The isolation and characterization of Ca\(^{++}\)-accumulating subcellular membrane fractions from cerebral arteries

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A study was undertaken using differential centrifugation methods to isolate from rabbit cerebral arteries the subcellular microsomal protein fractions capable of actively sequestering Ca\(^{++}\). One isolated protein fraction displayed a relatively large adenosine triphosphate (ATP)-dependent Ca\(^{++}\)-accumulating capacity that was completely inhibited by NaN\(_3\), and was therefore designated the "mitochondrial fraction." Electron microscopy confirmed that this fraction consisted of numerous mitochondrial elements. Another isolated membrane fraction possessed a Ca\(^{++}\)-accumulating capacity dependent on ATP and oxalate and only partially sensitive to NaN\(_3\). In the presence of mersalyl acid or the Ca\(^{++}\) ionophore, A23187, Ca\(^{++}\) uptake by this fraction was inhibited 98.0% and 87.4%, respectively. Electron microscopy revealed that this fraction consisted of numerous membrane vesicles, and measurements of Na\(^+\)-K\(^+\)-ATPase (adenosine triphosphatase) activity indicated minimal plasma membrane contamination. It was concluded that this microsomal fraction consisted primarily of sarcoplasmic reticulum. At physiological free [Ca\(^{++}\)] levels, Ca\(^{++}\) uptake by this fraction was inhibited by norepinephrine through a process sensitive to tolazoline but not propranolol. The effects on Ca\(^{++}\) uptake of added cyclic adenosine monophosphate (cAMP) alone or with rabbit or bovine protein kinase were inconclusive. The organic Ca\(^{++}\) channel blockers, nifedipine and verapamil, significantly inhibited Ca\(^{++}\) uptake by sarcoplasmic reticulum.

KEY WORDS □9 vasospasm □9 calcium channel blockers □9 protein fraction □9 sarcoplasmic reticulum □9 mitochondrial elements

To further elaborate the mode of action of vasoactive agents that primarily rely on intracellular stores of Ca\(^{++}\) necessitates a more complete understanding of the role of intracellular Ca\(^{++}\) storage compartments in cerebrovascular smooth muscle. To our knowledge, isolation of sarcoplasmic reticulum from cerebral blood vessels has not been reported. We have therefore undertaken the isolation from rabbit cerebral arteries of a subcellular fraction consisting primarily of sarcoplasmic reticulum, and have characterized some of its Ca\(^{++}\) transport properties.

Materials and Methods

New Zealand White rabbits were sacrificed with an intravenous overdose of sodium pentobarbital (50 mg/kg) and the brains were placed in ice-cold Hanks' buffer without Ca\(^{++}\) or Mg\(^{++}\). Under low-power magnification with a Zeiss operating microscope, all accessible cerebral arteries were removed and transferred to a centrifuge tube containing ice-cold Hanks' buffer. Approxi-
mately 0.4 to 0.8 gm of wet arterial tissue was collected from four to six rabbit brains for each isolation procedure. All succeeding manipulations were performed at 4°C. After centrifugation at 60 G for 5 minutes, the loosely packed pellet was homogenized with a handheld glass tissue homogenizer in approximately 8 vols of 30 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid)-300 mM sucrose buffer titrated to pH 7.2 with 1 M Tris. Three fractions, designated P1, P2, and P3, were obtained by differential centrifugation, as outlined in Fig. 1. Fractions P1 to P3 were resuspended in 30 mM HEPES-Tris-100 mM KCl (pH 7.2) and stored at −80°C for no more than 2 days. The protein yield for P3, the presumptive sarcoplasmic reticulum fraction, averaged 2.9 mg protein/gm of wet arterial tissue, as determined by the method of Moore and Stein.

Calcium uptake assays were performed at 37°C by modification of previously described methods. Uptake was measured in a final volume of 1 ml containing 30 mM HEPES-Tris buffer (pH 7.2), 5 mM MgCl2, 5 mM NaCl, 0.5 mM Na-oxalate, 5 mM NaN3, 0.5 mM Na-ATP (adenosine triphosphate), 100 mM KCl, 10 to 100 μM CaCl2 (0.8 μCi 45Ca++), and 35 to 70 μg protein. Modifications of the standard compositional Ca++ uptake assay buffer are indicated in the Results section. Membrane fractions were preincubated in the standard assay buffer for 10 minutes before initiating uptake with the addition of Ca++. Uptake was terminated by filtration through 0.5-μm cellulose acetate filters. Each filter was washed with 10 ml of ice-cold HEPES-Tris buffer and dissolved in Filtron-X scintillation fluid.

