vesicles, the small density differences between SR and some parts of the plasma membrane, *e.g.* regions to which contractile proteins are attached, and the lack of suitable marker enzymes for the SR. In the present work, these difficulties were minimized as follows.

The tendency to aggregate of crude microsomes of different smooth muscles was compared by centrifugation at low speed of the carefully resuspended microsomal fractions and by measuring the amount of protein which is sedimented. As seen in Table I the amount of pelleted protein is smallest for the pig gastric smooth muscle. Apparently, the microsomal vesicles of gastric smooth muscle remain better separated than those of the other muscles tested.

The problem of small density differences between the SR and other membranes was eliminated by loading of the crude microsomes with Ca in the presence of oxalate, which induces the formation of intravesicular calcium oxalate deposits in the SR vesicles (see above and ref. [15]). Evidently, leaky SR vesicles are not isolated by this procedure. The loaded vesicles can be sedimented by low speed differential centrifugation. Table I shows the values of the intravesicular Ca content and of the enrichment in Ca content of the SR fractions obtained in this way from different muscles. The highest values for the Ca uptake in the presence of oxalate are found for the rabbit uterus, followed by the pig stomach muscle. In view of the lower degree of aggregation of the microsomal vesicles of the latter preparation, and because it is easily available in large quantities,

Table I. Comparison of different smooth muscles with respect to the aggregation of the microsomal vesicles and with respect to the yield and the enrichment of the vesicles loaded with calcium oxalate. The left column shows the protein content of the pellet sedimented by low speed centrifugation (Sorvall SS-34 rotor, 14 000 rpm, 15 min) of the resuspended crude microsomal fraction, expressed as the percentage of the protein content of the crude microsomal fraction. "Ca oxalate pellet" refers to the material sedimented by low speed centrifugation of the crude microsomal fraction following Ca uptake for 60 min. The enrichment is the ratio of the intravesicular ^{45}Ca content ($\text{nmol} \cdot \text{mg protein}^{-1}$) of the Ca oxalate pellet over that of the crude microsomes. The yield is the total amount of intravesicular ^{45}Ca in the Ca oxalate pellet expressed as the percentage of the total amount of intravesicular ^{45}Ca in the crude microsomal fraction.

	Protein content of pellet [%]	^{45}Ca content of Ca oxalate pellet [$\mu\text{mol} \cdot \text{mg}^{-1}$]	Enrichment	Yield in terms of ^{45}Ca content [%]
pig stomach	5	2.2	20	45
bovine main pulmonary artery	40	1.5	7	20
pig coronary artery	40	0.58	6	15
rabbit uterus	24	4.2	3	53

the smooth muscle of the pig stomach was selected for further study of the isolated SR.

Characterization of the isolated sarcoplasmic reticulum of gastric smooth muscle

Morphology. Fig. 3 shows an electron micrograph of a thin section of a pellet of the sarcoplasmic reticulum enriched fraction isolated from the smooth muscle of the pig stomach. The fraction consists of vesicles of varying size and shape. Several nodular deposits of calcium oxalate can be observed. The number of vesicles which contain oxalate crystals is much greater in untreated preparations because fixation, staining and embedding induces an outflow of calcium [15, 18]. Several large deposits were removed during sectioning, leaving holes in the section.

A replica of a freeze cleaved and etched SR fraction is shown in Fig. 4. Intramembrane particles are present on the protoplasmic as well as on the exoplasmic fracture faces. Their packing density is $165/\mu\text{m}^2$ which is about 30 times lower than in SR of skeletal muscle [16]. In the latter, the particles are

