# Role of Calcium Binding by Sarcoplasmic Reticulum in the Contraction and Relaxation of Uterine Smooth Muscle

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ABSTRACT The binding of calcium by isolated sarcoplasmic reticulum from cow uterus was studied. Sarcoplasmic reticulum was prepared by differential centrifugation. Three fractions were obtained: I, sedimented between 2,500-15,000 × *g*; II at 40,000 × *g*; and III, at 150,000 × *g*. Fraction II was further purified on a sucrose density gradient. All three fractions contained considerable amounts of intrinsic calcium, mostly in fraction I. Calcium binding in the presence of ATP[1] and Mg also was greatest in fraction I, followed by fraction II, with less in fraction III. Without ATP no calcium was taken up. 5 and 10 mM sodium azide partially inhibited calcium binding in fraction I, but not in fraction II, suggesting the presence of some mitochondria or mitochondrial fragments in fraction I. Calcium binding in fraction II was completely inhibited by 3 mM salyrgan; this fraction thus appears to be sarcoplasmic reticulum. ATPase activity was found in all three fractions, highest in fraction II. It is computed that calcium binding in fractions I and II, on the basis of a 50% yield of protein, is sufficient to elicit contraction by supplying calcium to the contractile proteins of the smooth muscle cell and to regulate relaxation and contraction.

## INTRODUCTION

The release and storage of calcium in the sarcoplasmic reticulum regulate relaxation and contraction in skeletal and heart muscle (for review see references 1 and 2). In mammalian smooth muscle, no such mechanism has been demonstrated. A well-developed sarcoplasmic reticulum is found surrounding the skeletal myofibril, predominantly in fast and thick muscles (1). Hill (3, 4) calculated that the time required for calcium to diffuse from outside into the

[1] Abbreviations used: ATP, adenosine 5′-triphosphate; ATPase, adenosine triphosphatase; salyrgan, salicyl-hydroxymercuri-methoxypropyl-amidoorthoacetate; EGTA, ethyleneglycol bis(β-aminoethylether)-*N*,*N*-tetraacetic acid.

skeletal myofibril would be too long for activation. An extensive sarcoplasmic reticulum serving as storage site would considerably shorten the pathway of calcium diffusion. The uterine smooth muscle cell is only approximately 10 $\mu$ in diameter and full activation may take as long as 5–10 sec (5). The sarcoplasmic reticulum is scanty and its physiological importance is questioned (6). Estimates based on the equation given by Hill (3) show no necessity for an intracellular calcium storage site. Speculation has envisioned such a system, its form and function (7).

We have now isolated fragmented sarcoplasmic reticulum from uterine smooth muscle. In the present communication the preparation and partial purification of the sarcoplasmic reticulum are described. The sarcoplasmic reticulum was characterized with respect to calcium binding and ATPase activity. It was found that calcium binding is considerably lower than that of a similar preparation from cardiac muscle (8, 9), but may well be sufficient to be of physiological importance.