For each experiment, a control procedure without membranes was performed in an identical manner. The radioactivity on control filters was taken to represent Ca++ nonspecifically bound to the Millipore filters (less than 2% of total radioactivity per filter) and was subtracted from experimental results to obtain net Ca++ uptake.

Because of the presence of several Ca++ chelators such as oxalate in the incubation medium, the added [Ca++] and the actual free [Ca++] differed substantially. Free [Ca++], designated [Ca++]f, was determined according to the method of Schatzmann by means of a Ca++ electrode. The added [Ca++] for most experiments was either 10 or 50 μM, which was estimated to be equivalent to 0.10 or 0.22 μM [Ca++]f, respectively.

The Na+-K+-ATPase (adenosine triphosphatase) activity was assayed at 37°C by a modification of previously described methods. The assay buffer, 20 mM HEPES (pH 7.0), in a total volume of 0.5 ml, contained 2 mM MgSO4, 1 mM EGTA (ethyleneglycol-bis-[aminoethyl ether] N,N'-tetra-acetic acid), 1 mM

ATP (1 to 2 μCi 32P-ATP per assay), 0.01% deoxycholate, approximately 20 μg protein, and either 120 mM NaCl alone or 100 mM NaCl and 20 mM KCl. Inclusion of the detergent, deoxycholate, in the assay mixture was necessary to measure total Na+-K+-ATPase activity, presumably because plasma membranes, during homogenization, form sealed right-side-out vesicles. Titration with deoxycholate over the concentration range of 0.001% to 1.0% indicated maximal activity with 0.01% detergent. The Na+-K+-ATPase activity was measured as the difference in ATP hydrolyzed in assays with or without 20 mM KCl.

After 10-minute preincubation of the protein with the assay components, ATP was added to start the reaction. The reaction was terminated by adding 100-μl aliquots to 500 μl of 1.2% sodium dodecyl sulfate. After mixing, 400 μl of 20 mM ammonium molybdate in 1.25N H2SO4 was added, the phosphomolybdate complex was extracted with 3 ml of butyl acetate, and phase separation was achieved by centrifugation at 73 G for 5 minutes. A 1-ml aliquot of the organic phase was added to 4 ml of Hydrofluor scintillation fluid.

For morphological analysis, each of the three pellets obtained in the isolation procedure was fixed in Trumps fixative (glutaraldehyde/paraformaldehyde), postfixed in 2% osmium tetroxide/0.1 M sodium cacodylate buffer (pH 7.3), stained with uranyl acetate, dehydrated through graded ethanols and propylene oxide, and embedded in Embed-812. Thin sections (45 μm) were taken from multiple areas of each pellet, stained with lead citrate, and examined using a Philips 300 electron microscope.

FIG. 1. The differential centrifugation procedure for isolating subcellular membrane fractions.

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* EH filters manufactured by Millipore Corp., Bedford, Massachusetts.
† Filtron-X manufactured by National Diagnostics, Somerville, New Jersey.
‡ F2112 Ca++-Selectrode manufactured by Radiometer, Copenhagen, Denmark.
§ Hydrofluor manufactured by National Diagnostics, Somerville, New Jersey.

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Throughout this study, statistical analyses were computed using one- or two-tailed Student t-tests, with \( p < 0.05 \) being considered as the level of statistical significance.

Results

Characterization of Subcellular Fractions

A morphological examination of the isolated subcellular fractions revealed that the \( P_1 \) fraction contained primarily a mixture of erythrocytes, membrane fragments and vesicles, and other cell debris (Fig. 2). Approximately 45\% of the \( P_2 \) fraction consisted of mitochondria, both intact and disrupted: the remainder of this pellet consisted of membrane fragments and vesicles plus some cell debris (Fig. 3). The \( P_3 \) fraction was composed of membrane vesicles and some fragments: an occasional mitochondrial fragment was observed (Fig. 4).

Fig. 2. Electron micrographs of representative sections through the \( P_1 \) fraction. Left: A mixture of cell debris, membrane fragments and vesicles, and erythrocytes can be seen. \( \times 2900 \). Right: The nucleus with intact nuclear membrane and prominent nucleolus, erythrocytes, membrane fragments and vesicles, and cell debris are shown. \( \times 5900 \).

