Control of intracellular Ca\(^{2+}\) activity in rat myometrium

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In rat myometrium, the control of intracellular Ca\(^{2+}\) activity is important for the regulation of muscle contractions. Ca\(^{2+}\) uptake properties of the plasma membrane (PM), smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), and mitochondria were isolated from estrogen-dominated rat myometrium. Ca\(^{2+}\) uptake by these fractions was studied in order to estimate the relative potential of the corresponding organelles for controlling intracellular Ca\(^{2+}\) activity. Ca\(^{2+}\) uptake properties of the PM, SER, and RER fractions were similar except that potentiation by oxalate was observed in the PM and PM > SER > RER. However, studies with the ionophores X-537A and A23187 suggested that Ca\(^{2+}\) was transported into the lumen of membrane vesicles of these fractions. Unlike that of skeletal muscle sarcoplasmic reticulum, Ca\(^{2+}\) uptake by the myometrial fractions was not supported by high-energy compounds other than ATP. Mitochondria took up much less Ca\(^{2+}\) at low, and much more Ca\(^{2+}\) at high, free Ca\(^{2+}\) concentrations than did the other fractions. The amount of Ca\(^{2+}\) taken up in 30 s from a 1 μM free Ca\(^{2+}\) solution in the presence of ATP was similar for all fractions. These results suggested that mitochondria may act as an important Ca\(^{2+}\) control system in rat myometrium when the intracellular Ca\(^{2+}\) concentration is near 1 μM or higher, whereas the PM, SER, and RER may be of major importance at Ca\(^{2+}\) levels of 0.3 μM or lower.

In this study, we have isolated subcellular fractions enriched in plasma membrane (PM), smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), and mitochondria from the myometrium of estrogen-dominated rats. We have studied the Ca\(^{2+}\)-uptake characteristics of these fractions and have determined the influence of the free Ca\(^{2+}\) concentration upon the amount of Ca\(^{2+}\) that can be taken up.

**Materials and Methods**

*Preparation of fractions.* The method used for the isolation of PM, SER, and RER is based on one previously described (29) for isolation of PM from rat myometrium. A number of modifications were employed to increase the purity of PM and to isolate SER and RER from the same sucrose gradient as PM.

Female Wistar rats weighing 160–200 g were injected subcutaneously with 200 μg diethylstilbestrol (dissolved in peanut oil) daily for 2 days and were killed on the third day by a blow on the head. The two uterine horns were rapidly removed and placed in ice-cold 250 mM sucrose, 40 mM histidine solution at pH 7.0 (pH adjusted with HCl); this buffer solution was used for all subsequent steps unless otherwise stated. The uterine horns were trimmed of fat and loosely bound connective tissue, slit open with scissors, and stripped of endometrium and most circular muscle; this entire process was carried out on a filter paper moistened with buffer and maintained at approximately 4°C on a cold plate (Thermoelectrics Unlimited, Inc.). All subsequent operations were carried out at 4°C unless otherwise stated. Myometria from 10 to 12 uteri were finely minced with scissors, suspended in 30 ml of buffer, and homogenized twice for 10 s at 15,000 rpm with a Polytron PT20 (Kinematic GMBH).

Figure 1 shows the centrifugation procedures that were used. First, the homogenate was centrifuged at 1,000 × g (avg) for 10 min in order to remove nuclei.
contractile proteins, whole cells, and unbroken tissue. The supernatant from this centrifugation was centrifuged at 10,000 \times g (avg) for 10 min to yield a crude mitochondrial fraction (as the pellet) and a crude microsomal fraction (as the supernatant). This 10,000 \times g centrifugation was omitted in some experiments as indicated in Table 1.

Mitochondria to be used for studies of Ca\(^{2+}\) uptake were then prepared by suspending the crude mitochondrial pellet in buffer and centrifuging for 10 min at 1,000 \times g (avg). The supernatant was centrifuged at 10,000 \times g (avg) for 10 min, and the pellet was washed and resuspended in buffer. This latter centrifugation and washing was repeated once, and the final mitochondrial pellet was resuspended in sucrose-histidine buffer to yield a uniform suspension containing 1–5 mg protein ml\(^{-1}\). Teflon glass homogenizers were used for the suspension of all pellets.

