CALCIUM UPTAKE, RELEASE AND Mg-ATPase ACTIVITY OF SARCOPLASMIC RETICULUM FROM ARTERIAL SMOOTH MUSCLE

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Sarcoplasmic reticulum (SR) isolated from rat aorta exhibited ATP dependent Ca²⁺ uptake in the presence of Mg²⁺. The maximum capacity and apparent binding constant were 23 nmoles/mg and 1.8 x 10⁶ M⁻¹, respectively. The energy source of this active Ca²⁺ accumulation would be Mg-ATPase associated with the membrane. Ca²⁺ which was taken up inside of sarcoplasmic reticulum was released rapidly by washout with the medium containing no Ca²⁺. These facts suggest that sarcoplasmic reticulum in vascular smooth muscle cell may play an important role in the regulation of intracellular Ca²⁺ concentration.

Recent work of van Breemen et al.¹,² indicated that under physiological conditions, most of the Ca²⁺ for activation of contractile proteins of smooth muscle, as well as those of skeletal muscle³, was released from intracellular stores and relaxation was brought about by intracellular Ca²⁺ binding. These concepts were supported by the examination of fine structure of smooth muscle showing much of the vesicular structures inside the plasma membrane⁴,⁵ Devine et al.⁶ have suggested that vesicular structure separated from cell membrane system might be the Ca²⁺ storage site within a cell. Fitzpatrick et al.⁷ demonstrated that microsomes from aortic muscle exhibited Ca²⁺ pump activity. Hess and Ford⁷ have reported the Ca²⁺ binding properties and enzymatic characterization of sub-cellular fraction from vascular smooth muscles. Our preliminary work⁸ indicated that the microsomal fraction obtained from rat aorta was able to accumulate Ca²⁺ in the presence of ATP and Mg²⁺. However, to establish that the sarcoplasmic reticulum (SR) is the regulatory site for intracellular Ca²⁺ concentration in the vascular smooth muscle cell, measurements of maximum Ca²⁺ uptake ability and binding constant, quantitative relationship between ATPase activity and Ca²⁺ uptake is required.

To clarify the biological and enzymatic characteristics of SR isolated from vascular smooth muscle, the effect of ions, pH and temperature was studied on Ca²⁺ uptake, ATPase activity of SR. Also, Ca²⁺ release from the SR was measured to obtain information on the process of activation of the contractile system.

MATERIALS AND METHODS

Aorta, femoral and carotid arteries were isolated from Wistar rats (either sex, 20 to 30 weeks old) and transferred into ice cold 0.25 M mannitol-5 mM histidine solution at pH 7.0. Preparation of samples was performed by the method of Fitzpatrick et al.⁹: arterial tissues were cut into small pieces followed by gentle homogenization for 8 minutes in 20 volumes of 0.25M mannitol-5 mM histidine and 1 mM dithiothreitol (DTT) solution using siliconized glass-teflon Potter homogenizer at 0 to 2°C. SR was obtained as

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Fig. 1. Electron micrograph of sarcoplasmic reticulum isolated from rat aorta. Magnification, \( \times 40,000 \).

Fig. 2. Time course of \( \text{Ca}^{2+} \) uptake by sarcoplasmic reticulum. (M): (minutes). Conditions: 0.1M KCl, 5mM Mg\(^{2+}\) in 10mM tris-HCl containing \( 10^{-4}\)M CaCl\(_2\), pH 7.4, temperature 37°C.
\( \times \) without ATP; \( \triangle \) with 3mM ATP; \( \bigcirc \) with 3mM ATP and oxalate (5 mM). Numbers of experiments (N)=4, Mean \( \pm \) S.E.

the microsomal fraction of the homogenate at 1,500g for ten minutes centrifugation, supernatant at 27,000g for ten minutes and again the supernatant at 105,000g for 60 minutes. The pellet thus obtained was suspended in tris buffer (pH 7.4) solution.

Measurements of \( \text{Ca}^{2+} \) binding by SR were performed using Millipore filtration method. The medium contained various amounts of \( \text{Ca}^{2+} \) (Ca\(^{45}\)) in 0.1M KCl, 3mM ATP, 3mM MgCl\(_2\) and 10mM tris-HCl (pH 7.4). Addition of 0.1 ml sample suspension (2mg/ml) into the medium (1 ml) at 37°C was the initiation of the reaction. The reaction mixture was then applied on the millipore filter (pore size, 0.45 \( \mu \) ) under reduced pressure. Filtration was completed in about one second. Filter paper was removed, dried and subjected to radioactivity measurements utilizing the liquid scintillation counter (Packard tri-cub). The \( \text{Ca}^{2+} \) absorbed on the filter paper itself was controlled by subtracting radioactivity from the same medium without SR. The maximum binding and binding constants were estimated from
Fig. 3. Double reciprocal plots of free and bound Ca\(^{2+}\) to SR. Ca\(^{2+}\) bound to SR (1 mg) in various concentrations of Ca\(^{2+}\) in the medium for 10 minutes. Other conditions, same as in Fig. 2.