## MATERIALS AND METHODS

*Preparation of Sarcoplasmic Reticulum* Uteri were obtained at the slaughterhouse from freshly killed nonpregnant cows or heifers. Uterine horns only were used. The tissue was chilled on ice and dissected free of endometrium and blood vessels. Pieces approximately 1 inch square were homogenized in a Waring Blendor for 30–40 sec in 2 volumes of ice-cold 0.3 M sucrose containing 2 mM ascorbic acid, 0.02 M Tris buffer, pH 7.2. Approximately 800 g myometrial tissue were used and homogenized in three portions. In cows 6–7 yr of age this amounts to nine uterine horns. The homogenates were transported in ice to the laboratory and all further manipulations were carried out in a cold room at 2-3°C and in refrigerated centrifuges by procedures outlined previously with some modifications (8, 9). Routinely the homogenate was centrifuged in a Sorvall centrifuge for 15 min at 2500 × *g*, filtered through two layers of gauze, and the pellet discarded. The supernatant was again centrifuged at 15,000 × *g* for 20 min. This yielded pellet I (mitochondrial fragments and large vesicles). Centrifugation of the supernatant at 40,000 × *g* for 90 min resulted in pellet II (sarcoplasmic reticulum); centrifugation of this supernatant at 150,000 × *g* in a Spinco L2-50 centrifuge for 90 min yielded pellet III (small microsomes). Each of these pellets was suspended in 40% sucrose, homogenized by hand with a Teflon-coated homogenizer, and kept in ice. For further purification pellet II was suspended in 8–10 ml NaCl-oxalate (0.08 M NaCl, 0.005 M Na oxalate, pH 7.0) and placed on two sucrose density gradients each consisting of layers of 6 ml each of 35, 45, 55, and 70% sucrose. In later experiments the 70% sucrose layer was omitted and the remaining layers increased proportionately. Centrifugation at 25,000 rpm for 2 hr in a Spinco swinging bucket rotor No. 25.1 revealed three protein layers in positions similar to those obtained with heart muscle (8, 9): (1) a red layer on top, (2) the main layer between 35 and 45% sucrose, and (3) a narrow zone between 45 and 55% sucrose, which sometimes spread into the 55% sucrose layer (see Fig. 1). The protein layers were withdrawn with a syringe and stored on ice. Protein concentration was determined by the method of

Lowry et al. (10) using bovine serum albumin as a standard. In some experiments subfractions of pellet I were prepared by an additional centrifugation at 8000 × *g*.

*Experimental Procedures*

*Filtration Method* Incubation mixtures contained 0.5 mM $MgCl_2$, 0.5 mM ATP, 0.11 M KCl, 0.01 M NaCl, 0.04 M histidine buffer, pH 7.2, $10^{-5}$ M $CaCl_2$ containing 0.1 $\mu$c $^{45}Ca$, in a total volume of 10 ml. Vesicular protein concentration was 1.0 mg/ml incubation mixture. To achieve this the vesicles stored on ice were diluted to a concentration of 10 mg/ml with 40% sucrose, 1 ml being used to start the reaction. The reaction mixture thus contained 4% sucrose. The rate of calcium uptake was measured by filtering 2 ml aliquots at specified time intervals through Millipore filters (0.45 $\mu$ diameter) (8, 9, 11) using prefilters.

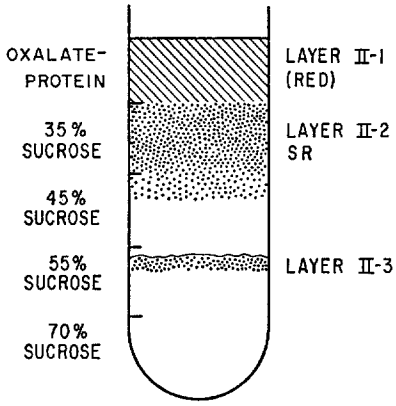


FIGURE 1. Fractionation of sarcoplasmic reticulum from cow uterus muscle on a sucrose density gradient. *SR*, sarcoplasmic reticulum.

Calcium binding was computed from the counts in the filtrates; no allowance being made for any intrinsic calcium in the preparations (8, 9, 11). Aliquots of the filtrates were counted in a Beckman LS-100 liquid scintillation counter using 0.1 ml filtrate, 1 ml 0.1 M HCl, and 10 ml Beckman cocktail D. (Cocktail D consists of 5 g Beckman "PPO" (2,5-diphenyloxazole) and 100 g "Baker Analyzed" naphthalene in 1 liter Baker Analyzed 1,4-dioxane.) Blanks not containing vesicular protein were run simultaneously and counted at the start and termination of each experiment. Centrifugation of filtrates at 150,000 × *g* for 1 hr did not result in a pellet and the counts before and after centrifugation were identical; hence no precipitation of calcium salts occurred at this low calcium concentration. Absorption of counts to the filters was minimal for which correction was made.

