Rate of Calcium Binding and Uptake in Normal Animal and Failing Human Cardiac Muscle

MEMBRANE VESICLES (RELAXING SYSTEM) AND MITOCHONDRIA

By Shoichi Harigaya, Ph.D., and Arnold Schwartz, Ph.D.

ABSTRACT

Cardiac relaxing system (CRS) and mitochondria were isolated by a modified rapid procedure from human, rabbit, and dog. Relaxing system (sarcoplasmic reticulum) was also isolated from white and red skeletal muscles for comparative purposes. Rapid kinetic measurements were made with a dual-beam spectrophotometric assay procedure. Maximum calcium binding (absence of oxalate), expressed as nmoles calcium/mg protein in 5 minutes at 25°C, for rabbit heart and red and white skeletal muscles were approximately 40, 58 and 170, respectively. The calcium binding constant for rabbit CRS was $2 \times 10^{-6}$ M$^{-1}$. The estimated initial binding rates (nmoles calcium/mg protein/minute) of cardiac, white and red skeletal muscle relaxing systems were 256, 1440 and 182, respectively. The rate constant (M$^{-1}$ sec$^{-1}$) of CRS was about 2400, which falls between white and red skeletal muscle preparations. Human cardiac muscle from recipients obtained at the time of transplantation yielded CRS with slower rates of accumulation of calcium and very little calcium release, compared to normal animal heart preparations. ATP was the most effective of four nucleoside triphosphates in supporting calcium binding and uptake. Calcium binding and release and calcium uptake of CRS were temperature-sensitive. The energies of activation of binding and uptake were 10.5 kcal mole$^{-1}$ and 22.5 kcal mole$^{-1}$, respectively. Under specific conditions, heart mitochondria accumulated calcium at a rate faster than CRS. Mitochondria could also release accumulated calcium.

ADDITIONAL KEY WORDS

sarcoplasmic reticulum rabbit murexide method heart failure dog

It has been suggested that the basic mechanism for relaxation of cardiac muscle (1-6) is qualitatively the same as that of skeletal muscle (7-12); that is, relaxation is probably due to the removal of calcium from the myofibrillar region by internal membranes (sometimes termed sarcoplasmic reticulum, or microsomal fraction). Precise kinetic studies of calcium binding or uptake, however, have not been carried out in preparations from cardiac muscle. Furthermore, while mitochondria have been implicated in contraction-relaxation processes in heart (13-17), no definitive studies have thus far been published.

The present study describes a rapid method for the isolation of a highly active cardiac relaxing system from different species, including human. This preparation, and a relatively purified mitochondrial fraction, both actively bind calcium. A dual-beam spectrophotometric assay procedure, instead of the usual Millipore filtration technique, was employed for measuring rapid calcium binding and uptake kinetics. Red and white skeletal muscles were included for comparison. Since preliminary experiments from this laboratory (18) and others indicate the possible importance of calcium metabolism in human heart failure, the roles of intracellular membrane...
systems in active binding and release of this divalent cation may be of importance.

Abbreviations

The following abbreviations are used in this article:

- CRS = cardiac relaxing system
- GEDTA = glycoletherdiaminetetraacetic acid
- ATP = adenosine triphosphate
- ITP = inosine triphosphate
- GTP = guanosine triphosphate
- TTP = thymidine triphosphate
- Tris = tris-(hydroxymethyl) aminomethane
- RS = relaxing system

Methods

Preparation of Membrane Vesicles from Cardiac Muscle

Young male rabbits weighing 2 to 2.5 kg were killed by a blow on the head; the heart was immediately removed and washed with ice-cold isotonic saline. After removal of fatty and connective tissue, approximately 3 g of ventricular muscle was cut into small pieces with a scissors and placed in a Sorvall centrifuge tube containing 4 to 5 volumes of 10 mM sodium bicarbonate with 5 mM sodium azide. The suspension was homogenized with a Polytron (PT-20 Brinkman Instruments Co.), with a rheostat setting of 2, for 5 seconds, three times, with a rest interval of about 15 to 20 seconds. The entire procedure was carried out in crushed ice. The pH of the solution was maintained at about 7.0. The resulting homogenate was centrifuged at 8700 × g for 20 minutes. The supernatant fluid was centrifuged at 8700 × g for 20 minutes, yielding a second supernatant fraction which was centrifuged at 37,000 × g for 30 minutes. The precipitate was suspended thoroughly in a glass homogenizer with a Teflon pestle in 3 to 4 volumes of 20 mM Tris-maleate buffer (pH 6.8), containing 0.6M KC1. The resulting suspension was then centrifuged at 37,000 × g for 20 minutes to remove solubilized actomyosin. The harvested precipitate was then suspended in a small volume of 20 mM Tris-maleate buffer (pH 6.8) containing 5 mM calcium chloride; 0.01 μcuries/ml of membrane protein (CRS) in a total volume of 1 ml.

