Quantitative Aspects of Dog Cardiac Microsomal Calcium Binding and Calcium Uptake

By Arnold M. Katz, M.D., and Doris I. Repke, C.T.

ABSTRACT

Crude microsomes prepared from dog ventricular myocardium by differential centrifugation bound 0.029 μmole of Ca/mg of microsomal protein in the absence of oxalate, and accumulated 1.92 μmole Ca/mg in the presence of oxalate. Purified cardiac microsomes, obtained by sucrose density-gradient ultracentrifugation of the crude microsomes, bound 0.026 μmole Ca/mg and accumulated 2.32 μmole Ca/mg. The total Ca-binding capacity of the myocardium, based on the yields and Ca-binding activities of the crude microsomes, was 0.050 μmole/g wet weight of the ventricle. This value is very nearly equal to the amount of Ca believed to be removed from the actomyosin during relaxation, suggesting that myocardial relaxation may be effected by Ca binding to the sarcoplasmic reticulum. The binding constant for Ca binding to cardiac microsomes is approximately 10^5 M^-1. Purified cardiac microsomes lowered free Ca from 10^-5 M to approximately 10^-7 M in the presence of oxalate, but Ca uptake proceeded much less rapidly than did Ca binding. Calcium uptake followed first order kinetics. A typical rate constant was 0.24 sec^-1 mg microsomal protein^-1, giving a rate constant of 0.047 sec^-1, for the initial uptake from a solution of 10^-5 M CaCl2 by 1 g of heart based on the yields of purified microsomes. This rate is significantly lower than that of relaxation in the intact ventricle, and remains so even if the higher yields of crude microsomes are used in the calculation.

ADDITIONAL KEY WORDS

sarcoplasmic reticulum actomyosin excitation-contraction coupling muscle heart muscle relaxation

The central role of Ca^2+ in the excitation-contraction coupling of skeletal muscle has been suggested by several lines of evidence (1-7). A homologous calcium-accumulating mechanism within the heart is suggested by the presence in cardiac muscle of a sarcoplasmic reticulum qualitatively similar to that of skeletal muscle (8, 9), and the finding that the cardiac contractile proteins, like those of skeletal muscle, are sensitive to changing Ca^2+ concentration (10-12). Preparation of microsomal calcium-accumulating vesicles from cardiac muscle has proven difficult however, and these fractions have generally been found to be unstable and to have relatively little ability to take up calcium (10, 13-16). For this reason, we have examined the characteristics of a highly active cardiac microsomal preparation that was isolated by a slight modification of the conventional method of differential centrifugation, followed by fractionation on a sucrose density-gradient. The possible relationship of the calcium-binding and calcium-accumulating abilities of these microsomal preparations to relaxation of the intact myocardium were examined. In the present study, calcium binding refers to the ability of microsomes to bind calcium in the absence of oxalate; calcium uptake or calcium accumulation signify the binding of calcium in solutions containing 2.5 mM oxalate.
Methods

Hearts obtained from dogs anesthetized with pentobarbital were opened, washed with cold water and immediately chilled on ice. The atria, valves, great vessels and most of the epicardial fat were removed and, within 5 min after excision, the ventricles were ground in an Oster Model 516 Grinder and then homogenized in a Waring Blender for 40 sec with 2/3 volumes of ice cold 0.3 M sucrose, 5 mM Tris oxalate and 5 mM histidine at pH 7.4. After centrifugation for 30 min at 13,000 × g, the supernatant was filtered twice through 4 to 6 layers of gauze and centrifuged for 90 min at 105,000 × g. The pellet was taken up in 0.3 M sucrose, 1 mM Tris oxalate and 5 mM histidine at pH 7.4, and homogenized by 10 gentle strokes of a glass Potter-Elvehjem homogenizer fitted with a Teflon pestle. Two milliliters of the homogenate, containing approximately 50 mg of crude microsomes, was applied to the top of a sucrose gradient. The gradient usually consisted of 14 ml of 35% sucrose and 14 ml of 20% sucrose, layered the same day in a cellulose centrifuge tube. The gradient tubes were centrifuged for 2 hr at 25,000 rpm in an SW-25 swinging bucket rotor (Beckman Instrument Co., Spinco Div.). The purified microsomes appeared in one prominent layer (Fig. 1B). In earlier experiments three prominent bands were seen on a gradient made up of 9 ml each of 25, 45, and 60% sucrose (Fig. 1A).