*Centrifugation Method* The binding of calcium by the isolated fractions was measured by atomic absorption spectroscopy using the method of Carvalho (12). The reaction mixtures contained 0.01 M KCl, 0.5 mM ATP, 0.5 mM $MgCl_2$, $2 \times 10^{-5}$ M $CaCl_2$, 0.02 M imidazole buffer, pH 7.0, and various reagents as indicated in the tables and figures, in polyallomer tubes. The reaction was started by adding 10-20 mg protein in 2 ml 40% sucrose, bringing the final volume to 10 ml. The tubes were spun for 30 min at 150,000 × *g* in the 50 Ti rotor of the Spinco L2-50 ultracentrifuge. After

centrifugation the pellets and walls of the tubes were rinsed with deionized water, and the pellets resuspended in 10 ml 0.02 M imidazole buffer, pH 7.0. The suspensions were spun again for 30 min at 150,000 × *g*. In order to determine initially bound calcium, samples of protein were carried through the centrifugation and washing procedures. The adequacy of imidazole buffers has been discussed by Carvalho (12). The pellets were resuspended in 5 ml deionized water and the protein concentration redetermined. Analyses for calcium were carried out in deproteinized solutions containing 5% trichloroacetic acid, 0.5% $La^{+++}$, and 5 mM CsCl (12, 13) with a Perkin-Elmer absorption spectrophotometer (model 303). Duplicate samples were carried through the entire procedure.

ATPase activity was measured in separate experiments using the medium of the centrifugation method and 0.2 mg protein per ml. 2 ml aliquots were removed into 2 ml 10% trichloroacetic acid at specified time intervals. Inorganic phosphate was determined by the method of Rockstein and Herron (14). Cytochrome oxidase activity was assayed according to Cooperstein and Lazarow (15).

For electron microscopy vesicle suspensions were centrifuged (1 hr) at 100,000 × *g*, fixed in 2% glutaraldehyde, followed by 1% $OsO_4$, dehydrated in ethanol, released from the plastic centrifuge tube by 100% acetone, and embedded in Araldite 502 (Ciba Pharmaceutical Company, Summit, N. J., Plastics Division). Sections were cut at 500 A and 1000 A, examined unstained for crystalline inclusions, and stained (with $KMNO_4$ and lead citrate) for vesicle morphology.

*Materials* All chemicals used were reagent grade. ATP (Sigma Chemical Co., St. Louis, Mo.) and sucrose (Mann Research Laboratories, N. Y., Special Enzyme grade) were purified by treatment with Dowex 50-x8-$H^+$ and $Na^+$, respectively, as previously described (8, 16). Salyrgan was dissolved in a minimum amount of NaOH, treated with Dowex 50-x8-$Na^+$, and diluted to volume. Addition of the salyrgan solutions to the reaction mixtures caused no rise in pH.

## RESULTS

By analogy with similarly prepared fractions from heart muscle (8, 9), fraction II can be identified as consisting mainly of vesicles derived from the sarcoplasmic reticulum. From Table I it is seen that the yield is only four-tenths that obtained from dog heart. In preliminary experiments it was established that, as in skeletal muscle (17), calcium binding depends on ATP and Mg and is maximal in this fraction at a concentration of 0.5 mM ATP and 0.5 mM $Mg^{++}$. The rate of calcium binding in this fraction measured with the filtration method was very rapid; binding was complete in 1 or 2 min as shown in Fig. 2. Partial inhibition of calcium binding with a low concentration of salyrgan and no inhibition with sodium azide further substantiate the resemblance of this fraction to sarcoplasmic reticulum from skeletal and heart muscle.