Calcium uptake was measured in the presence of 5 mM sodium oxalate in the above medium; calcium, however, was increased to 100 μM and the membrane protein was decreased to 20 to 50 μg/ml. Inesi and Watanabe (22) pointed out that calcium oxalate only begins to precipitate at 0.25 mM calcium concentration and at 5°C. Using the conditions described above, our preliminary experiments with either the Millipore method or the dual beam spectrophotometric method, showed that there was no precipitation of calcium oxalate. This is in agreement with the data of Inesi and Watanabe.

The reaction was started by the addition of ATP after a 1-minute incubation period. After incubation at selected temperatures and times, an aliquot (usually 0.8 ml) was placed on a Millipore filter (HA 0.45 μ) connected via flasks to a vacuum pump. Using a liquid scintillation counter, calcium binding and uptake were estimated from the radioactivity of the filtrate and filter. Both were suspended in a medium containing 10% naphthalene, 0.6% PPO (2, 5- diphenoxyazole) and 2% ethylene glycol-mono-

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Human Heart Preparations

Entire hearts (or most of the ventricular muscle mass) were obtained from five recipient patients undergoing transplantation at this institution, at the time of surgery. The hearts were placed immediately in ice and transported to this laboratory within 5 minutes. In general, the patients had severe atherosclerotic coronary occlusive disease with several previous myocardial infarctions. Digitalis was administered in a full digitalizing dose during the operation. The full details of the operation, condition of the patients and physiological and pharmacological studies have recently been published (19, 20).

Mitochondria

Mitochondria were prepared from rabbit heart according to the method of Sordahl and Schwartz (21). The final pellet was suspended in 0.25M sucrose-10 mM Tris-Cl, pH 7.4.

Assay of Ca²⁺-Binding Activity

Millipore Filter Method.—Calcium binding by membrane vesicles was measured using ⁴²Ca. The reaction mixture consisted of 100 mM KC1, 10 mM MgCl₂, 20 mM Tris-maleate buffer (pH 6.8), 10 μM CaCl₂ containing ⁴²Ca (0.01 μcuries/ml), 2 mM Tris-ATP and 100 to 200 μg/ml of membrane protein (CRS) in a total volume of 1 ml.

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ethyl ether, in dioxane. ATP-dependent Ca\(^{2+}\) binding was calculated from the differences of the values in the presence and absence of ATP. Thus, the presence of small amounts of soluble \(^{45}\)Ca remaining on the filter is practically negligible. The Ca\(^{2+}\)-binding value calculated by the use of the filtrate alone was not significantly different from that using the above procedure. The amount of calcium contained in the incubation medium and in CRS was measured by the colorimetric method of Yanagisawa (23) and by the use of an atomic absorption spectrophotometer. The calcium content of CRS was in a range of 10 to 20 nmoles/mg protein in rabbit and dog and approximately 3 nmoles/mg in human. Contaminating calcium in the reaction mixture was about 10 nmoles/ml. These values were included in the calculations.

**Dual-Beam Spectrophotometric Method.**—Calcium-binding velocity was measured by the dual-beam spectrophotometric procedure of Ohnishi and Ebashi (24) (Aminco-Chance Dual-Beam Spectrophotometer, American Instrument Co., Silver Spring, Md.). This method employs a dye, murexide,\(^1\) which binds calcium in solution at pH 7.0. As calcium is accumulated by the CRS, there are small but significant changes in light transmission and a shift in the absorption maximum. The reaction mixture consisted of 100 mM KCl, 10 mM MgCl\(_2\), 20 mM Tris-maleate buffer (pH 6.8), 0.3 mM murexide and 0.4 to 1.3 mg/ml of membrane protein; the total volume was 3 ml. Thirty \(\mu\)litters of 3 mM CaCl\(_2\) (final concentration of 30 \(\mu\)M) and 15 \(\mu\)liters of 40 mM ATP solution (final concentration of 0.2 mM) were added, consecutively, into the cuvette. The change in transmission difference between 507 \(\text{m\mu}\) and 542 \(\text{m\mu}\) was recorded with respect to time. Although the change caused by ATP was very small in the presence of MgCl\(_2\) (24), this change was measured in the absence of protein in each experiment and was subtracted from the experimental values. The change caused by ATP did not exceed the change caused by the same volume of water in the above experimental condition. Since this procedure is designed to measure rapid reaction rates, consideration must be given to whether the observed kinetics are in any way influenced by the rate of mixing by diffusion. We have carried out a series of studies involving GEDTA and calcium in the absence of relaxing system. The response time, using a plunger, is extremely rapid (less than 1 second), but to insure that diffusion problems do not interfere with the measurements, we routinely mixed thoroughly after each addition and commenced reading 3 seconds later. In more recent experiments (manuscript submitted for publica-