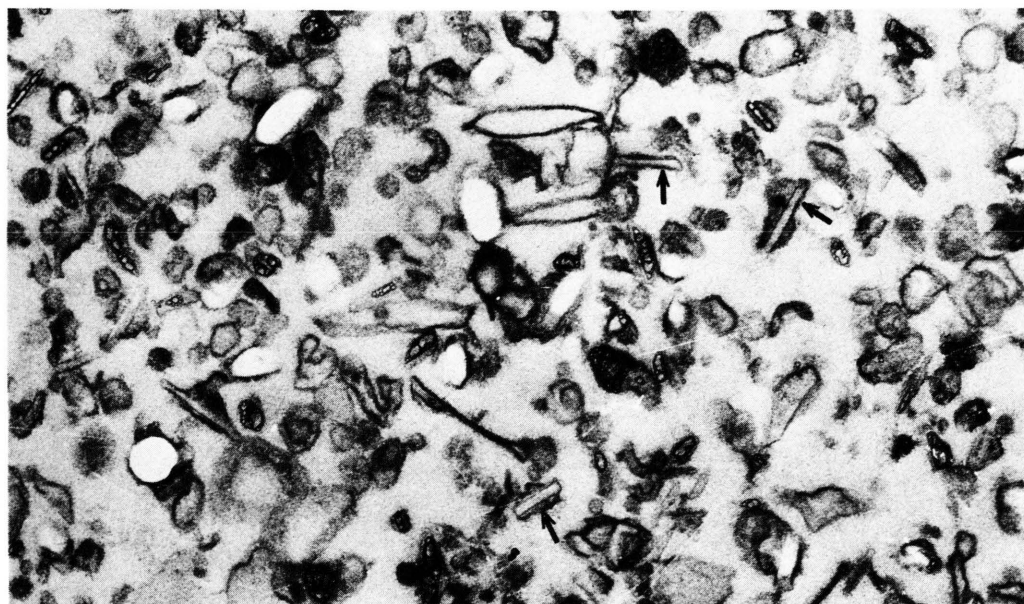


Fig. 3. Electron micrograph of a thin section of a pellet of calcium oxalate loaded SR vesicles of pig stomach smooth muscle. The vesicles are heterogeneous in size and shape. Many vesicles contain calcium oxalate crystals, but most of them are empty because the fixation, staining and embedding induces an efflux of Ca. Holes are seen in the section where deposits have fallen out during cutting. Some membrane profiles present a very regular shape (arrows), probably due to stretching around the growing crystal. $\times 42\,000$.

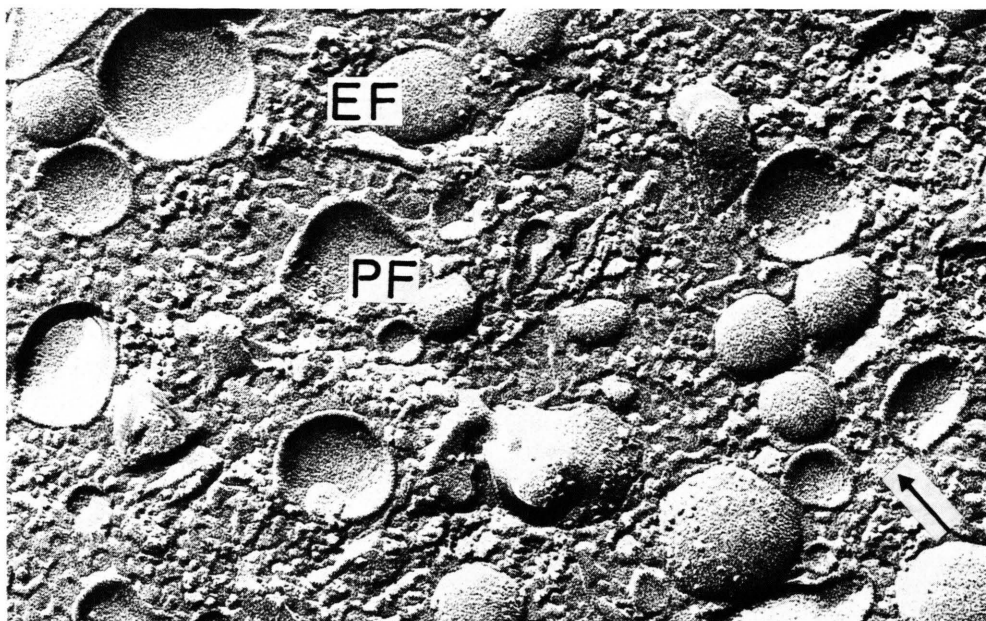


Fig. 4. Freeze-cleaved vesicles of isolated SR vesicles of smooth muscle. Particles are present on the protoplasmic (PF) as well as on the exoplasmic (EF) fracture faces. The number of particles per unit membrane area is much less than in SR of skeletal muscle. The arrow shows the direction of shadowing. $\times 90\,000$. (This figure is a courtesy of Dr. B. Agostini.)

known to correspond to Ca transport sites. The small number of intramembrane particles is in agreement with the low number of phosphorylated Ca transport sites in this preparation (see below). When a fractured specimen of the smooth muscle SR fraction is shadowed with a rotary gun instead of by conventional unidirectional shadowing, the particles are seen to be composed of subunits similar to those observed in SR of skeletal muscle [16].

Biochemistry. Although the SR vesicles isolated according to the present procedure contain calcium oxalate deposits, the vesicles are able to accumulate an additional amount of Ca. The time course of this Ca uptake in the presence of 5 mM oxalate is compared to that in the crude microsomal fraction in Fig. 4. The rate of Ca uptake is about 20 times faster in the purified SR fraction than in the crude microsomes. It should be noted however that this high enrichment is partly due to the removal of contractile proteins from the vesicles [15]. The rate of Ca uptake by the SR vesicles in the presence of oxalate is also shown when it is energized by different nucleoside triphosphates. Their order of potency is $\text{ATP} > \text{UTP} \approx \text{dATP} > \text{ITP} > \text{GTP} \approx \text{CTP}$. This sequence differs from that seen with

the SR of skeletal muscle, which is $\text{ATP} > \text{ITP} > \text{GTP} > \text{CTP} > \text{UTP}$ [19].