When it had been established that sarcoplasmic reticulum could be obtained from uterine smooth muscle and that the velocity of calcium binding was

TABLE I

CALCIUM BINDING BY SUBCELLULAR FRACTIONS FROM COW UTERUS AND DOG HEART

| Preparation | Yield protein | Initial calcium | Final calcium | Calcium binding | Calcium binding capacity |
|---|---|---|---|---|---|
| | *mg/g tissue* | | *μmoles/g protein* | | *mμmoles/g tissue* |
| Cow uterus | | | | | |
| I 2,500-15,000 × *g* | 0.15 | 67.3 | 94.0 | 26.7 | 4.01 |
| II 15,000-40,000 × *g* | 0.41 | 17.6 | 18.8 | 1.2 | 0.49 |
| III 40,000-150,000 × *g* | 0.80 | 16.1 | 16.4 | 0.3 | 0.24 |
| II-1 red layer (sucrose-purified) | 0.06 | 3.5 | 4.4 | 0.9 | 0.05 |
| II-2 SR* (sucrose-purified) | 0.19 | 17.2 | 19.8 | 2.6 | 0.50 |
| Ia 2,500-8,000 × *g* | 0.07 | 74.3 | 106.8 | 32.5 | 2.28 |
| Ib 8,000-15,000 × *g* | 0.07 | 46.0 | 59.0 | 13.0 | 0.91 |
| Dog heart | | | | | |
| II 15,000-40,000 × *g* | 1.03 | — | — | 20.0 | 20.6 |
| II-2 SR* (sucrose-purified) | 0.56 | — | — | 24.0 | 13.5 |

* SR, sarcoplasmic reticulum.

adequate, all further experiments were carried out with the centrifugation method.

The isolated washed sarcoplasmic reticulum (fraction II) contained 16-21 μmoles calcium/g protein; in fraction I calcium content was as high as 55-70 μmoles/g protein. This calcium remained bound after washing and

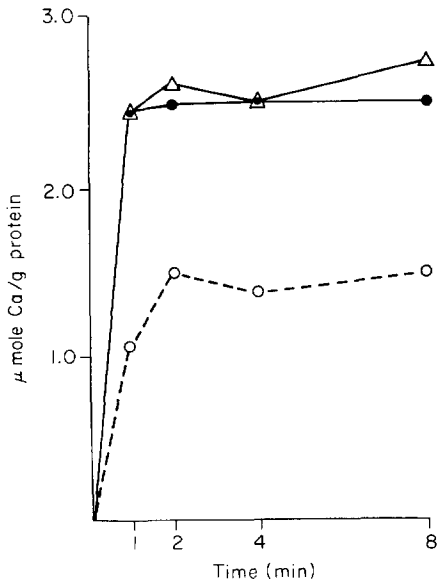


FIGURE 2. Calcium binding by purified sarcoplasmic reticulum, filled circles; $5 \times 10^{-4}$ M sodium azide, open triangles; $5 \times 10^{-4}$ M salyrgan, open circles.

appears to be part of the vesicular membranes. It will be referred to here as initial or "intrinsic" calcium. The additional calcium bound by the preparations in the presence of ATP also remained tightly bound and will be referred to as bound calcium. Calcium "binding" thus measures the calcium taken up in the presence of ATP, compared with washed preparations.

Fraction I proved to be extremely unstable and storage overnight caused the calcium binding to decline 40–55%. Hence calcium binding was measured as soon as fraction I had been prepared. Fractions II and III were stable in sucrose until the next day. Fraction I showed the greatest calcium binding, the small microsomes (III) the lowest (Table I). On purification in a sucrose density gradient, the maximum calcium binding of the isolated

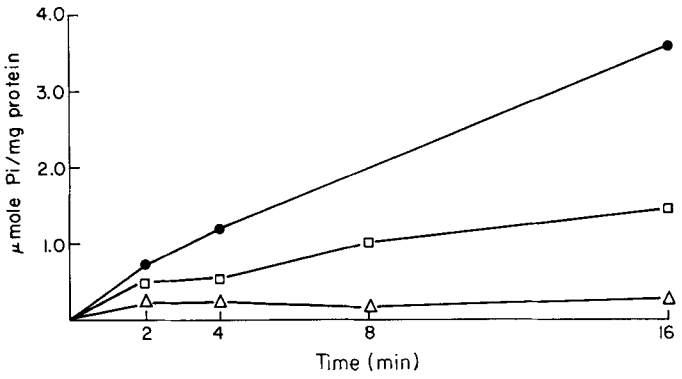


FIGURE 3. Rate of ATP splitting: fraction I, open squares; fraction II, filled circle; fraction III, open triangles.

sarcoplasmic reticulum (II-2) more than doubled, but still was much lower than that of fraction I.