The crude microsomal fraction was sedimented by centrifugation at 113,000 \times g (avg) for 30 min, and the pellet was suspended in 3 ml of buffer. The resulting suspension was carefully layered on the sucrose gradient. The gradient was composed of, from the bottom of the tube upwards, 4 ml of 45% sucrose and 3 ml each of 33.0 and 28.0% sucrose, respectively (Fig. 1). The sucrose concentrations were checked with a refractometer. The density gradient preparation was centrifuged at 112,000 \times g (avg) for 120 min in a swinging bucket rotor (Beckman SW40) after which protein bands were carefully removed from the gradient tube by Pasteur pipettes. Individual fractions obtained were slowly diluted with buffer and deionized distilled water to yield a final sucrose concentration of 8%. The resulting suspensions were centrifuged at 10,000 \times g (avg) for 10 min and the resulting supernatants were centrifuged at 102,000 \times g (avg) for 30 min to yield the final pellets that were used to prepare uniform suspensions of 0.2–1 mg protein ml\(^{-1}\). As shown in Fig. 1, fractions designated PM, SER, and RER were obtained from protein bands located at the 8–28% interface, within the 33% sucrose band, and at the 33–45% interface, respectively.

**Enzymatic determinations.** The following were used as plasma membrane marker enzymes (11, 14): 5'-nucleotidase (44); K\(^{+}\)-activated; ouabain-sensitive phosphatase assayed as described by Kidwai and colleagues (29), except that pH 7.4 and 1 mM ouabain were used; and phosphodiesterase I (46). Nonspecific phosphatase was estimated using \(\beta\)-glycerophosphate and glucose 6-phosphate as substrates (16). Nonspecific phosphatase activity was found to be less than 10% of the 5' nucleotidase activity in all fractions, and therefore no correction for it was made.

Mg\(^{2+}\)-ATPase activities were measured in similar solutions to those used for Ca\(^{2+}\) uptake, except that all Ca\(^{2+}\) was omitted, and 5 mM ATP and 0.15 mM ethylene glycol bis (\(\beta\)-aminoethyl)ether)-N,N',N'-tetraacetic EGTA were present. Incubation media for Mg\(^{2+}\)-ATPase measurements were the same as those for Mg\(^{2+}\)-ATPase, except that 0.1 mM CaCl\(_2\) was added. The ATPase reactions were started by the addition of 0.1 ml of protein (to give a final volume of 1 ml) and were stopped after a 10-min incubation at 37°C by addition of 1 ml of cold 10% trichloroacetic acid (TCA). Inorganic phosphate was measured by the method of Fiske and Subbarow (19) using 2,4-diaminophenol as the reducing agent (41). Protein was determined as described by Lowry et al. (33), and cytochrome c oxidase activity was measured by the method of Cooperstein and Lazarow (10).

**Electron microscopy.** Pellets were fixed in buffered glutaraldehyde (pH 7.1), postfixed in osmium tetroxide, and embedded in epon. Sections were stained with uranyl acetate and lead citrate, and photographed on a JEOL 7A electron microscope. Several sections from each of several different preparations were examined.

**Measurement of Ca\(^{2+}\) uptake.** Ca\(^{2+}\) uptake was measured using an adaptation of the filtration technique of Martonosi and Feretos (34) and was always done on freshly prepared fractions. The reaction mixture contained: 100 mM KCl; 5 mM MgCl\(_2\); 0.1 mM CaCl\(_2\), labelled with 0.4 \(\mu\)Ci ml\(^{-1}\)\(\cdot\)45Ca\(^{2+}\), 40 mM imidazole; and 10–50 \(\mu\)g of protein per final milliliter of reaction mixture, depending on the fraction used and the conditions of the assay. Where indicated in the text, 5 mM sodium ATP, 0.5 mM sodium azide or 5 mM potassium oxalate, variable amounts of potassium EGTA, and various substrates were added and dissolved directly in the incubation medium. The drug, X-537A and A23187, however, had to be added in an ethanol vehicle in such a way that the final concentration of ethanol in the incubation medium became 1%. The reaction mixture was buffered at pH 7.0 with 40 mM imidazole that would prevent changes in the free Ca\(^{2+}\) concentration under our conditions due to changes in pH. Uptake reactions were carried out at 37°C in a shaking-water bath unless otherwise indicated. Reactions were started by addition of protein (0.1 ml for every 1 ml of final reaction mixture) accompanied by rapid mixing with a Vortex-Genie after a preincubation time of 5 min at 37°C. The reaction
was stopped by withdrawing 0.8 ml with a Pipettman (Gilson Medical Electronics, Inc.) automatic pipette and filtering through a 25-mm-diam filter with pore size of 0.45 μm (Matheson-Higgin Co.) that was held on a Millipore 3025 filtration manifold. Filtration took 2–3 s and was followed by a wash with 10 ml of 250 mM sucrose-40 mM imidazole solution at pH 7.0 which took 8–12 s. Prior to use, all filters were washed with 10 ml of 100 mM KCl solution followed by 10 ml of sucrose-imidazole solution; this procedure was required for low background counts.

Filters were dissolved in 10 ml of Bray’s solution (6) and were counted to 2% accuracy. Appropriate blanks (no protein added), controls, and aliquots of reaction mixtures from blank tubes were counted for each experiment. No quench correction was required.