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>Ca(^{2+}) Binding (nmoles Ca(^{2+})/mg protein)</th>
<th>Uptake (nmoles Ca(^{2+})/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25°C (5 min)</td>
<td>37°C (1 min)</td>
</tr>
<tr>
<td>Heart muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>39.5 ± 2.5 (9)*</td>
<td>41.7 ± 4.6 (8)*</td>
</tr>
<tr>
<td>Dog</td>
<td>76</td>
<td>78</td>
</tr>
<tr>
<td>Human</td>
<td>45</td>
<td>38</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit white</td>
<td>170</td>
<td>160</td>
</tr>
<tr>
<td>Rabbit red</td>
<td>58</td>
<td>83</td>
</tr>
</tbody>
</table>

The reaction mixture consisted of 100 mM KCl, 10 mM MgCl\(_2\), 20 mM Tris-maleate buffer (pH 6.8), 200 \(\mu\)g/ml (heart) or 100 \(\mu\)g/ml (white muscle) of membrane protein, 2 mM Tris-ATP, and 10 \(\mu\)M CaCl\(_2\) containing \(^{45}\)Ca, in the absence of oxalate. In the presence of sodium oxalate (5 mM), the Ca\(^{2+}\) content was 100 \(\mu\)M and the membrane protein was 50 \(\mu\)g/ml (rabbit heart and red muscle), 30 \(\mu\)g/ml (dog heart) and 20 \(\mu\)g/ml (white muscle). Ca\(^{2+}\) accumulation was measured by the Millipore filter method. The dog heart preparation was incubated at 37°C for 30 seconds (in absence of oxalate) instead of the usual 1 minute.

*Standard error of the mean. The number of experiments (different animals) is indicated in parentheses. The values without a standard error represent the average of at least three experiments from different hearts, with the exception of the human heart, whose values were obtained from the least gross pathological areas of the myocardium (see text) of one heart. All other specimens from the human heart exhibited low activity (ref. 18 and Table 3).

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\(^1\)Ammonium purpurate, Matheson Company, East Rutherford, New Jersey.
tion), both a rapid mixing device, and a stop-flow apparatus with oscilloscope recording were employed, and the results confirm the data presented in this paper. In the uptake experiments, we previously determined that, under our conditions, calcium oxalate does not precipitate.

The results using the dual-beam method were consistent with those obtained by the Millipore filter method. Throughout this paper, binding refers to accumulation of calcium in the absence of oxalate or phosphate, while uptake refers to accumulation in the presence of oxalate or inorganic phosphate.

**Results**

**Calcium-Accumulating Capacity of Muscle Membrane Vesicles**

The maximal accumulation of Ca$^{2+}$ by membrane preparations from rabbit, dog, and human cardiac muscle were compared to white and red skeletal muscle. Note that an active preparation was obtained from what appeared to be, at least grossly, a "normal" area of human heart (Table 1).

The Ca$^{2+}$-binding activity by CRS agrees with that of other investigators (1, 4). However, the Ca$^{2+}$-binding activity of rabbit CRS was higher than that reported by investigators using this species (25, 26); the activity of dog CRS was much higher than that from rabbit.