When maximum binding is multiplied by yield of protein, the total capacity of fraction I as seen in Table I, is still eight times that of fraction II. Inasmuch as fraction I is a crude fraction and contains mitochondrial fragments and sarcoplasmic reticulum, as borne out by the experiments using sodium azide (see below) and by electron microscopy, a quantitative comparison between mitochondria and sarcoplasmic reticulum is not possible. Cytochrome oxidase activity, characteristic of mitochondrial preparations, was found in fraction I only. The data given in Table I are those obtained in a typical experiment with cow uterus. The calcium-binding capacity in fraction II was 0.49 mμmole/g uterine muscle. Through purification on the sucrose density gradient, less active protein was removed and calcium uptake doubled; total calcium-binding capacity was constant at 0.50 mμmole/g uterine muscle.

Small amounts of calcium were retained in the small microsomes (III)

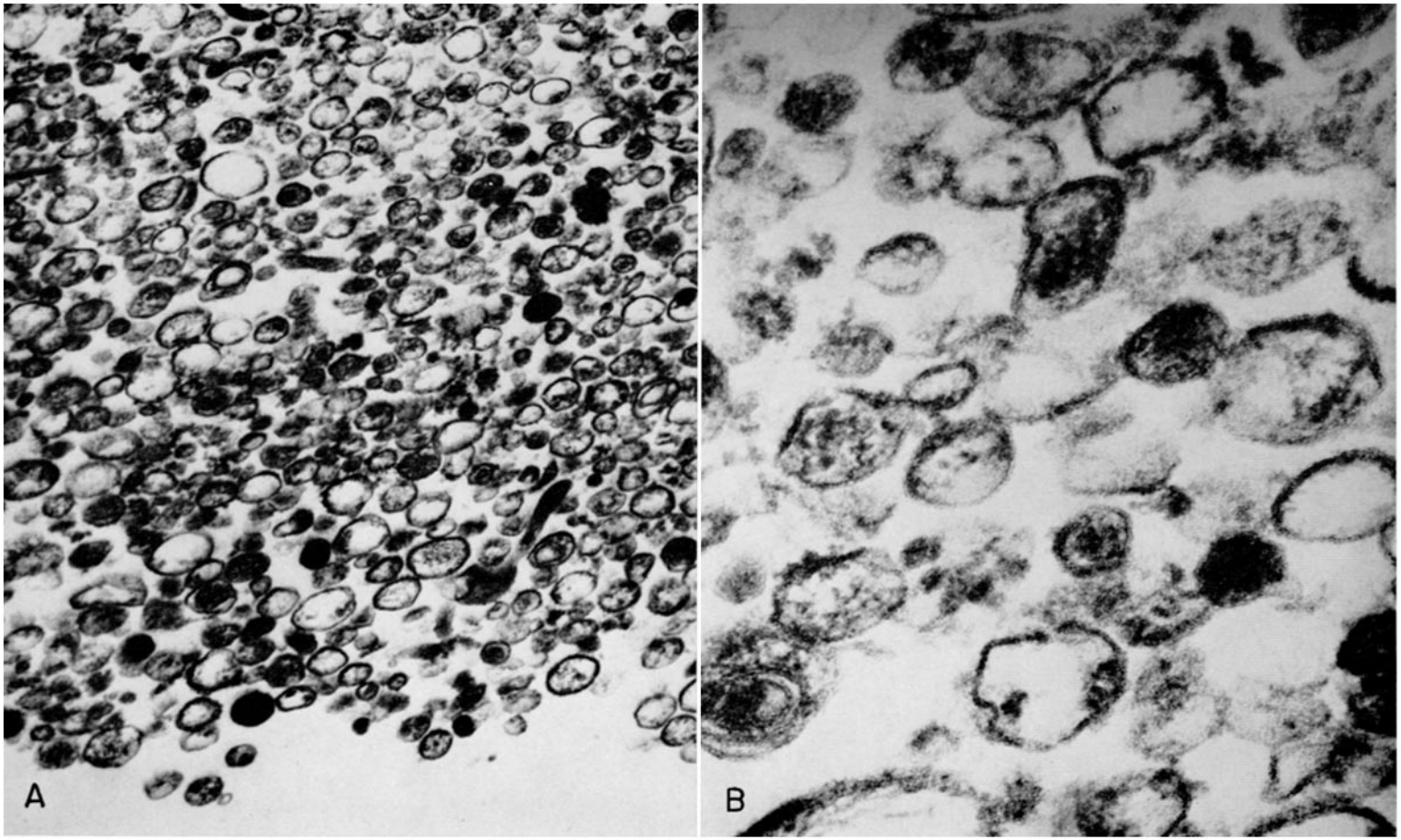


FIGURE 4. Electron micrographs of fragmented sarcotubular vesicles after sucrose gradient centrifugation. 1000 A thick section, stained with $KMNO_4$ and Pb citrate, showing bottom surface of centrifuged pellet of uterine sarcotubular vesicles. × 40,500 (A) and × 136,000 (B). Electron microscopy performed by Dr. M. K. Reedy, Department of Physiology, University of California at Los Angeles.

and in the red layer of the sucrose density gradient (II-I) as seen in Table I. The contribution of the former to the binding is based primarily on its large yield. Binding by fraction II-3 could not be measured, as the amount of protein was too small to be recovered from the sucrose gradient tube.