Free Ca2+ concentration. The free Ca2+ concentration in the reaction mixture was controlled by the use of EGTA. The concentrations of EGTA required to produce desired free Ca2+ concentrations were calculated from the stability constants used by Godt (22) by means of the equations of Katz et al. (30).

Materials. Distilled water was deionized to a specific resistance greater than 16 MΩ cm–2. Organic compounds were of the highest purity available from Sigma Chemical Co.; 45CaCl2 was obtained from Amersham/Searle Corp.

Terminology. We have used Ca2+ uptake as an operational term to include all the processes whereby 45Ca2+ becomes associated with membrane vesicles. Ca2+ transport is used when we refer specifically to processes leading to formation of a gradient of Ca2+ across the vesicle membrane. We think the term binding should be used for chemical association between Ca2+ and a membrane site.

RESULTS

All of the protein from the sucrose gradient shown in Fig. 1 was removed as 11 fractions. The enzymatic activities, Ca2+ uptake in the presence of azide, and morphology of these fractions were studied to determine which fractions best represented plasma membrane, smooth endoplasmic reticulum, and rough endoplasmic reticulum (unpublished studies). From these studies, fractions designated PM, SER, and RER (Fig. 1) were chosen for further study. The activities of these fractions were compared with those of the 1,000 × g supernatant (which was free of nuclei, collagen, and cells) and the mitochondrial fraction isolated by differential centrifugation. Table 1 shows the enzymatic activities of these fractions.

The PM exhibited a 14- to 45-fold enhancement in the specific activity of plasma membrane marker enzymes compared with the 1,000 × g supernatant. Fractionation of this fraction by centrifugation before the sucrose gradient step was omitted. Numbers in parentheses are the number of independent preparations tested.

Table 1. Enzymatic activities of fractions from rat myometrium

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fraction</th>
<th>PM</th>
<th>SER</th>
<th>RER</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-Nucleotidase</td>
<td>7.8 ± 0.6</td>
<td>111.5 ± 3.4</td>
<td>28.0 ± 1.5</td>
<td>14.4 ± 1.1</td>
<td>16.7 ± 1.3</td>
</tr>
<tr>
<td>(10)</td>
<td>(32)</td>
<td>(30)</td>
<td>(21)</td>
<td>(19)</td>
<td></td>
</tr>
<tr>
<td>K+-stimulated, oxalated,</td>
<td>0.07 ± 0.13</td>
<td>3.16 ± 0.25</td>
<td>0.25 ± 0.28</td>
<td>0.20 ± 0.11</td>
<td>0.50 ± 0.09</td>
</tr>
<tr>
<td>sensitobiotic phosphatase</td>
<td>(4)</td>
<td>(6)</td>
<td>(6)</td>
<td>(6)</td>
<td>(4)</td>
</tr>
<tr>
<td>Phosphodiesterase I</td>
<td>0.120 ± 0.08</td>
<td>1.67 ± 0.54</td>
<td>0.292 ± 0.08</td>
<td>0.252 ± 0.14</td>
<td>0.131 ± 0.13</td>
</tr>
<tr>
<td>(7)</td>
<td>(13)</td>
<td>(12)</td>
<td>(12)</td>
<td>(3)</td>
<td></td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>1.0 ± 0.2</td>
<td>13.5 ± 1.4</td>
<td>29.5 ± 0.6</td>
<td>31.4 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>(12)</td>
<td>(12)</td>
<td>(9)</td>
<td>(9)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Specific activities are expressed as micromoles phosphate released milligram–1 protein hour–1 except in the case of cytochrome c oxidase activity that is expressed as a percent of the specific activity of the inner mitochondrial membrane fraction obtained from the sucrose gradient tube; in these experiments the 10,000 × g centrifugation before the sucrose gradient step was omitted. Numbers in parentheses are the number of independent preparations tested.

Representative electron micrographs of the PM, SER, and RER are shown in Fig. 2. Triple layer membrane structures were seen at higher magnifications of all fractions (not shown). PM and SER consisted mainly of vesicles not bounded by ribosomes, and RER was composed almost entirely of vesicles with attached ribosomes. Mitochondrial fragments were seen in the RER fraction but not in PM or SER. The RER fraction was designated as such on the basis of its appearance in all electron micrograph examinations and its low contamination with plasma membrane marker enzymes (Table 1). The SER best represented material derived from smooth endoplasmic reticulum because it consisted mainly of smooth vesicles that exhibited low activity of both plasma membrane and mitochondrial marker enzymes (Table 1). The lack of established marker enzymes for smooth muscle endoplasmic reticulum makes this designation tentative. Similarly, the extent to which this fraction contaminated others is not known.