Preparation of the sucrose density-gradient tubes a day in advance, which would produce a more gradual concentration gradient within the tube, was attempted, but because the microsomes spread into a broader band the sharper sucrose density-gradient was chosen. Prolonged homogenization of the ventricles in the Waring Blender, or more extensive homogenization of the crude microsomes prior to application to the sucrose density-gradient greatly reduced the calcium-accumulating activity. The hearts of obese animals with greater amounts of epicardial fat appeared to give less active microsomal preparations. If, at any time, the microsomes approached room temperature, the preparation became inactive.

Calcium binding was measured at microsome concentrations of 0.05 to 0.20 mg/ml in 0.12 M KCl, 5 mM MgATP and 10 mM histidine at pH 7.0. At zero time an appropriate amount of 1 mM 45CaCl2, 0.05 μc/ml, was added. Samples taken after 1 min were subjected to Millipore filtration and the calcium remaining in solution was determined by counting the filtrate in a liquid scintillation counter. Preliminary experiments showed that calcium binding had reached a stable value after 15 sec. Measurements at shorter intervals were not possible with the present method.

The calcium uptake by 0.005 to 0.02 mg/ml microsomes was usually measured at 25°C in 2.5 mM Tris oxalate, 5 mM MgATP and 10 mM histidine at pH 7.0. Five minutes before addition of 45CaCl2 the microsomes were added to the remaining ingredients of the reaction mixture and allowed to "warm up" at 25°C. At zero time an appropriate amount of 1 mM 45CaCl2, 0.05 μc/ml, was added. Samples were taken with a disposable plastic syringe (Becton, Dickinson and Co.) and a microsome-free filtrate was obtained by ejecting the contents of the syringe through a Swinny Hypodermic adapter (Millipore Filter Corp.) containing a 13-mm Ha (0.45 μ) Millipore filter (17). One half-milliliter portions of the filtrate were added to 15 ml of Bray's Solution (18) and counted in a liquid scintillation counter.

That the Millipore filters effectively filtered out all of the cardiac microsomes was demonstrated by total removal of 45Ca at high microsome concentrations.

At the concentrations of calcium and oxalate employed in these experiments, the solubility product for calcium oxalate in water was exceeded. Hasselbach (14) found that ATP greatly increases the solubility of calcium oxalate however, and control experiments in which microsome-free reaction mixtures were passed through Millipore filters showed no loss of 45Ca attributable to formation of insoluble calcium oxalate.

Adenosine triphosphatase activities were measured by following the rate of liberation of inorganic phosphate, measured by the method of Taussky and Shorr (19). Reactions were started

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1 Abbreviations: Tris = tris (hydroxymethyl) amino methane.
TABLE 1
Calcium Binding by Crude and Purified Cardiac Microsomes

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Yield mg/g heart wt</th>
<th>Calcium binding μmole/mg microsomal protein</th>
<th>Calcium binding μmole/g heart wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude microsomes</td>
<td>1.71 ± 0.21*</td>
<td>0.029 ± 0.011*</td>
<td>0.050 ± 0.021*</td>
</tr>
<tr>
<td>Purified microsomes</td>
<td>0.39 ± 0.08*</td>
<td>0.026 ± 0.004*</td>
<td>0.010 ± 0.004*</td>
</tr>
</tbody>
</table>

Reactions were carried out at a microsome concentration of 0.1 mg/ml in 50 μM ⁴⁶CaCl₂, 5 mM MgATP, 0.12 M KCl and 10 mM histidine at pH 7.0.

*One standard deviation.