ATPase activity was found in all three fractions. It was highest in fraction II (Fig. 3), in contrast to calcium binding which was highest in fraction I. In fact, the inorganic phosphate liberated by the vesicular ATPase slightly exceeded the amount of ATP added: this may indicate the presence of myokinase.

TABLE II

EFFECTS OF INHIBITORS ON CALCIUM BINDING IN SUBCELLULAR FRACTIONS FROM COW UTERUS

| Fraction | Additions | Binding | Inhibition |
|---|---|---|---|
| | | *μmoles/g protein* | % |
| I 2,500-15,000 × *g* | 0 | 23.9 | |
| | 5 mM azide | 15.8 | 34* |
| | 10 mM azide | 11.7 | 51* |
| II-2 SR (sucrose-purified) | 0 | 1.9 | |
| | 5 mM azide | 2.2 | 0‡ |
| | 10 mM azide | 2.1 | 0‡ |
| | 0 | 2.1 | |
| | 3 mM salyrgan | −0.9 | 100* |
| | 0 | 3.3 | |
| | No ATP | −0.5 | 100‡ |

* Means of six experiments.
‡ Means of three experiments.

Electron microscopy of fraction II-2 established the vesicular nature of the material (Fig. 4). On close inspection at high magnification the triple layered vesicular membrane can be seen as observed in skeletal muscle vesicles (18). After incubation of uterine vesicles in the presence of 0.2 mM $CaCl_2$ and 5 mM sodium oxalate, followed by centrifugation at 100,000 × *g* for 1 hr and embedding, no crystalline deposits of calcium oxalate could be detected. Similarly treated cardiac sarcoplasmic reticulum showed such deposits. This is consistent with the much higher calcium uptake into cardiac sarcoplasmic reticulum. Further work on this aspect is in progress and will be reported.