Calcium binding by RS of rabbit muscle at 25°C was about 40 nmoles/mg protein for heart, 58 nmoles/mg for red muscle and 170 nmoles/mg for white muscle. The activity of cardiac and red muscle membrane preparations was almost of the same order. Maximal binding by red muscle vesicles was greater at 37°C than at 25°C, while maximal binding by both white and cardiac preparations was only slightly changed by the increase in temperature (see later). The 1- and 5-minute periods at 37°C and 25°C, respectively, were selected because maximal accumulation occurred at these times. The Ca$^{2+}$-binding constant of rabbit CRS (i.e., the reciprocal value of the concentration of Ca$^{2+}$ at which half-maximal binding occurs) was $2 \times 10^9$ M$^{-1}$ (Fig. 1).

**Calcium-Binding Velocity of Cardiac Membrane Vesicles**

The kinetics of calcium binding by membrane vesicles of cardiac, white, and red muscle.
TABLE 2
Rate of Ca\(^{2+}\) Binding by Relaxing System from Various Rabbit Muscles

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Rate constant (\text{M}^{-1} \text{sec}^{-1})</th>
<th>Initial rate (\text{nmoles Ca}^{2+}/\text{mg} \cdot \text{min}^{-1})</th>
<th>Half-maximal time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac muscle</td>
<td>2375</td>
<td>256</td>
<td>10</td>
</tr>
<tr>
<td>White muscle</td>
<td>3450</td>
<td>1440</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Red muscle</td>
<td>1270</td>
<td>182</td>
<td>20</td>
</tr>
</tbody>
</table>

Each value was calculated from the results presented in Figure 2. Initial rates were estimated from the value at 3 seconds.

skeletal muscle can be measured much more accurately by dual-beam spectrophotometry at room temperature (about 23°C) than by the usual Millipore procedure. In Figure 2 the data of B were recorded at a speed 12 times that of A.

The patterns of Ca\(^{2+}\) binding by preparations isolated from white, red, and cardiac muscles were almost the same, although the Ca\(^{2+}\)-binding velocity and capacity of membrane vesicles from white muscle were faster and greater, respectively, than that from cardiac muscle (Fig. 2). It is noteworthy that red muscle did not release Ca\(^{2+}\) within 10 minutes, while both cardiac and white muscle readily did so.

A summary of the Ca\(^{2+}\)-binding properties of various membrane preparations is presented in Table 2. The rate constant of Ca\(^{2+}\)-binding was calculated according to the method of Harigaya et al. (28), assuming that the rate of binding is linearly related to the concentration in the medium and to the remaining available sites for binding. The value of the rate constant, 1270 \(\text{M}^{-1} \text{sec}^{-1}\) of red muscle, was consistent with previously published results (28). The value of the cardiac muscle preparation, 2400 \(\text{M}^{-1} \text{sec}^{-1}\), falls between white and red skeletal muscle.

The initial rate of Ca\(^{2+}\)-binding was calculated from the binding value at 3 seconds. Since the minimum time required from the addition of ATP to the actual recording was 2 to 3 seconds, we could not reliably measure activities at shorter time periods. In many other experiments, under the same conditions, the Ca\(^{2+}\)-binding rate of rabbit CRS at 3 seconds ranged from 220 to 350 nmoles/mg/min.

It has been reported that sarcoplasmic reticulum of skeletal muscle binds Ca\(^{2+}\) in two separate stages as ATP concentration is increased (29, 30), viz., Ca\(^{2+}\)-binding increases after the addition of 10\(^{-7}\) to 10\(^{-6}\) M ATP (the first stage) and then again after addition of 10\(^{-4}\) to 10\(^{-3}\) M ATP (the second
TABLE 3

Rate of Ca\(^{2+}\) Binding by Relaxing System from Failing Human Cardiac Muscle

<table>
<thead>
<tr>
<th>Human heart</th>
<th>Ca(^{2+}) binding rate (nmoles mg(^{-1}) min(^{-1}))</th>
<th>Half-maximal time (sec)</th>
<th>Maximal Ca(^{2+}) binding (nmoles mg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36.4</td>
<td>20</td>
<td>24.2</td>
</tr>
<tr>
<td>2</td>
<td>34.8</td>
<td>70</td>
<td>35.2</td>
</tr>
<tr>
<td>3</td>
<td>34.3</td>
<td>33</td>
<td>25.6</td>
</tr>
<tr>
<td>4</td>
<td>69.6*</td>
<td>32</td>
<td>42.3</td>
</tr>
</tbody>
</table>

These values were calculated from data in Figure 3. The Ca\(^{2+}\) binding rate was calculated from the measurement at the 10-second period. All hearts were obtained at the time of implant surgery and all were in failure.