The specific activities of the plasma membrane marker enzymes in the mitochondrial fraction isolated by differential centrifugation were 9–16% of those in the PM. In addition to intact mitochondria, some smooth and rough vesicles could be seen in electron micrographs of these fractions (not shown). Some indication of the contamination of this fraction by SER and RER is obtained from the effects of azide and of oxalate on Ca2+ uptake (Table 2). Nearly all of the Ca2+ uptake by the mitochondrial fraction is azide sensitive, and the small increase in Ca2+ uptake in the presence of oxalate is not statistically significant; both of these effects are in contrast to those seen in the other fractions.

The lack of inhibition by azide in the PM (Table 2) confirmed the results of Table 1 that indicated very little mitochondrial contamination in this fraction. The
presence of some mitochondrial membrane in SER and RER is indicated by the azide sensitivity of the Ca\(^{2+}\) uptake (Table 2) and by the cytochrome c oxidase activities (Table 1).

Because Ca\(^{2+}\) uptake by PM exhibits little potentiation by oxalate (Table 2), it appears that Ca\(^{2+}\) transport might not take place in this fraction; alternatively, the vesicles might not be permeable to oxalate. Similarly, the contribution made by Ca\(^{2+}\) transport to the total uptake in SER and RER was unknown. The results obtained by Scarpa et al. (38), who were able to abolish the Ca\(^{2+}\) gradient across membranes with the ionophoric antibiotics A23187 and X-537A, prompted us to use these substances in the myometrial system to determine whether or not Ca\(^{2+}\) gradients are created. The presence of 1% ethanol in the incubation medium had a negligible effect upon uptake by SER and RER although it produced a slight inhibition of uptake by PM. The presence of both A23187 and X-537A rendered PM, SER, and RER much less capable of retaining Ca\(^{2+}\) than they were.
in the absence of ionophore (Table 3). The residual Ca²⁺ uptake was similar to that in the absence of ATP (Table 2).

Table 4 shows the ATPase activities of the fractions. In the absence of Ca²⁺, the ATPase activity of PM was 250% greater than the activity of SER which was more than 150% higher than the activity of RER. When Ca²⁺ was added, all fractions showed increased splitting of ATP, but only that by PM and RER was shown to be significant.

Although the enzyme responsible for the Ca²⁺ transport in skeletal muscle sarcoplasmic reticulum (SR) has been termed ATPase, it has been clearly shown that inosine, guanosine, cytidine, or uridine triphosphate (ITP, GTP, CTP, or UTP, respectively) (24, 34), acetyl phosphate (12), and paranitrophenyl phosphate (28) can provide the energy for transport of Ca²⁺ in this system. Therefore we determined whether Ca²⁺ uptake by the myometrial systems had a similar or different substrate specificity. Table 5 shows that none of the alternative substrates that was used was capable of elevating Ca²⁺ uptake by PM, SER, or RER above that occurring in the absence of any substrate. In fact, paranitrophenyl phosphate decreased Ca²⁺ uptake by reticulum fractions (P < .05). When these experiments were repeated with 5 mM oxalate present in the incubation medium, essentially similar results were obtained (results not shown).

The time courses for Ca²⁺ uptake by the three vesicular fractions (PM, SER, and RER) were found to be very similar to each other (Fig. 3). They all showed very rapid uptake from 17 μM free Ca²⁺ for the first 30 s and reached steady-state values of 20–30 μmol Ca²⁺ g⁻¹ protein in 10–15 min. In contrast, mitochondria reached a steady-state value of about 100 μmol g⁻¹ protein in 5

TABLE 2. Calcium uptake by fractions from rat myometrium.

<table>
<thead>
<tr>
<th>Addition to Incubation Medium</th>
<th>Fraction</th>
<th>PM</th>
<th>SER</th>
<th>RER</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>5.2 ± 1.0</td>
<td>5.0 ± 0.6</td>
<td>4.9 ± 1.0</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>5 mM ATP</td>
<td></td>
<td>29.0 ± 9.7*</td>
<td>17 ± 1.8*</td>
<td>16.9 ± 1.9*</td>
<td>13.4 ± 24*</td>
</tr>
<tr>
<td>5 mM ATP + 5 mM oxalate</td>
<td></td>
<td>29.2 ± 3.6</td>
<td>35.5 ± 9.6*</td>
<td>48.3 ± 9.9*</td>
<td>144.9 ± 30</td>
</tr>
<tr>
<td>5 mM ATP + 0.5 mM azide</td>
<td></td>
<td>21.9 ± 3.6</td>
<td>11.9 ± 0.6</td>
<td>10.0 ± 2.5</td>
<td>7.4 ± 1.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. The units are micromoles Ca²⁺ gram⁻¹ protein. Conditions were as given in text.

TABLE 3. Effect of ionophores on Ca²⁺ uptake by fractions from rat myometrium.