Fig. 3. Electron micrographs of representative sections through the \( P_2 \) fraction. Left: Abundant mitochondria (both intact and disrupted), membrane whorls, intact membrane vesicles (arrowheads), and membrane fragments can be seen. \( \times 7500 \). Right: An intact mitochondrion and mitochondrial debris are shown. \( \times 35,900 \).
A potentially significant contaminant of isolated sarcoplasmic reticulum, in terms of Ca++ transport, is the plasma membrane. To determine the degree of plasma membrane contamination in the isolated membrane fractions, we monitored Na+-K+-ATPase activity (Table 1). Most of the Na+-K+-ATPase activity was found in the P1 fraction (> 40%). Only 4.4% of the total homogenate activity was localized to the P3 fraction.

**Ca++ Uptake in Subcellular Fractions**

The P1 fraction and the initial homogenate contained clumps of various cellular materials that interfered with the Ca++ transport assays. Therefore, characterization of Ca++ transport processes focused on membrane fractions P2 and P3.

Table 2 shows that at 50 μM added [Ca++] the P2 fraction has a relatively large Ca++ uptake capacity. Uptake was totally absent without ATP (−99.6%), and almost completely inhibited by 5 mM NaN3 (−98.6%), an inhibitor of mitochondrial Ca++ uptake. Further characterization of this mitochondrial fraction will be the subject of future studies.

The Ca++ uptake associated with the P3 fraction, the final pellet, in the presence of 50 μM added [Ca++] was found to be partially inhibited by 5 mM NaN3; without NaN3 it was 6.18 ± 0.19 nmoles Ca++/mg protein for four assays, and with NaN3 it was 4.18 ± 0.11 nmoles Ca++/mg protein for four assays following a 10-minute incubation period. Therefore, in all subsequent Ca++ uptake experiments with the P3 fraction, 5 mM NaN3 was present to eliminate the effects of contaminating mitochondria.

A typical time course for Ca++ uptake by the P3 fraction is shown in Fig. 5 left. The Ca++ uptake occurs mostly within the first 5 minutes and subsequently decreases to a slower rate. In some but not all experiments a final steady state was achieved within 30 min-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Isolated Fraction</th>
<th>Na+-K+-ATPase Activity (μmoles Pi/mg protein/min)†</th>
<th>Homogenate (%)</th>
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<tr>
<td>homogenate</td>
<td>0.090 ± 0.003</td>
<td>100.0</td>
</tr>
<tr>
<td>P1</td>
<td>0.040 ± 0.011</td>
<td>44.4</td>
</tr>
<tr>
<td>P2</td>
<td>0.023 ± 0.004</td>
<td>25.6</td>
</tr>
<tr>
<td>P3</td>
<td>0.004 ± 0.001</td>
<td>4.4</td>
</tr>
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* Values are means ± standard error of means for four assays.
† The amount of added [Ca++] was 50 μM. ATPase = adenosine triphosphatase.

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**TABLE 2**

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Ca++ Uptake (nmoles/mg protein/10 min)†</th>
<th>Decrease From Optimal Uptake (%)‡</th>
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</thead>
<tbody>
<tr>
<td>+ATP, −NaN3</td>
<td>185.85 ± 10.2</td>
<td>0</td>
</tr>
<tr>
<td>−ATP, −NaN3</td>
<td>0.67 ± 0.09</td>
<td>99.6</td>
</tr>
<tr>
<td>+ATP, +NaN3</td>
<td>2.68 ± 0.03</td>
<td>98.6</td>
</tr>
</tbody>
</table>

* Values are means ± standard error of means for four assays.
† The amount of added [Ca++] was 50 μM. Experiments were performed with (+) and without (−) ATP (adenosine triphosphate) and NaN3.
‡ Statistically significant at p < 0.05.
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Fig. 5. Graphs showing Ca++ uptake by the P3 protein fraction. The standard assay buffer was used in these experiments. Values are means ± standard error of the means for four assays at each time point. Left: Uptake with time. The added [Ca++] is 50 µM, and the protein concentration is 56 µg/ml. Right: Uptake as a function of [Ca++]. The incubation time is 1 minute, and the protein concentration is 50 µg/ml. The added [Ca++] for the uptake values shown is equivalent to the following: 10 µM added [Ca++] = 0.10 µM [Ca++]f; 25 µM added [Ca++] = 0.15 µM [Ca++]f; 50 µM added [Ca++] = 0.22 µM [Ca++]f; and 100 µM added [Ca++] = 0.35 µM [Ca++]f.

utes (data not shown). A plot of the initial rate (1-minute incubation) of Ca++ uptake as a function of added [Ca++] reveals a saturable process (Fig. 5 right). A Lineweaver-Burk analysis of these data yields a K_m (Michaelis constant) of approximately 25 µM. In terms of [Ca++], a K_m of 0.15 µM is calculated, which is well within the physiological [Ca++] range in vascular smooth muscle.