*The value calculated at the 3-second period is 112.0 (nmoles mg\(^{-1}\) min\(^{-1}\)), which is significantly lower than the values obtained for animal preparations from heart (see Table 2).

In the range of concentrations of ATP from 10\(^{-4}\) to 10\(^{-3}\) M, the capacity and rate of Ca\(^{2+}\) binding by CRS also increased slightly. The release of Ca\(^{2+}\) was more rapid at low ATP concentrations, than at higher concentrations. Furthermore, the release of accumulated Ca\(^{2+}\) occurred more rapidly at high protein concentrations of CRS. However, both the maximum amount and rate of Ca\(^{2+}\) binding were almost the same in all of the experiments. The rates and maximum binding values were from 330 to 360 nmoles Ca\(^{2+}\)/mg/min, and about 40 nmoles Ca\(^{2+}\)/mg, respectively, when protein concentration was 0.3 mg to 0.8 mg/ml. All of these results indicate the importance of ATP concentration for Ca\(^{2+}\) binding by membrane vesicles.

The velocity of Ca\(^{2+}\) binding of preparations isolated from failing human cardiac muscle is shown in Figure 3 and Table 3. The rate of binding was calculated from the value at 10 seconds. The characteristic pattern of Ca\(^{2+}\) binding, in general, indicated a slower rate of accumulation and slower or no release, compared to cardiac muscle preparations from normal animals. The pattern appeared rather similar to that of red muscle from rabbit. It should be emphasized that all of the hearts were in various stages of failure, usually secondary to severe coronary artery disease (19, 20) and all of the patients had received digitalis, anesthesia, and preanesthetic drugs. It is not known at this time whether the properties of these hearts are characteristic of the disease process, since normal human heart muscle preparations have not, as yet, been studied in detail. It should be noted, however, that even those human preparations which did exhibit maximal Ca\(^{2+}\) binding similar to rabbit cardiac preparations, also showed slower binding rates and slower release compared to the animal preparations (Fig. 3D and Table 3).

Calcium Uptake Velocity by Cardiac Membrane Vesicles from Rabbit Heart

In the presence of 5 mM sodium oxalate, the responses of murexide to various Ca\(^{2+}\) concentrations were completely linear, using the dual-beam spectrophotometric method, although the change in transmission was slightly lower than in that obtained in the absence of oxalate. After addition of ATP, Ca\(^{2+}\) accumulated rapidly, and then was taken up slowly for 3 to 45 seconds (Fig. 4A). The initial rate appeared to be almost the same as that observed under conditions that appeared to be optimal for Ca\(^{2+}\) binding (absence of oxalate) shown in the superimposed dotted line. After a lag time of 1 minute, the rate of Ca\(^{2+}\) uptake increased, presumably concomitant with the beginning of calcium oxalate precipitation within the vesicles. All of the calcium in the medium was taken up in 2 to 3 minutes (Fig. 4A). If additional calcium was added at this time, the preparation still accumulated Ca\(^{2+}\) but at a slower rate (Fig. 4B). However, the subsequent addition of ATP then caused a significant increase in rate (Fig. 4B). The rates of Ca\(^{2+}\) uptake at each time interval (a-h in Fig. 4) were calculated from the slope at each point. The rate of Ca\(^{2+}\) binding at the first stage, 260 nmoles/mg/min, was more than...
Ca\textsuperscript{2+} uptake by rabbit cardiac relaxing system measured by dual-beam spectrophotometry. The reaction mixture was as in Figure 2 plus 5 mM sodium oxalate and 0.4 mg/ml membrane protein. Calcium and ATP were added as indicated in the figure. Incubation at room temperature (about 23°C). The recording speed was different in A and B (see text). The dotted line represents calcium binding in the absence of oxalate. The letters a-h refer to rates calculated from the slope at each point: a = 260; b = 60; c = 39; d = 109; e = 45; f = 136; g = 121; h = 87 nmoles mg\textsuperscript{-1} min\textsuperscript{-1}.