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Fraction</th>
<th>PM</th>
<th>SER</th>
<th>RER</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM ATP</td>
<td></td>
<td>23.2 ± 5.0</td>
<td>17.5 ± 2.9</td>
<td>12.8 ± 2.5</td>
</tr>
<tr>
<td>5 mM ATP + 1% ethanol</td>
<td></td>
<td>17.3 ± 4.5</td>
<td>11.2 ± 2.4</td>
<td>14.6 ± 3.3</td>
</tr>
<tr>
<td>5 mM ATP + 1% ethanol + 10 μM</td>
<td></td>
<td>5.5 ± 4</td>
<td>4.7 ± 9.5</td>
<td>4.5 ± 4.7</td>
</tr>
<tr>
<td>A53187</td>
<td></td>
<td>7.5 ± 2.5</td>
<td>9.8 ± 4.2</td>
<td>4.1 ± 1.2</td>
</tr>
</tbody>
</table>

Values are means ± SE of the uptake of five preparations. The units are micromoles Ca²⁺ gram⁻¹ protein. Conditions were as given in Table 2.

TABLE 4. ATPase activities of the fractions from rat myometrium.

<table>
<thead>
<tr>
<th>Additions to Incubating Fraction</th>
<th>Fraction</th>
<th>PM</th>
<th>SER</th>
<th>RER</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM ATP + Mg²⁺ ATPase</td>
<td></td>
<td>116 ± 4</td>
<td>46 ± 3</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>MgATPase</td>
<td></td>
<td>104 ± 5</td>
<td>41 ± 3</td>
<td>26 ± 1</td>
</tr>
</tbody>
</table>

Difference = extra splitting caused by added Ca²⁺

Values are means ± SE of the activities of four preparations expressed as micromoles Pi released per milligram protein. Assay conditions are given in the text. * The difference is significant at P < .005 using a paired Student t test.

TABLE 5. Ca²⁺ uptake by subcellular fractions of rat myometrium in presence of various substrates.

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Fraction</th>
<th>PM</th>
<th>SER</th>
<th>RER</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>35 ± 5</td>
<td>36 ± 3</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>5 mM ATP</td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5 mM GTP</td>
<td></td>
<td>21 ± 5</td>
<td>28 ± 7</td>
<td>16 ± 10</td>
</tr>
<tr>
<td>5 mM ITP</td>
<td></td>
<td>26 ± 6</td>
<td>32 ± 7</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>5 mM AcP</td>
<td></td>
<td>27 ± 8</td>
<td>26 ± 11</td>
<td>25 ± 11</td>
</tr>
<tr>
<td>5 mM PNpP</td>
<td></td>
<td>25 ± 19</td>
<td>21 ± 4</td>
<td>11 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3 preparations and are expressed as a percent of Ca²⁺ uptake in the presence of 5 mM ATP. Conditions were as given in text.

![Fig. 3. Time course of Ca²⁺ uptake by subcellular fractions of rat myometrium.](http://apcell.physiology.org/)

In upper curve A represents RER, PM, and SER. Standard errors of means were less than 4 in all cases except for RER at 5.10 and 15 min at which points they were less than 12. Lower curve is mitochondria. Points are means from 6 experiments in all cases.
min under the same conditions. The effects of the free Ca\(^{2+}\) concentration upon Ca\(^{2+}\) uptake were studied using incubation times of 10 min. Under these conditions, less than 15% of the ATP present was hydrolyzed, and the amounts of calcium taken up could be accurately measured by our technique.

Just as the time courses for Ca\(^{2+}\) uptake by PM, SER, and RER were similar, so the dependences of Ca\(^{2+}\) uptake on the free Ca\(^{2+}\) concentration were similar for these three fractions (Fig. 4). Mitochondria, however, unlike the vesicular fractions, did not exhibit ATP-dependent uptake from solutions of 0.03 to 0.3 \(\mu\)M free Ca\(^{2+}\), but bound far more Ca\(^{2+}\) at 1 \(\mu\)M (Fig. 4) and higher Ca\(^{2+}\) concentrations (Fig. 5) than did the other fractions.

In order to compare the initial rates of Ca\(^{2+}\) uptake by the PM and mitochondrial fractions, total and ATP-dependent binding by these fractions was measured after a 30-s incubation at 20°C. The free Ca\(^{2+}\) concentration in these experiments (1 \(\mu\)M) was chosen to be similar to the intracellular Ca\(^{2+}\) activity of maximally contracted myometrium (see discussion). Total Ca\(^{2+}\) uptake was 1.3 ± 0.2 \(\mu\)mol g\(^{-1}\) protein for PM, and 0.7 ± 0.2 \(\mu\)mol g\(^{-1}\) for mitochondria, whereas ATP-independent uptake was 0.5 \(\mu\)mol g\(^{-1}\) for both PM and mitochondria. Three independent observations were made in all cases.