Maximal calcium binding by sarcoplasmic reticulum (fraction II-2) was as high as 3.9 μmoles/g protein in one experiment. Salyrgan, known to inhibit calcium uptake in skeletal (17) and cardiac (8) sarcoplasmic reticulum, inhibited completely the ATP-supported calcium binding (Table II), in some instances leading to values below those of initially bound calcium. In

the absence of ATP no binding occurred. Sodium azide, an inhibitor of calcium binding in cardiac mitochondria (19), decreased calcium binding in fraction I but not in fraction II, in concentrations of 5 and 10 mM (Table II). Significantly, fraction Ia, with the sedimentation characteristics of mitochondria, contributed more to the binding than Ib, as demonstrated in a separate experiment (Table I). These results suggest that binding in fraction I is largely due to mitochondrial fragments rather than to contamination with fragments of the sarcoplasmic reticulum.

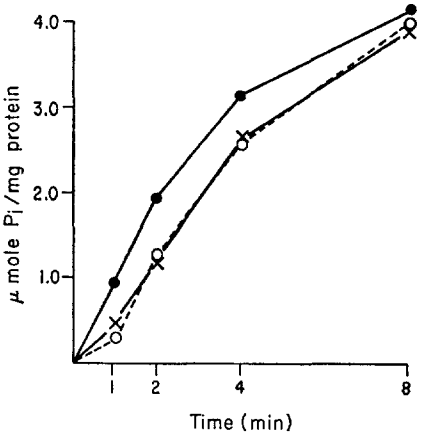


FIGURE 5. Rate of ATP splitting in fraction II-2, purified sarcoplasmic reticulum. 0.5 mM ATP, 0.5 mM $MgCl_2$, 0.02 M imidazole buffer, pH 7.0, 0.01 M KCl, $10^{-5}$ M $CaCl_2$, 0.2 mg protein/ml, filled circles; $10^{-3}$ M salyrgan added, open circles; no calcium, $10^{-3}$ M EGTA added, ×.

The rate of ATP hydrolysis by the purified sarcoplasmic reticulum was lowered in the presence of salyrgan or in the virtual absence of free calcium obtained by addition of a high concentration of EGTA (Fig. 5).

Substitution of 2 mM dithiothrytol for the ascorbic acid in the buffer used for homogenizing the tissue resulted in similar retention of calcium.

## DISCUSSION

It is appropriate to open the discussion with an evaluation of the methods used in this investigation. The filtration method suffers from the uncertainty of (*a*) possible contamination of the assay mixtures with extraneous calcium, and of (*b*) "binding" reflecting to an unknown extent exchange of radioactive calcium with calcium already present in the sarcoplasmic reticulum. (*a*) We have satisfied ourselves that with the precautions taken (deionized water, treatment of reagents with ion exchangers, use of plastic containers instead of glassware, and washing of all equipment with dilute HCl), contamination with extraneous calcium becomes serious only at concentrations

of $10^{-6}$ M or less, but not with the concentration used here ($10^{-5}$ M). (*b*) Comparison of Fig. 2 and Table I shows that there is complete agreement as to the amount of calcium bound, measured with both methods, and that we are assured of measuring net uptake of calcium.

*The results presented clearly indicate that vesicles originating from the sarcoplasmic reticulum can be isolated from smooth muscle of the uterus.* These preparations bind calcium in the presence of ATP and $Mg^{++}$ and thus exhibit properties similar to those of their analogues from skeletal and heart muscle. Calcium binding was observed in all three fractions isolated by differential centrifugation. Although it was found that more calcium is retained in fraction I than in fraction II, much of our work was done with fraction II because this fraction could be obtained in relatively pure form; i.e., apparently free of mitochondrial fragments. Indeed under the electron microscope, the isolated vesicles (Fig. 4) closely resemble those isolated from skeletal and heart muscle. Furthermore, this fraction can be directly compared with preparations from heart muscle which have been prepared in the same manner (8, 9).