TABLE 2
Calcium Uptake by Crude and Purified Cardiac Microsomes

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Yield mg/g heart wt</th>
<th>Calcium uptake μmole/mg microsomal protein</th>
<th>Calcium uptake μmole/g heart wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude microsomes</td>
<td>1.88 ± .19*</td>
<td>1.92 ± .17*</td>
<td>3.61 ± .45*</td>
</tr>
<tr>
<td>Purified microsomes</td>
<td>0.38 ± .08*</td>
<td>2.32 ± .06*</td>
<td>0.87 ± .17*</td>
</tr>
</tbody>
</table>

Reactions were carried out at a microsome concentration of 0.005 mg/ml in 0.1 mM ⁴⁶CaCl₂, 2.5 mM Tris oxalate, 5 mM MgATP and 10 mM histidine at pH 7.0.

*One standard deviation, n = 7.

Results

The calcium binding by the crude microsomes amounted to approximately 0.029 μmole/mg microsomal protein. When the average yield of crude microsomes in this series of experiments is taken into account, the estimated calcium-binding capacity of 1 g of ventricle is 0.050 μmole (Table 1).

The crude microsomal preparation took up an average of 1.92 μmole of calcium/mg of microsomal protein in the presence of 0.1 mM CaCl₂ and 2.5 mM oxalate (Table 2). These data indicate the calcium-accumulating capacity of the crude microsomes to be approximately 3.6 μmole/g wet weight of ventricular myocardium.

When the microsomes were fractionated on a sucrose density-gradient ranging in concentration from 25 to 60% sucrose, three layers usually appeared below the top reddish layer (Fig. 1A). The latter corresponded to the applied sample, which contained 10% sucrose.
In terms of calcium uptake, the upper microsomal layer (Fig. 1A) was the most active, having a calcium uptake of 1.87 μmole/mg in 0.12 M KCl, the middle layer was less active, having a calcium uptake of 0.52 μmole/mg, while the bottom layer did not take up significant amounts of calcium.

When the sucrose gradient was changed to 20 and 35% sucrose, it was possible to pack Layers II and III in the pellet at the bottom of the tube (Fig. 1B) and isolate the purified microsomes in Layer I; these contained 2.32 μmole calcium uptake/mg of microsomal protein in 0.1 mM CaCl₂ (Table 2). The average calcium uptake recovered in the purified cardiac microsomes, approximately one quarter of the total calcium uptake of the crude microsomes, corresponds to a calcium-accumulating capacity for the myocardium of 0.87 μmole/g wet wt.

Maximal calcium binding, seen at microsomal concentrations of approximately 0.2 mg/ml, occurred at calcium concentrations above 25 mM (Fig. 2). The average for maximal calcium binding by the purified microsomes, 0.028 μmole/mg, did not differ significantly from that for crude microsomes (Table 1), but the wide experimental variability could have obscured small differences. The total calcium-binding capacity measured in the purified microsomal preparation was 0.010 μmole/g wet wt. Like calcium uptake, this was only about one fifth of that found in the crude microsomal preparation.

The purified microsomes could be stored for several days on ice with little loss of activity, whereas the crude microsomes rapidly lost the ability to take up Ca²⁺. Contamination of the microsomes by both soluble and mitochondrial enzymes, estimated by malic dehydrogenase determinations, was reduced by fractionation on the sucrose gradient. In a typical experiment the malic dehydrogenase activity associated with crude microsomes was 0.84 unit/mg while that in the purified microsomes was 0.09 unit/mg.

The calcium uptake of both crude and purified microsomes was inhibited by 10 to 20%
Hydrolysis of ATP by crude cardiac microsomes. The liberation of $P_i$ by 0.1 mg/ml crude microsomes in the absence (open symbols) and presence (closed symbols) of 5 mM sodium azide was measured in 5 mM MgATP, 2.5 mM Tris oxalate, 0.12 mM KCl and 10 mM histidine at pH 7.0. The basic ATPase activity was measured in the absence of added calcium (o, •), and the calcium-activated ATPase was measured after addition of calcium to a final concentration of 0.1 mM (o, •), left-hand panel. The extra ATPase was calculated by subtracting the basic from the calcium-activated ATPase activities (o, • right-hand panel).

in 5 mM sodium azide (Fig. 3). Although the sucrose gradient enriched the calcium-accumulating microsomal fraction, there was little reduction of azide sensitivity following such fractionation. The basic ATPase of the crude microsomes, measured in the absence of CaCl$_2$, was significantly inhibited by sodium azide (Fig. 4A). However, this mitochondrial ATPase inhibitor has little effect upon the "extra" ATPase associated with calcium uptake (Fig. 4B). Because of the low yield, it was not possible to examine the effects of azide on the ATPase activity of purified cardiac microsomes.