Effect of Nucleoside Triphosphates

The effects of nucleoside triphosphates other than ATP on Ca\textsuperscript{2+} binding by rabbit CRS were studied using the dual-beam spectrophotometric method. As shown in Figure 5 and Table 4, the effect of GTP and ITP was about half that of ATP in supporting the rate of Ca\textsuperscript{2+} binding, and two-thirds that of ATP in supporting amount of maximum binding. The effect of TTP was minimal. The specificity of ATP was clearer when Ca\textsuperscript{2+} uptake was measured as shown in Figure 6. Ca\textsuperscript{2+} uptake, supported by 10 times higher concentrations of GTP and ITP (2 mM), was still much lower and slower than that in the presence of 0.2 mM ATP. After the slow Ca\textsuperscript{2+} uptake in the presence of GTP or ITP, the
Rate of Ca\(^{2+}\) Binding by Relaxing System from Rabbit Cardiac Muscle in the Presence of Various Nucleotides

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Ca(^{2+}) binding rate (nmoles mg(^{-1}) min(^{-1}))</th>
<th>Maximal Ca(^{2+}) binding (nmoles mg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>250</td>
<td>35.3</td>
</tr>
<tr>
<td>GTP</td>
<td>140</td>
<td>20.3</td>
</tr>
<tr>
<td>ITP</td>
<td>130</td>
<td>22.0</td>
</tr>
<tr>
<td>TTP</td>
<td>12</td>
<td>5.6</td>
</tr>
</tbody>
</table>

These results were calculated from the data in Figure 5.

addition of ATP restored the usual rapid uptake (Fig. 6).

**Temperature Effect**

Ca\(^{2+}\) binding and release and its uptake by rabbit cardiac membrane vesicles (studied by the Millipore filter method) were temperature-sensitive (Figs. 7 and 8). The energies of activation of Ca\(^{2+}\) accumulation, calculated from Figures 7 and 8, according to Inesi and Watanabe (22) were 10.5 kcal mole\(^{-1}\) in the absence of oxalate, and 22.5 kcal mole\(^{-1}\) in its presence, respectively. These values were similar to those obtained for skeletal muscle "microsomes" (22).

**Ca\(^{2+}\) Uptake by Rabbit Cardiac Mitochondria**

Azide strongly inhibited Ca\(^{2+}\) uptake by mitochondria, but had no effect on uptake or binding by CRS under any of the conditions employed (Table 5). Oxalate in concentrations greater than 1 mM, markedly stimulated Ca\(^{2+}\) uptake by CRS but had no effect on mitochondria. Low concentrations of phosphate, however, strongly stimulate uptake by mitochondria, but had only minimal effects on CRS (Fig. 9). Higher phosphate levels stimulated the uptake by CRS.

Using optimal assay conditions for calcium binding by membrane vesicles, mitochondria...
TABLE 5

Effect of Azide on Ca\(^{2+}\) Binding and Uptake by Relaxing System and Mitochondria from Rabbit Cardiac Muscle

<table>
<thead>
<tr>
<th>Azide in assay medium (5 mM)</th>
<th>Cardiac relaxing system</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>40</td>
<td>650</td>
</tr>
<tr>
<td>50 uu</td>
<td>42</td>
<td>790</td>
</tr>
<tr>
<td>None, Inorganic phosphate (10 mM)</td>
<td>1010</td>
<td>1890</td>
</tr>
<tr>
<td>None, Oxalate (5 mM)</td>
<td>110</td>
<td></td>
</tr>
</tbody>
</table>

Ca\(^{2+}\) accumulation was measured by the Millipore filter method. The reaction mixture for the relaxing system was the same as in Table 1, except that when 10 mM inorganic phosphate was present, 100 \(\mu\)g/ml of protein and 100 \(\mu\)M of added Ca\(^{2+}\) were used. The reaction mixture for mitochondria consisted of 100 mM KCl, 10 mM MgCl\(_2\), 20 mM Tris-maleate buffer (pH 6.8), 5 mM succinate, 4 mM phosphate and 100 \(\mu\)g/ml of mitochondrial protein, 100 \(\mu\)M added Ca\(^{2+}\) and 2 mM ATP. The reactions were carried out at 37°C for 1 minute (membrane vesicles in absence of oxalate) and for 30 minutes (membrane vesicles and mitochondria in presence of phosphate or oxalate).

Accumulated calcium more slowly than the vesicular preparation (Fig. 10). Note that, as usual, CRS released calcium after 1 minute (37°C), while mitochondria continued to bind it with no apparent release (Fig. 10).

Using assay conditions which appear to be more adequate for mitochondria, however, the initial rate and total amount of calcium...
accumulated by mitochondria was faster and greater, respectively, than the membrane vesicles (Fig. 11). Calcium accumulation was also measured by the dual-beam spectrophotometer and is illustrated in Figure 12. In the absence of succinate and ATP, mitochondria did not accumulate any calcium; the addition of succinate, however, caused a rapid uptake. After a few minutes, Ca\(^{2+}\) was gradually released, as shown by the dotted line. The addition of ATP prevented the release, and Ca\(^{2+}\) uptake continued.