Fig. 4. Effect of Mg\(^{2+}\) on Ca\(^{2+}\) uptake of SR. The conditions were the same in Fig. 2 and 3 except for Mg\(^{2+}\) concentrations. × without ATP; △ with 3 mM ATP; ○ with 3 mM ATP and 5 mM oxalate. N=3.

double reciprocal plots of free and bound Ca\(^{2+}\).

Ca\(^{2+}\) release from SR was achieved by quick dilution of the suspension of SR according to the method described by Kasai and Miyamoto.\(^9\) SR was loaded with Ca\(^{2+}\) by incubating in the medium for 15 minutes at 37°C. The reaction mixture was then diluted 20 times with the same medium except ATP and Ca\(^{2+}\). 2 ml of aliquot was withdrawn from the mixture at 30 sec, 1, 3, 5, 10 and 15 minutes, and filtered. Radioactivity of each filter paper was measured according to the method described above.

ATPase activity was measured in 1 ml of medium containing 0.1 M KCl, 3 mM ATP in 20 mM tris-HCl buffer, pH at 7.4. A specified amount of Mg\(^{2+}\) and/or Ca\(^{2+}\) was added. Reaction was started with the addition of ATP and stopped with 10% TCA. Pi liberated from ATP was determined by modified Allen's method\(^10\) and expressed as Pi μmol./mg protein/min. Concentrations of SR suspension were expressed as mg protein per ml., determined by Lowry's method\(^11\).

Electron microscopic examination of the samples was performed after fixation with 3% glutaraldehyde and 2% osmic tetraoxide. Actual procedures were described in a previous report.\(^12\)

**RESULTS**

**Morphological examination**

Under electron microscopic examination, vas-
molecular muscle microsomes appeared, like skeletal muscle, with enclosed vesicular structure as shown in Fig. 1. Main contaminations other than SR particles appear to be fragmented mitochondria and fat particles.

**Ca²⁺ uptake by SR**

SR from arterial smooth muscle exhibited ATP dependent Ca²⁺ uptake. 5 mM oxalate in the medium facilitated its Ca²⁺ uptake to a large extent. However, time required to reach the optimum level was much longer than that of skeletal SR, as shown in Fig 2. The maximum bindings and binding constants in the presence of Mg²⁺ and ATP, obtained from double reciprocal plots of bound and free Ca²⁺ in various concentrations in the medium (Fig. 3), were 23.8 nmol./mg and 1.8 x 10⁴M⁻¹, respectively.

Ca²⁺ uptake by SR required Mg²⁺ in the medium. As shown in Fig. 4, presence of 5 mM Mg²⁺ gave the maximum ATP dependent Ca²⁺ uptake either with or without oxalate. High concentration of Mg²⁺ tended to inhibit Ca²⁺ uptake, probably due to a competition between the two ions at the binding site.

**ATPase activity of SR**

ATPase activity of SR was greatly accelerated by either Mg²⁺ or Ca²⁺ in a similar manner (Fig. 5 a,b) and the maximum activation was achieved at 2 mM in either ion. When its ATPase activity was maximally activated by one of these ions, addition of the other ion caused inhibition of the activity rather than activation. Extra splitting of ATP by addition of Ca²⁺ in Mg-ATPase was not observed in SR from this vascular smooth muscle. Measurement of ATPase activity in the presence of sodium azide (5 mM) resulted in approximately 35% inhibition of Mg-ATPase activity but Ca-ATPase was not affected at all. However, Ca²⁺ uptake of SR in the presence of Mg²⁺ and ATP was not affected by the azide in spite of inhibitory action of its ATPase activity.

**Effect of temperature and pH on Ca²⁺ uptake and ATPase activity of SR**

Ca²⁺ uptake and Mg²⁺ activated ATPase activity in the presence of Ca(10⁻⁴M) exhibited large temperature dependency in parallel manner as shown in Fig. 6.
pH dependence of Ca$^{2+}$ uptake and ATPase activity is shown in Fig. 7. In the presence of oxalate, Ca$^{2+}$ uptake exhibited a relatively sharp optimum at around pH 6.5 where as without oxalate, a broad optimum was observed from pH 7.0 to 8.0.

Ca$^{2+}$ release from SR

Rapid release of Ca$^{2+}$ from SR occurred immediately after initiation of the reaction, as shown in Fig. 8. Approximately 70% of Ca$^{2+}$ in SR was released within ten seconds (Fig. 8b). If ATP and Mg$^{2+}$ were present in the medium, released Ca$^{2+}$ was taken up by SR again slowly (Fig. 8, a). When the reaction was started in the medium containing 2 mM EGTA, rapid release of 90% bound Ca$^{2+}$ followed by gradual loss were observed (Fig. 8, a,b). Release of bound Ca$^{2+}$ was also facilitated by high temperature and exhibited rather small pH dependence.