A double reciprocal plot was used to determine the binding constant for calcium binding to purified cardiac microsomes (Fig. 5). The use of this graphical analysis is based on the standard Langmuir adsorption isotherm (20). The rate of calcium binding \( k_2 \) [Ca] [S - B], where [Ca] is the free calcium concentration, [S] is the total number of binding sites and [B] is the number of such sites to which calcium is bound. The rate of calcium release \( k_1 \) [B]. $k_2$ and $k_1$ are rate constants for calcium binding and calcium release by the microsomes, respectively. At equilibrium, $k_1 [B] = k_2 [Ca] [S - B]$, so

\[
\frac{1}{[Ca]} = \frac{k_2 [S]}{k_1 [B]} - 1
\]

and

\[
- \frac{k_2}{k_1} = \frac{1}{[Ca]} - \frac{k_2 [S]}{k_1 [B]},
\]

at the intercept where $\frac{1}{[Ca]} = 0$,

\[
- \frac{k_2}{k_1} = - \frac{k_2 [S]}{k_1 [B]}
\]

and [B] = [S]. This permits evaluation of [S], the number of binding sites. At the in-
FIGURE 5

Double reciprocal plot of calcium binding versus calcium concentration. The intercept where
\[ \frac{1}{[Ca^2+]_{bound}} = 0 \]
represents the number of binding sites, which was 0.015 μmole calcium/mg microsomal protein for this preparation. The binding constant, calculated from the intercept where
\[ \frac{1}{[Ca^2+]_{bound}} = 0, \]
was 1.15 × 10^5 M⁻¹. Results were obtained with 0.2 mg/ml purified microsomes in 5 mM MgATP, 0.12 M KCl and 10 mM histidine at pH 7.0.

The ability of the purified cardiac microsomes to reduce free Ca²⁺ concentration in the presence of oxalate was examined by adding the microsomes to a solution containing 10⁻⁵ M CaCl₂. The cardiac microsomes (0.018 mg/ml) were able to lower the free Ca²⁺ concentration to almost 10⁻⁷ M (Fig. 6), a level that might be expected to bring about cardiac relaxation (11, 12). This view is supported by the finding that the Mg²⁺-activated ATPase activity of a Ca²⁺-sensitive reconstituted cardiac actomyosin (12) was markedly inhibited by these microsomes.

The kinetics of calcium uptake were studied by measuring the initial rate of ⁴⁶Ca disappearance from solutions containing 10⁻⁵ M CaCl₂. Calcium uptake followed first order kinetics at the start of the reaction. The rate constant, \( K \), in the equation:
\[ Kt = -\ln \frac{C(t)}{C(o)} \]
where \( t \) = time in seconds, \( C(t) \) = free calcium concentration at time \( t \), and \( C(o) \) = initial calcium concentration, was estimated by plotting
\[ -\ln \frac{C(t)}{C(o)} \]
against time (Fig. 7). The rate constant calculated in this manner from the data in Figure 7 was 0.24 sec⁻¹ mg microsomal protein⁻¹. When the yield of purified microsomes per gram wet heart weight is considered, the rate constant for the initial uptake of calcium from a solution of 10⁻⁵ M CaCl₂ by 1 g of heart muscle is 0.047 sec⁻¹. This means that
Reduction of free calcium concentration by purified cardiac microsomes. The amounts of calcium remaining in solution after \(^{45}\text{CaCl}_2\) was added to 0.018 mg/ml microsomes to give a final \(\text{Ca}^{2+}\) concentration of \(10^{-5}\) M are plotted. The experiment was carried out at 25°C in 2.5 mM Tris oxalate, 5 mM MgATP, 0.12 M KCl and 10 mM histidine at pH 7.0.