**DISCUSSION**

Intracellular Ca\(^{2+}\) activity of cells must be carefully controlled because Ca\(^{2+}\) plays a key regulatory role in a variety of cellular processes (32). In muscle cells, Ca\(^{2+}\) enters or is released from internal stores to initiate contraction and must be removed to allow relaxation. There is general agreement that the SR is the major regulator of Ca\(^{2+}\) activity during contraction in white skeletal muscle (27), whereas mitochondria may be the most important system in cells such as those of liver, kidney (4) and nerve (1) that do not have to remove large quantities of the ion so rapidly. There is some dispute as to the relative regulatory contributions of SR and mitochondria in red skeletal and heart muscle (32, 42), but the work of Scarpa and Graziotti (39) indicates that Ca\(^{2+}\) uptake by mitochondria is grossly inadequate for the regulation of the beat-to-beat Ca\(^{2+}\) cycle of mammalian heart. It is established that the plasma membrane of nerve (1), kidney (36), and erythrocytes (40) can transport Ca\(^{2+}\), although the role of this organelle in lowering intracellular Ca\(^{2+}\) after contraction of skeletal (45) and smooth muscle (26) is not well established because it is not known if these preparations can transport Ca\(^{2+}\) from a solution of low Ca\(^{2+}\) activity at a reasonable rate. In smooth muscle, no internal organelle exists similar to the sarcoplasmic reticulum of striated muscle in organization or in amount of membrane. It has been suggested (5, 43) that mitochondria and endoplasmic reticulum play important roles in regulation of intracellular Ca\(^{2+}\) in vascular smooth muscle. Alternately, an outwardly directed pump for Ca\(^{2+}\) located in the plasma membrane has been suggested as a major mode of Ca\(^{2+}\) regulation in myometrium (49).

In this study we have isolated membrane fractions enriched in plasma membrane, simultaneously with fractions somewhat enriched in smooth and rough endoplasmic reticulum and mitochondria from the longitudinal muscle of rat uterus. Furthermore, we have investigated the ability of these fractions to remove Ca\(^{2+}\) from solutions of physiological Ca\(^{2+}\) concentrations.

The PM fraction that we isolated was at least 14 times enriched in plasma membrane marker enzymes as compared to the 1,000 \(\times g\) supernatant, and this represents a considerable improvement in purity over the plasma membrane fraction from this tissue previously isolated by Kidwai et al. (29). Table 1 shows that the SER and RER fractions had very low plasma membrane marker activity; however, contamination of these two fractions by mitochondrial material was quite substantial. SER had 42% of its ATP-dependent Ca\(^{2+}\) uptake inhibited by the presence of azide (Table 2) and 42% of the specific
activity of cytochrome c oxidase present in the mitochondrial fraction (Table 1). The RER had a similar cytochrome c oxidase activity to that of the mitochondrial fraction, and its ATP-dependent Ca\(^{2+}\) uptake was inhibited 58\% by azide. In the case of both SER and RER, however, total ATP-dependent Ca\(^{2+}\) uptake was much less than that predicted from the specific activity of cytochrome c oxidase activity relative to Ca\(^{2+}\) uptake by the mitochondrial fraction. There was a large azide-insensitive component in each case and their Ca\(^{2+}\) uptake properties were also markedly different (Figs. 3, 4, and 5). This suggests that the mitochondrial contamination of both SER and RER resulted mainly from damaged particles that did not fully contribute to Ca\(^{2+}\) uptake.

Thus much of the material in these fractions is derived from the endoplasmic reticulum. However, the lack of an established marker for smooth muscle endoplasmic reticulum (50) made it impossible to determine either the purification of endoplasmic reticulum in these fractions, or the contamination of these fractions in PM. Morphological evidence, however, strongly supports our conclusions that the fraction designated RER is composed substantially of material derived from the rough endoplasmic reticulum of the muscle. An estimate of the amount of endoplasmic reticulum in each fraction might be made from the effect of oxalate on ATP-dependent uptake in each fraction. The mitochondrial fraction had an azide-insensitive ATP-dependent uptake of about 5 \(\mu\)mol Ca\(^{2+}\) g\(^{-1}\) protein under the standard assay conditions (see Table 2) and an increase in uptake due to oxalate of about 28 \(\mu\)mol Ca\(^{2+}\) g\(^{-1}\). The respective numbers for the RER fraction were 5 and 31, indicating that there may have been as much material of rough endoplasmic reticulum origin in the mitochondrial fraction as was in RER. If this really was the case, cytochrome c oxidase activities and azide sensitivities will give an overestimate of the contamination in other fractions due to mitochondria. However, there is no independent evidence that in smooth muscle an "oxalate effect" is unique to the endoplasmic reticulum, and the increase with this anion in the mitochondrial fraction was not statistically reliable.