**Discussion**

Active vesicular fractions that accumulate calcium are far more difficult to prepare from cardiac muscle or red skeletal muscle than from white skeletal muscle. One explanation is that the sarcoplasmic reticulum, or other nonmitochondrial membrane areas involved in calcium metabolism, are less well developed in cardiac and red muscles than in white skeletal muscles. Another is the labile nature of preparations from red and cardiac muscles. It is also well known that red skeletal and cardiac muscles contain many more mitochondria than white skeletal muscle, which suggests that either some metabolic functions or activities of mitochondria make it more difficult to obtain active vesicular fractions from these muscles, or that mitochondria may play some role in calcium accumulation. Azide was added to the isolation medium, during homogenization, to minimize mitochondrial activity. In fact, more active preparations of CRS with respect to calcium binding were obtained in the presence of this inhibitor. By the use of the Polytron for muscle homogenization, more active preparations were obtained than when other homogenizing vessels or devices were used (data not shown). The advantages of this device are that muscles can be quickly homogenized directly in the centrifuge tube, damage to membranes and mitochondria appear to be minimal (as determined by electron microscopy), and active vesicular preparations from both cardiac and skeletal muscle can be prepared.

The possibility that the Ca\(^{2+}\) uptake by our preparations is partially due to contamination by intact mitochondria can be vitiated by the comparative data of Ca\(^{2+}\) accumulation by CRS and mitochondria, and by the effects of azide. However, the possibility that "denatured" mitochondrial membranes contribute to the results should still be considered.
The Ca$^{2+}$ binding constant of cardiac vesicles (in absence of oxalate) is about 2 x 10$^6$ M$^{-1}$, which is higher (i.e., the dissociation constant is lower) than the value of 10$^5$ M$^{-1}$ reported by Katz and Repke (5) for dog cardiac microsomes. It is possible that procedures for isolation are responsible for the differences. However, in recalculation, Katz and Repke have now found a binding constant of 7 x 10$^5$ M$^{-1}$ (personal communication), which is more consistent with our observations. The Ca$^{2+}$ binding constants of microsomes from white skeletal muscles are 7 x 10$^6$ M$^{-1}$, 4 x 10$^7$ M$^{-1}$ and 10$^7$ M$^{-1}$, according to Ebashi et al. (6, 31, 32), Weber et al. (29) and Inesi et al. (30), respectively. The Ca$^{2+}$ binding constant of our cardiac preparation (2 x 10$^6$ M$^{-1}$) is almost one-fourth that of skeletal muscle, obtained under the same experimental conditions. Recently, Ebashi et al. (33) showed that the Ca$^{2+}$ binding constant of cardiac troponin, which may be the receptor protein for Ca$^{2+}$ in contractile systems, is 3.4 x 10$^6$ M$^{-1}$, which is about one-third that of the binding constant for skeletal muscle troponin (9.3 x 10$^6$ M$^{-1}$). If one assumes that membrane vesicles (sarcoplasmic reticulum) must remove Ca$^{2+}$ from troponin to trigger muscle relaxation, the relationship of the Ca$^{2+}$ binding constants of RS to the binding constants of cardiac and skeletal troponin might have an important physiological significance.