The initial rates of calcium uptake by purified cardiac microsomes plotted as \(-\ln \frac{C(t)}{C(0)}\) at microsome concentrations of 0.011 mg/ml (○), 0.022 mg/ml (●), 0.065 mg/ml (▲), 0.110 mg/ml (☆), 0.165 mg/ml (△) and 0.220 mg/ml (□). All reactions were carried out at 25°C in 0.12 M KCl, 5 mM MgATP, 2.5 mM Tris oxalate, and 10 mM histidine at pH 7.0. After 5 min were allowed for “warming up,” \(^{45}\text{CaCl}_2\) was added to give a final \(\text{Ca}^{2+}\) concentration of \(10^{-5}\) M.

Discussion

The present findings confirm previous reports that the myocardium is provided with an intracellular system capable of binding and accumulating large amounts of free calcium. The maximal calcium uptake by cardiac microsomes in 0.1 mM \(\text{CaCl}_2\) reported by Fanburg et al. was less than 1 \(\mu\)mole/mg microsomal protein (15). Using \(\alpha\)-tocopheral to inhibit hematin-catalyzed peroxidation of lipid, Inesi et al. (21) obtained a calcium uptake of 560 \(\mu\)mole/g dry weight in 0.065 mM \(\text{CaCl}_2\) and 2.5 mM oxalate. This value corresponds to an uptake of approximately 1 \(\mu\)mole/mg of microsomal protein. Lee, who used methods similar to those developed by Inesi et al. measured calcium uptake of 0.5 \(\mu\)mole/mg microsomal protein in 0.1 mM \(\text{CaCl}_2\) and of 2.5 \(\mu\)mole/mg in 0.5 mM \(\text{CaCl}_2\) (22), but these high levels of activity were found only in the presence of 6 mM phosphocreatine and phosphocreatinekinase. Such a requirement for an ATP-regenerating system was not observed in the present study. Values for calcium uptake similar to those reported here were recently reported by Weber et al. (23).
The use of sucrose density gradients to purify calcium-binding fractions of muscle, introduced by Hasselbach and Makinose (24) and Seraydarian and Mommaerts (25), has been applied by Carsten (26) to obtain stable rabbit cardiac microsomes. Carsten collected her microsomes from a layer in the gradient between 35 and 45% sucrose. This layer, which was less active than the purified microsomes obtained in the present study, probably corresponds to Layer II (Fig. 1), which took up only 0.52 \( \mu \text{mole calcium/mg of microsomal protein} \) in the present experiments. In the present study, enrichment of the calcium uptake by sucrose density-gradient centrifugation was slight (Table 2). However, this procedure has the advantage of stabilizing the microsomes and greatly reducing the azide-sensitive ATPase activity (Fig. 4).

Although it cannot be established that these calcium-accumulating microsomal fractions are derived exclusively from the sarcoplasmic reticulum, it is unlikely that mitochondrial contamination contributes significantly to their observed calcium uptake. While the "basic" (not \( \text{Ca}^{2+}\)-activated) ATPase of the crude microsomes is significantly inhibited by azide, an inhibitor of both the ATPase and calcium uptake of mitochondria (15, 27, 28), the "extra" (\( \text{Ca}^{2+}\)-activated) ATPase (Fig. 4) and calcium uptake (Fig. 3) of the crude microsomes are inhibited by less than 20%. It is possible that the slight inhibition of calcium uptake reflects azide sensitivity of the microsomal fraction itself because further purification of the calcium-accumulating fraction, while lessening malic dehydrogenase contamination did not reduce azide sensitivity significantly (Fig. 3). This view is supported by the finding that the calcium uptake of microsomes prepared by similar methods from rabbit white skeletal muscle, which contains few mitochondria, is also slightly inhibited by azide (B. B. Rubin and A. M. Katz, unpublished observations).