The fact that the presence of the ionophorous antibiotics in the incubation medium lowered the Ca\(^{2+}\) uptake by PM, SER, and RER to values close to those obtained in the absence of ATP (Tables 2 and 3) suggests that a major fraction of the ATP-dependently accumulated Ca\(^{2+}\) is transported across the vesicle membrane and that a Ca\(^{2+}\) concentration gradient is formed in all three cases. Thus the lack of effect of oxalate in augmenting Ca\(^{2+}\) uptake by PM (Table 2) appears to be due to a lack of permeability of the PM to oxalate rather than to either the absence of transport by PM or a higher leakiness of PM vesicles to Ca\(^{2+}\). In this discussion it is assumed that these ionophores act by allowing Ca\(^{2+}\) diffusion across membranes (38), but not by inhibiting Ca\(^{2+}\) binding or exchange (17). Direct evidence that net uptake rather than exchange of Ca\(^{2+}\) occurred is not yet available.

The apparent presence of an extra-splitting of ATP due to added Ca\(^{2+}\) shown in Table 4 seems to support the concept that a Ca\(^{2+}\)-stimulated ATPase is responsible for the Ca\(^{2+}\) transport by these fractions. Closer examination of the data reveals that such support is at best very tenuous. Under the conditions of ATPase assay that we used, the free Ca\(^{2+}\) in the medium upon addition of Ca\(^{2+}\) was 1 \(\mu\)M (30, 22). From a 1 \(\mu\)M Ca\(^{2+}\) solution, PM, SER and RER all take up approximately 8 \(\mu\)mol Ca\(^{2+}\) g\(^{-1}\) protein in 10 min (Fig. 4). Thus if the stoichiometry that pertains in SR of skeletal muscle, whereby two Ca\(^{2+}\) ions are transported while one ATP molecule is hydrolized (27), pertains also in PM, SER, and RER, we should expect to see only 0.004 \(\mu\)mol P\(_{i}\) mg\(^{-1}\) protein released under those conditions. Clearly such a small amount is not measurable with the techniques we have used. The much larger amount of P\(_{i}\) released in response to added Ca\(^{2+}\) may indicate that a large proportion of the ATPase normally responsible for Ca\(^{2+}\) transport in PM, SER, and RER has become "uncoupled" or that there exist Ca\(^{2+}\)-stimulated ATPases not involved in Ca\(^{2+}\) transport. Moreover the ATPase involved in Ca\(^{2+}\) transport by our membrane vesicles is clearly different from that of skeletal muscle SR because it can utilize only ATP (Table 5) in contrast to the spectrum of substrates that can support Ca\(^{2+}\) transport in the latter (24, 34, 25, 12). Myometrial membranes resemble heart SR (17) and membranes from mesenteric artery (51) as well as microsomes from several other types of cells (35, 36, 37) in this regard. The stoichiometry of Ca\(^{2+}\) transport by this ATPase is unknown.

Our procedure requires only 5-6 h from the death of the rats to the complete fractionation of the tissue without the need for high salt extraction, thus allowing early study of Ca\(^{2+}\) uptake. Preliminary studies indicated that both SER and mitochondria lost some Ca\(^{2+}\)-sequestering ability after storage. Thus despite appreciable mutual contamination of endoplasmic reticulum and mitochondrial fractions, new information was obtained.

Compared to skeletal muscle SR (27), the vesicular fractions from rat myometrium exhibited a lower rate of Ca\(^{2+}\) uptake, a lower net uptake of Ca\(^{2+}\) at all Ca\(^{2+}\) concentrations tested, and a much lower enhancement of Ca\(^{2+}\) transport by oxalate and a more stringent substrate requirement.

In SR, isolated from rat skeletal muscle by the same techniques as used here takes up 5 times as much Ca\(^{2+}\) in 30 s from a solution of 0.3 \(\mu\)M Ca\(^{2+}\) as PM does in 10 min under otherwise identical conditions (unpublished studies). This difference is consistent with the relative rates of relaxation of these two types of muscle. However, the apparent rates of binding and the maximum amount of Ca\(^{2+}\) that can be bound by the myometrial fractions might be reduced as a result of vesicle leakiness and/or the presence of vesicles with the wrong orientation with respect to the direction of transport. Nevertheless, the Ca\(^{2+}\) uptake by the myometrial vesicular fractions from solutions of high Ca\(^{2+}\) concentrations was similar in magnitude to that reported for rabbit aorta (29) and guinea pig intestine (26). The results suggest that the mechanisms of Ca\(^{2+}\) transport of PM, SER, and RER are similar to each other but different from that of the SR of skeletal muscle.
The Ca\(^{2+}\)-transporting properties of mitochondria were markedly different from those of the other fractions, not only with regard to azide sensitivity and the lack of effect of oxalate (Table 2), but also with regard to Ca\(^{2+}\) dependence (Figs. 4 and 5). The amount of Ca\(^{2+}\) taken up by mitochondria in 10 min from 1 \(\mu\)M free Ca\(^{2+}\) is greater than that taken up by PM, SER, or RER, but the latter fractions take up much more Ca\(^{2+}\) from 0.3 \(\mu\)M Ca\(^{2+}\).