The ATPase activity, the phosphate turnover and the phosphoprotein formation in the isolated vesi-

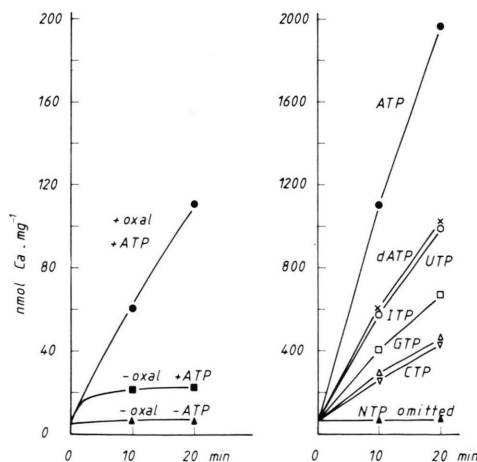


Fig. 5. Time course of the Ca uptake in the crude microsomal fraction (Fig. A) and in the isolated SR fraction (Fig. B) from the smooth muscle of the pig stomach. Note the different ordinate scales in Fig. A and B. In Fig. A the effect of the omission of ATP and oxalate is shown. Fig. B shows the time course of the Ca uptake in the presence of different nucleoside-5'-triphosphates. The curves are the mean of two experiments.

cles has been measured previously [20]. The Ca uptake and the Ca-stimulated ATP hydrolysis are both half-maximally activated at 7×10^{-7} M Ca^{2+} as in SR of skeletal muscle. The Ca uptake is accompanied by the Ca-dependent formation of an acid-stable hydroxylamine-sensitive phosphoprotein and by an ATP-ADP exchange. The turnover rate of the Ca-pump, calculated from the ratio of the ATPase activity to the steady-state level of phosphoprotein is similar to that in SR of skeletal muscle. The ATP-ADP exchange is only partially sensitive to Ca and its rate is much slower than that seen with SR of skeletal muscle [20].

The backward reaction of the Ca pump can be demonstrated in the isolated smooth muscle SR as the incorporation of inorganic phosphate into the ATP fraction. This ATP- P_i exchange is abolished by a calcium ionophore because a high intravesicular Ca concentration is required to drive the backward reaction [20].

Conclusions

The observation on chemically skinned smooth muscle cells that the formation of calcium oxalate deposits can be induced in the sarcoplasmic reticulum has demonstrated that the latter is able to accumulate Ca. It should be noted that the present method for the ultrastructural localization of Ca differs from the calcium-precipitation techniques commonly applied to smooth muscle [12, 13, 21, 22]. In the latter, the free Ca^{2+} concentration of the solutions is not controlled. The cells are made permeable to allow oxalate or other precipitating anions to enter and the inflowing anion precipitates at the sites where the Ca concentration exceeds the solubility product. In the present technique, the

Ca^{2+} concentration of the oxalate-containing solution which enters the cytoplasm is buffered with EGTA at a concentration which is below the precipitation threshold. Precipitation of calcium oxalate will only be possible in compartments which are separated from the cytoplasm and in which the Ca concentration is increased by an active pump mechanism. The finding that the SR of smooth muscle is able to accumulate Ca by an ATP-dependent Ca pump extends the observations of Somlyo and Somlyo [23] who demonstrated the accumulation of strontium in the SR, and of Somlyo *et al.* [11] who showed by X-ray microprobe analysis that the SR *in situ* contains a high Ca concentration.

The findings on skinned fibers and on the isolated SR suggest several similarities with SR of skeletal muscle, such as the presence of an ATP-dependent Ca pump, the permeability to oxalate, the affinity of the Ca pump to Ca, its turnover rate and the possibility to run the pump backwards. Moreover, recent findings made by gel electrophoresis of the phosphorylated Ca pump show that smooth muscle microsomes contain a Ca^{2+} -ATPase of a molecular weight of about 100 000, as the Ca pump of SR of skeletal muscle [24]. However, the two Ca transport systems differ in the relative abilities of different energy yielding substrates to support the Ca uptake.

The ability of the SR of smooth muscle to accumulate Ca suggests that it may play an important role in the removal of Ca from the cytoplasm during relaxation and that it may represent an internal store of Ca which is released during excitation. Further work is needed to clarify the regulation of the sarcoplasmic reticulum Ca pump and to find out how its role in the cellular metabolism of Ca is interrelated with the action of other Ca regulatory sites such as the plasmalemmal Ca pump.

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