The recovery of myocardial calcium-accumulating activity in the crude microsomal fraction is similar to that seen in the intact myocardium after brief glycerol treatment (5). This calcium uptake greatly exceeds the amount of calcium that must be removed from the heart's contractile proteins to account for the relaxation of the intact heart. The total calcium-accumulating ability recovered in the crude microsomes is over 3 \( \mu \text{mole/g wet weight of ventricle} \), while that in the purified microsomes is approximately 0.9 \( \mu \text{mole/g wet weight} \) (Table 2). The myosin content of the myocardium has been estimated to be between 0.05 and 0.08 \( \mu \text{mole/g wet weight} \), and the amount of calcium needed to fully activate cardiac actomyosin is less than 2 mole/mole of myosin (see ref. 12 for details of these calculations). From these data the maximum amount of free calcium that must be removed to inactivate the cardiac actomyosin in situ would be 0.10 to 0.16 \( \mu \text{mole/g wet weight} \). This is approximately 5% of the calcium-accumulating capacity of the crude microsomes and less than 20% of the total calcium-accumulating capacity of the purified microsomes. Furthermore, these purified microsomes can lower the free \( \text{Ca}^{2+}\) concentration from \( 10^{-5}\text{M} \), where cardiac actomyosin is fully active (12), to approximately \( 10^{-7}\text{M} \) (Fig. 5) at which concentration the ATPase activity of cardiac actomyosin is lowered to the myosin level (12). In accord with previous findings (10, 16), we have found that these cardiac microsomes inhibit the ATPase activity of calcium-sensitive actomyosins. However, the rate of calcium uptake by purified cardiac microsomes in vitro is less than that of relaxation in the intact heart. Estimates of the half-time for calcium uptake at a starting level of \( 10^{-5}\text{M} \) \( \text{Ca}^{2+} \), range around 10 to 15 sec, whereas in the intact myocardium, in which large amounts of calcium must be removed from the actomyosin, relaxation requires less than 1 sec. Even if the higher yields of crude microsomes are used in this calculation, the predicted half-time for relaxation at this temperature would be over 3 sec.

The present findings are consistent with the view that microsomal calcium binding, which proceeds much more rapidly than does calcium uptake (27), could itself account for
relaxation in the heart. The total calcium-binding capacity recovered in the crude microsomal preparations averaged 0.050 μmole/g of ventricle (Table 1), which is approximately half that needed to remove 2 moles of calcium from each mole of cardiac myosin. Although the rate of calcium binding was too great to be measured by the methods used in the present study, this process appears to be extremely rapid. The present data can be compared to those obtained by Ohnishi and Ebashi (27), who found the calcium binding of skeletal microsomes to be approximately 0.2 μmole/g, based on a calculated loss during preparation of 50%. Weber et al. obtained a value of 0.053 μmole/mg for calcium binding in their best preparation of chicken heart microsomes (23). This value is similar to the highest value of 0.045 μmole/mg obtained in the present study. If we assume a 50% loss in the case of the crude cardiac microsomes, the calculated calcium binding of the ventricular myocardium would be approximately 0.1 μmole/g, or half that of skeletal muscle. This figure is in accord with the finding that the myosin content of the heart is one third to one half that of skeletal muscle (30). The yield of purified microsomes was significantly lower than that of crude microsomes, approximately 0.010 μmole/g of ventricle, clearly reflecting losses that occurred during the purification procedure. It must be emphasized that these calculated values for the recovery of calcium-binding capacity are much less than those obtained in the case of the intact ventricle (5, 14).

The cardiac microsomes had a very high affinity for Ca²⁺ in the presence of ATP. The binding constant of approximately 1 × 10⁶ M⁻¹ corresponds to a pKm of 5.0 (Fig. 5), which differs little from the pKm for Ca²⁺ of 4.7 measured by Carvalho (31) for skeletal microsomes in the absence of ATP. This value is similar to the binding constant of skeletal microsomes of 1.0–3.0 × 10⁶ M⁻¹ determined under similar conditions by Ebashi and Endo (32), but less than the value of approximately 5 × 10⁶ M⁻¹ estimated by Weber et al. (23).

The present data are thus consistent with the view that a process analogous to the rapid binding of calcium to cardiac microsomes may account for relaxation in the intact heart, both in terms of the rate of calcium binding and the total amount of calcium bound. Although addition of oxalate, an agent that permits trapping of calcium within the microsomes as insoluble calcium oxalate, enhances calcium uptake to a level 100 times greater than that of calcium binding, the in vitro accumulation of calcium proceeds much less rapidly than does relaxation of the intact heart at a comparable temperature.

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References


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