The Ca$^{2+}$ binding rate of white skeletal muscle membranes was 1400 nmoles/mg/min, which is in fairly good agreement with the results of Weber et al. (29), but is slower than that obtained by Ohnishi and Ebashi (34).
This might be due to estimation at the 3-second period. Faster measurements at earlier time periods should yield higher values. Figure 13 illustrates the theoretical Ca^{2+} binding rate of cardiac vesicles, assuming a rate constant of 2375 M^{-1} sec^{-1} (Table 2). In the range of 10 to 30 seconds, the theoretical values coincide with the experimental values. Prior to 5 seconds, however, there is a discrepancy between the experimental and theoretical values. This suggests that the rate constant within 5 seconds is probably much higher than previously assumed. The amount of Ca^{2+} binding at 200 msec, for example, may be as high as 3 to 8 nmoles Ca^{2+}/mg (Fig. 13). Ebashi et al. (33) reported that the amount of Ca^{2+} bound by skeletal troponin is about 40 nmoles/mg protein; the amount of Ca^{2+} bound by cardiac troponin is about one-half that of skeletal troponin. These investigators also estimated that the troponin content in white skeletal muscle is about 3 mg/ml. If the assumption is made that the content of cardiac troponin is approximately the same as skeletal muscle, the amount of Ca^{2+} required for cardiac muscle contraction is estimated to be about 60 nmoles/g muscle, which is lower but similar to calculations of Katz and Repke (5). The contraction-relaxation cycle of cardiac muscle is much slower than that of skeletal muscle. Langer (35) suggested that the relaxation time for cardiac muscle is not less than 200 msec at 70 to 80 beats/min and at 20°C. The yield of cardiac membrane vesicles in our rabbit preparation is about 1 to 1.5 mg/g of muscle. Therefore, the rate of Ca^{2+} binding in the present study is estimated at 4.5 to 12 nmoles Ca^{2+}/g muscle/200 msec at room temperature (about 23°C). Although this value is somewhat lower than the amount of Ca^{2+} bound by troponin (60 nmoles/g muscle), it still is reasonable to explain relaxation of cardiac muscle if one considers that (a) the content of sarcoplasmic reticulum (or whatever morphological entity from which the membrane vesicles were derived) in the total heart probably is larger than our yield; (b) the in-vivo activity is probably higher because of the labile nature of isolated cardiac membrane preparations (6); (c) the above extrapolated value may be higher; and (d) at 37°C, the binding may be significantly higher. The levels of ATP may also play an important role.

The membrane fraction from both cardiac and white skeletal muscle released Ca^{2+} rapidly in the absence of oxalate, and this release was found to be highly sensitive to temperature. One of the reasons may be an increased ATP hydrolysis, since Ca^{2+} was again accumulated by the further addition of ATP. It is however, inconceivable that all the ATP was hydrolyzed at the time of Ca^{2+} release, since, at that time, Ca^{2+} can still be rapidly accumulated in the presence of oxalate. A specific concentration of ATP may prevent Ca^{2+} release from the vesicular membrane by some type of conformational change. It is interesting in this regard that the vesicles derived from red skeletal muscle, which have a higher ATPase activity (28, 36) than those from white muscle, do not release Ca^{2+}, or release it very slightly, within 10 minutes.

The relaxation of cardiac muscle may be explained, at least in part, by the initial rapid Ca^{2+} binding. It is attractive to postulate that this rapid binding might be caused by a conformational change of the membrane induced by ATP, as suggested by Ohnishi and Ebashi (34), by Ebashi (37), and, more recently, by Landgraf and Inesi (38). The subsequent slower Ca^{2+} uptake might be due to active transport associated somehow with ATP hydrolysis. Since Ca^{2+} release does not occur in the presence of oxalate, it seems likely that it depends on free Ca^{2+}, or readily exchangeable calcium in the vesicle, as well as on ATP concentration. There are two proposed mechanisms for Ca^{2+} accumulation by the sarcoplasmic reticulum: the active transport hypothesis of Hasselbach (39) based on the stoichiometry of ATP splitting and Ca^{2+} uptake, and the two-stage, binding and active transport process suggested by Ebashi and Ohnishi (34, 37). The present results do not exclude either hypothesis, although the latter
mechanism appears to be more consistent with the data.

It was shown by Hasselbach (40) that of the various nucleoside triphosphates, only ATP and CTP support the relaxation of skeletal muscle myofibrils. It is interesting that the specificity of ATP on Ca^{2+} binding and uptake by CRS is much greater than by the vesicles of skeletal muscle (41-43).

The experiments employing mitochondria indicate the importance of investigating appropriate isolation and assay conditions. Our results reinforce the suggestions of Chance (16) and more recently of Patriarca and Carafoli (13), that these organelles might participate in cardiac muscle relaxation. Further studies along these lines would be helpful, particularly since there is some discrepancy between Ca^{2+}-binding capacity and rate of cardiac membrane vesicles and muscle relaxation. The results using human preparations suggest the attractive possibility of altered calcium metabolism in heart failure. Future experiments along these lines would be helpful in determining if the slow binding rate and release phenomenon is an early characteristic of the disease process.

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Rate of Calcium Binding and Uptake in Normal Animal and Failing Human Cardiac Muscle: MEMBRANE VESICLES (RELAXING SYSTEM) AND MITOCHONDRIA
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