In the experiments with inhibitors the sarcoplasmic reticulum, isolated in fraction II-2, is shown to be similar to reticulum obtained from skeletal and heart muscle. Salyrgan completely inhibited the ATP-supported calcium binding (Table II), in some instances leading to values slightly below those of initially bound calcium. Salyrgan also leads to partial inhibition of the ATPase activity; the inhibition being equal to that in the virtual absence of calcium. Thus there appear in smooth muscle as in skeletal muscle the calcium-sensitive or "extrasplitting" ATPase (17) and a basic ATPase, the latter activated by $Mg^{++}$ only, not to exclude the possible occurrence of other ATPases. As in skeletal and heart muscle sarcoplasmic reticulum, free SH groups are required for calcium binding and for extrasplitting ATPase activity, both functions being inhibited by salyrgan (17, 8).

Fraction I is a mixture of elements of the sarcoplasmic reticulum and mitochondrial fragments; it does not contain intact mitochondria. The partial inhibition of calcium binding with sodium azide in fraction I, the large extent of binding in the low speed fraction (subfraction Ia), and the rapid deterioration of calcium-binding activity indicate a significant mitochondrial contribution to the calcium binding. It is tempting to suggest that some differences between smooth and striated muscle such as the slowness of contraction may possibly be explained by calcium storage in the mitochondria. Their contribution to the relaxation-contraction cycle in systems in which small amounts of calcium are involved and relaxation is slow has been considered (20).

As seen in Table I, the binding by smooth muscle vesicles (fraction II-2) is approximately one-tenth of that found in cardiac sarcoplasmic reticulum; the binding capacity of fraction II is only one-twenty-seventh to one-fortieth.

The actomyosin content of smooth muscle is estimated to be 6 mg/g wet weight of cow uterus (21, 22) whereas that of the dog heart is given as 54–57 mg (23); i.e., the actomyosin content of the cow uterus is one-nineth to one-tenth that of the dog heart. When allowance is made for 25% actin and tropomyosin, one computes a myosin content of 4.5 mg/g wet weight of uterus or $0.9 \times 10^{-5}$ mmole on the basis of a molecular weight of approximately 500,000 for myosin. If 1-2 molecules of calcium per myosin molecule are needed for activation (24), 9-18 mμmoles calcium should be needed for full activation of 1 g uterine muscle. Only 0.50 mμmole calcium can be supplied from storage in the sarcoplasmic reticulum, if we base our calculation entirely on fraction II-2, the purest fraction. If our yield represents 10% or less of the total sarcoplasmic reticulum present in the myometrium, one-half to one-fourth the calcium needed for maximum contraction would be immediately available from intracellular calcium stores. Alternatively, one may consider that additional binding in fraction I is as high as 4 mμmoles/g uterine muscle. When a 50% yield for all fractions combined (a conservative estimate) is assumed, one gets well within the range in which vesicular calcium binding would play an important role in contraction and relaxation of the myometrium. This interpretation is advanced in view of the increased activity of our preparations as compared with those discussed in a preliminary report (25).

It has recently been suggested that calcium activates myofibrillar contraction by binding to troponin (26). For skeletal muscle the troponin content was given as about 8% of the myofibrillar proteins (27); 22 μmoles of calcium were bound/g troponin (26). This would correspond to 1.8 μmoles of calcium/g myofibrillar protein. If 1 g muscle contains 100 mg myofibrillar protein (28) and smooth muscle 10% of this, again 18 mμmoles calcium would have to be bound by 1 g uterine muscle and the same calculation applies.

The necessity for calcium in maximum contraction has been established in experiments with glycerinated uterine smooth muscle fibers (29, 30). *Our investigation presents evidence for the existence of a calcium binding and storage site in the sarcoplasmic reticulum of the uterus.* As in skeletal and heart muscle, calcium binding would bring about relaxation and calcium release would be quantitatively adequate to trigger contraction. Further work will be aimed at establishing the role of the sarcoplasmic reticulum in pregnancy and labor.

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