In contrast to our results, Batra (2) has reported that mitochondria, but not microsomes isolated from human myometrium, are capable of taking up Ca\(^{2+}\) from low Ca\(^{2+}\) solutions at a sufficient rate to account for relaxation. These mitochondria could take up significant amounts of Ca\(^{2+}\) from solutions of 0.09 and 0.6 \(\mu\)M free Ca\(^{2+}\). Because Scarpa and Graziotti (39) have also noted a threefold difference in the half maximal rate of Ca\(^{2+}\) transport between frog heart and rat heart mitochondria, it seems likely that a species difference in the affinity of mitochondria for Ca\(^{2+}\) may well exist.

There was some variability in Ca\(^{2+}\) uptake from preparation to preparation, and because of this we were unable to determine the rank order of Ca\(^{2+}\) uptake at any given Ca\(^{2+}\) concentration among PM, SER, and RER (compare Figs. 2 and 3) although differences between these three fractions and the mitochondrial fractions were very clear (compare Figs. 4 and 5). Studies on glycerinated smooth muscles (5, 18) suggest that maximal contractions of these muscles occur when the free cytoplasmic Ca\(^{2+}\) concentration is 1-4 \(\mu\)M. Our results suggest the possibility that, when the longitudinal smooth muscle of the rat myometrium is relaxing from a maximal contraction, the plasma membrane, smooth and rough endoplasmic reticulum, and mitochondria may contribute equally (on a weight-to-weight of protein basis) to the necessary Ca\(^{2+}\) sequestration. However, the relative contribution of each of the organelles in relaxation will depend upon the relative amount of that organelle present in the cell. Wallach and Lin (50) have discussed the many problems involved in estimating the purity and yield of membrane fractions in studies of this type. The only satisfactory method by which the total amount of each organelle present in the cell can be estimated is a carefully controlled quantitative electron microscope study.

The complete relaxation of the uterus might possibly require that the free myoplasmic Ca\(^{2+}\) concentration be lowered to below 10\(^{-7}\) M (5, 18); if such were the case, then the relative importance of the various organelles would change at different stages of relaxation. Such changes in importance are indicated by the facts that at Ca\(^{2+}\) concentrations of 0.3 \(\mu\)M and less, only PM, SER, and RER bind significant amounts of Ca\(^{2+}\), whereas at concentrations of 1 \(\mu\)M and higher, the mitochondrial Ca\(^{2+}\) binding becomes of increasing significance.

Several factors are unfortunately unknown about the systems we have studied, those of major importance being the intracellular pH and the intracellular concentrations of MgATP. The ability of the various fractions to transport Ca\(^{2+}\) is undoubtedly dependent upon these two variables, and Godt (22) has clearly shown that the Ca\(^{2+}\) dependence of skinned frog muscle fibres is dependent upon the MgATP concentration. Thus it is impossible to determine precisely the ranges of Ca\(^{2+}\) concentrations (or states of contraction) over which the various organelles are most important for relaxation. Additionally, it has been suggested that functional relationships might exist in smooth muscle between closely associated plasma membrane, endoplasmic reticulum, and mitochondria (15, 23); such relationships are necessarily lost in subfractionation studies of this type.

Since the completion of our study, Moore et al. (35) found that rough as well as smooth endoplasmic reticulum from rat liver was able to actively take up Ca\(^{2+}\) although the latter took up Ca\(^{2+}\) more rapidly than the former. It is not surprising that both types of ER exhibit this activity because few or no qualitative functional differences exist between rough and smooth microsomes with the exception of the ability to carry out protein synthesis (13). Also we have found in sodium dodecyl sulfate-gel electrophoresis studies that the membrane proteins of the two fractions from myometrium show many similar but some different proteins (A. Murray, and E. E. Daniel unpublished studies). Ford and Hess (21) and Vallieres et al. (47) have recently reported that mitochondria from vascular smooth muscle did not rapidly take up Ca\(^{2+}\) from solutions of low Ca\(^{2+}\) activity. This conclusion is in agreement with our results on myometrial mitochondria. Of particular importance is their demonstration that mitochondria from vascular smooth muscle retain and tolerate high Ca\(^{2+}\) loads without loss of oxidative phosphorylation (47).

In summary the results of our studies provide the first direct evidence for a role for the plasma membrane in the regulation of Ca\(^{2+}\) activity of smooth muscle. Furthermore, they provide independent support for electron probe X-ray microanalysis studies (43) which suggest a potential role for mitochondria and endoplasmic reticulum in this process.

REFERENCES


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