Several criteria were tested to determine whether the Ca++ transport activity of the P3 fraction is similar to that of sarcoplasmic reticulum of other tissues. For these experiments, Ca++ uptake was terminated after 10 minutes of incubation. First, without ATP, Ca++ uptake was markedly reduced (−79.2%). Second, the Ca++ ionophore, A23187 (10 µM), prevented Ca++ accumulation (−87.4%). Third, mersalyl acid (5 µM), a known inhibitor of sarcoplasmic reticulum Ca++ uptake, totally blocked Ca++ uptake (−98.0%). Finally, Ca++ uptake increased with increasing oxalate, maximizing at an oxalate concentration of 5 mM. (In all other experiments, an oxalate concentration of 0.5 mM was used, which allowed adequate Ca++ uptake and minimized chelation by oxalate of incubation medium components.)

Additional experiments were performed with the P3 fraction to further define its Ca++ transport properties. In vascular smooth muscle, cyclic adenosine monophosphate (cAMP) and protein kinases are thought to alter intracellular Ca++ metabolism. At 50 µM added [Ca++], statistically significant increases in Ca++ uptake were found in the presence of 10 µM cAMP with 0.1 mg/ml bovine protein kinase (+11.0%). However, at 10 µM added [Ca++], significant decreases in Ca++ uptake were found in the presence of rabbit protein kinase alone (−12.5%), bovine protein kinase alone (−15.6%), and 10 µM cAMP with bovine protein kinase (−18.9%).

Figure 6 shows the effects of several pharmacological agents known to influence vascular smooth-muscle contraction on uptake of Ca++ by the P3 fraction. In the presence of 50 µM added [Ca++], none of these agents altered the Ca++ uptake as compared to controls (Fig. 6 upper). With 10 µM added [Ca++], significant decreases in Ca++ uptake were found in the presence of both 10 µM norepinephrine (−35.0%) and 10 µM norepinephrine with 20 µM of the beta blocker, propranolol (−34.1%) (Fig. 6 lower). However, 20 µM tolazoline, an alpha receptor blocker, abolishes the inhibition obtained with norepinephrine. Neither epinephrine (10 µM) nor serotonin (10 µM) was found to have any significant effect on Ca++ accumulation.

The effects of the organic Ca++ channel blockers, nifedipine (10 µM) and verapamil (10 µM), on the Ca++ accumulation by the P3 fraction were also examined (Fig. 7). Both agents significantly inhibited uptake at 50 µM added [Ca++].

Discussion

The first goal of our investigation was to isolate from rabbit cerebrovascular arterial tissue a membrane fraction consisting primarily of sarcoplasmic reticulum membrane elements capable of actively accumulating
Potential contaminants of this isolated microsomal protein fraction include both plasma membrane and mitochondrial membrane elements. Plasma membrane of vascular smooth muscle has its own Ca++ metabolic transport properties, but there are no known selective inhibitors to quantify its relative contribution to total Ca++ uptake in an isolated microsomal protein fraction. However, as shown in Table 1, only 4.4% of the total plasma membrane, as judged by the Na+-K+-ATPase activity, is present in P3, the final isolated microsomal fraction. In addition, plasma membrane, when homogenized, tends to vesiculate with a right-side-out membrane orientation and thus, in terms of ATP-dependent Ca++ transport activity, this small degree of contamination is also probably "silent." 27,38

Morphological examination of the P3 protein fraction revealed a minimal amount of mitochondrial contamination, but the partial inhibition of Ca++ uptake with NaN3 indicates significant mitochondrial Ca++ uptake. Therefore, for the present studies, NaN3 was included. We are presently exploring the use of density gradients to further purify the P3 fraction.