Most of Ca$^{2+}$ released from SR which was previously loaded with Ca$^{2+}$ in the presence of 5 mM oxalate, occurred immediately after the initiation of washout. Since oxalate facilitated Ca$^{2+}$ uptake of SR by formation of a complex with Ca$^{2+}$ inside the vesicle, these experiments therefore, indicate that Ca$^{2+}$ which forms complex with oxalate inside the SR also can be released rapidly.

**DISCUSSION**

Results obtained from these experiments confirmed that SR from rat aorta, like that from rabbit$^{6,13,14}$ or cattle aorta,$^7$ was able to accumulate Ca$^{2+}$ from the medium accompanied by ATPase activity. The maximum amount of Ca$^{2+}$ taken up per mg of SR (protein content) without oxalate was 20-25 nmol.; this value is in a range

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of agreement with those reported by other workers using different materials\(^5,7,13,14\). Characteristic features of SR from smooth muscle, especially aorta, compared with those of skeletal muscle, can be described as follows: (i) low Ca\(^{2+}\) uptake expressed by the maximum capacity (about 1/10 of skeletal), (ii) slow rate of Ca\(^{2+}\) uptake with relatively high ATPase activity, (iii) 5 mM oxalate facilitates Ca\(^{2+}\) accumulation only by a small extent.

The isolated SR from smooth muscle seems to be much more labile than that from skeletal muscle. We recognized that Ca\(^{2+}\) uptake ability of aorta SR fell off to about a half within 24 hours whereas no appreciable change in the ATPase activity was observed. Homogenization using siliconized glass-tellon homogenizer at 0\(^\circ\)C to 2\(^\circ\)C is necessary to obtain active sample which exhibits ATP-dependent Ca\(^{2+}\) uptake.

Acceleration of Mg-ATPase activity by addition of Ca\(^{2+}\) – extra splitting – which might be a direct evidence of energy linked Ca\(^{2+}\) transport by ATP hydrolysis, was hardly observed in this preparation of SR. Baudouin et al\(^13\) once reported the activation of Mg-ATPase of aortic microsomes by a small amount of Ca\(^{2+}\). However, they corrected this statement in the later report\(^15\) and postulated that Ca\(^{2+}\) uptake by SR may be one of the many other functions of membrane ATPase, so that it was difficult to observe direct linkage between Ca\(^{2+}\) uptake and ATPase activity of SR. Verty and Bevan\(^16\) observed inhibition, rather than activation, of Mg-ATPase of SR with addition of Ca\(^{2+}\). Lack of Ca\(^{2+}\) activation of Mg-ATPase activity was also recognized in plasma membrane from liver by Chambouret al\(^17\) and they pointed out the possibility that Ca\(^{2+}\) transport through the membrane might not be coupled with membrane ATPase activity directly. Thus, the coupling between Ca\(^{2+}\) transport poor compared with that of skeletal one, but at the present time.

The present experiments indicated that most of bound Ca\(^{2+}\) to SR were released readily by lowering Ca\(^{2+}\) concentration in the medium. The similar procedure can release the Ca\(^{2+}\) from skeletal muscle SR, however, the rate and amount of release are much less\(^9\) Baudouin et al\(^13,14\) also reported that Ca\(^{2+}\) release from aortic microsome was rapid. These facts indicated that membrane of SR from aorta may be leaky and maintenance of Ca\(^{2+}\) in high concentration inside the vesicle is achieved only at the expense of the energy supply at high rate, liberated by ATP hydrolysis (apparent low efficiency).

It is a central question whether SR in smooth muscle cell play a principal role in the control of the contractile system by Ca\(^{2+}\) binding and release. Ca\(^{2+}\) uptake of smooth muscle SR is poor compared with that of skeletal one, but the maximum amount of ATP dependent Ca\(^{2+}\) uptake is in the order of 20 nmol/mg protein of SR. If we assume that SR occupies about 5% of vascular smooth muscle cell\(^9\) and 1 mg of SR corresponds to 1 ml of volume\(^18\) release of bound Ca\(^{2+}\) from SR can easily explain the increase of intracellular Ca\(^{2+}\) concentration necessary to activate the contractile system of smooth muscle. Shibata et al\(^19\) demonstrated that superprecipitation of actomyosin from aorta was inhibited about 50% by SR obtained from aorta. Ca\(^{2+}\) uptake ability of SR in the living cell should be considered greater than in isolated samples. Thus, SR in aorta and also probably in other smooth muscle cells, may play a principal role in the regulation of muscle activity, together with other mechanisms.

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