According to morphological criteria, the membrane fraction P2 was composed predominantly of mitochondria and also exhibited a substantial ATP-dependent Ca++-accumulating capacity that was almost completely inhibited by NaN3. These findings agree with previous investigations of isolated subcellular fractions from vascular smooth-muscle tissue, but there are no known selective inhibitors to quantify its relative contribution to total Ca++ uptake in an isolated microsomal protein fraction. However, as shown in Table 1, only 4.4% of the total plasma membrane, as judged by the Na+-K+-ATPase activity, is present in P3, the final isolated microsomal fraction. In addition, plasma membrane, when homogenized, tends to vesiculate with a right-side-out membrane orientation and thus, in terms of ATP-dependent Ca++ transport activity, this small degree of contamination is also probably "silent." 27,38

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the slowing of Ca++ uptake may also be a characteristic of isolated sarcoplasmic reticulum from cerebrovascular smooth-muscle tissue, since, to the best of our knowledge, cerebrovascular sarcoplasmic reticulum has never been isolated and used in Ca++ uptake experiments. Nonetheless, the overall Ca++ uptake properties associated with the P3 fraction closely agree with the Ca++ uptake characteristics of sarcoplasmic reticulum fractions isolated from vascular smooth muscle in other studies.

The sources of Ca++ for vascular smooth-muscle excitation-contraction coupling include extracellular Ca++ and bound or stored intracellular Ca++,4 Which source or combination of sources is utilized by the vascular smooth-muscle cell is thought to vary according to type of tissue or stimulus involved. Several in vitro studies have shown that cAMP and cAMP-dependent protein kinase stimulate an increase in Ca++-dependent ATPase activity and Ca++ uptake into isolated microsomes from cardiac and aortic tissue. In the present study, although scattered statistically significant differences in the Ca++ transport of the P3 fraction were found under various conditions in the presence of CAMP and protein kinases, we believe that no definite conclusions can be drawn from the present data regarding the role of CAMP in the regulation of the intracellular Ca++ concentration in cerebrovascular smooth muscle by sarcoplasmic reticulum. Likewise, it has been suggested that serotonin exerts an effect on Ca++ stored intracellularly. However, in our study, no significant effect of serotonin on the Ca++ uptake by P3 vesicles could be demonstrated.

Norepinephrine, which contracts vascular smooth muscle and is implicated as a possible spasmonic agent involved in the development of cerebrovascular vasospasm, has been shown to activate the release of Ca++ from intracellular stores if norepinephrine does cause a release of intracellularly accumulated Ca++, one would expect to see a decrease in Ca++ uptake by isolated membrane vesicles in the presence of norepinephrine. Our results show that at 0.10 µM [Ca++]o, Ca++ accumulation by the isolated P3 protein is significantly inhibited by norepinephrine. This inhibition appears to involve an alpha receptor site since tolazoline, an alpha receptor blocker, abolishes the inhibition, whereas propranolol, a beta receptor blocker, has no effect. It has been suggested that alpha receptors are involved with Ca++ transport, and evidence to support this suggestion has come from work with both isolated rabbit aorta and bovine carotid artery microsomes. The present data suggest that, at least with cerebral arteries, alpha receptors may also be localized to the sarcoplasmic reticulum. Our findings cannot be attributed to contamination by plasma membrane vesicles, since the very small amount of contamination present (4.4% of total plasma membrane) would appear insufficient to account for the very significant inhibition observed (35.0%). The lack of significant inhibition of Ca++ uptake by norepinephrine, with an increase of [Ca++]o to 0.22 µM, is difficult to explain except that physiologically one would expect vascular smooth-muscle tissue to be sensitive to small changes in [Ca++]o.

Several studies have shown that Ca++ channel blockers diminish vascular smooth-muscle contraction by inhibiting extracellular Ca++ fluxes. Others have concluded that the inhibition of vascular smooth-muscle contraction by treatment with Ca++ channel blockers was due to an interference with the release of Ca++ from intracellular storage compartments. In the present study, verapamil and nifedipine significantly inhibited the ATP-dependent Ca++ uptake of the isolated P3 protein, the sarcoplasmic reticulum fraction of cerebrovascular smooth-muscle tissue. Whether the effects of organic Ca++ channel blockers on intact vascular smooth-muscle tissue can be caused by Ca++ blockers crossing the muscle cell membrane and directly acting on intracellular Ca++ storage compartments remains to be established. The possibility that the lycophilic organic Ca++ channel blockers cross cell membranes and directly influence an intracellular site has been suggested elsewhere.

Conclusions

It is apparent that additional research into the Ca++ metabolism involved with vascular smooth-muscle contraction, particularly in cerebrovascular smooth muscle, is needed. We hope that the results reported in the present study, which demonstrated, for the first time to our knowledge, an isolatable intracellular microsomal fraction in cerebrovascular tissue capable of actively sequestering Ca++, will generate further investigation into the normal Ca++ physiology associated with the contraction and relaxation of cerebral arteries and will ultimately lead to an understanding of the pathological state of cerebral vasospasm.

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References


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