Structural and Functional Characterization of Dog Heart Microsomes

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ABSTRACT

Several procedures for the preparation of microsomes from heart tissue were compared. Sucrose was found to be the most effective addition to preserve the activity of the sarcoplasmic reticulum vesicles.

Electron microscopy of sectioned pellets shows uniform vesicles, but negative staining of microsomal suspensions allows recognition of mitochondrial fragments, which are found in cardiac microsomal fractions in greater amounts than in skeletal muscle preparations. The quantity of mitochondrial contaminations in different fractions was estimated by measuring enzymatic activities.

A comparison of Ca²⁺ accumulation, Ca²⁺ efflux, and ATPase activity shows identical features in preparations from the heart and from skeletal muscle, although the specific activity is approximately ten times lower in those from the heart. The apparent affinity of sarcoplasmic reticulum for Ca²⁺ in vitro justifies the assumption that sarcoplasmic reticulum lowers the intracellular Ca²⁺ concentration in the in-vivo heart below the level required for contraction. However, in a weight unit of fresh tissue, the ratio between the maximal Ca²⁺ accumulated by SR and the amount of contractile protein is much lower in the heart than in skeletal muscle.

Caffeine and, more markedly, propranolol and tetracaine increase efflux of accumulated Ca²⁺ and thereby reduce net uptake by fragmented sarcoplasmic reticulum. Isopropylmethoxamine is not effective at millimolar concentrations.

ADDITIONAL KEY WORDS sarcoplasmic reticulum calcium release mitochondria fragments calcium accumulation effect of drugs

Preparations of fragmented sarcoplasmic reticulum have been obtained from heart tissue with considerably more difficulty than from skeletal muscle (1-5). In addition, cardiac sarcoplasmic reticulum is less active, and whether the lower activity is due to more rapid inactivation or to the presence of inert contaminants is uncertain. This knowledge is important if one wants to estimate the activity and the functional role of the sarcoplasmic reticulum in the heart in vivo.

Cardiac and skeletal preparations have qualitative similarities, inasmuch as they both inhibit model contraction by sequestering Ca²⁺ from the reaction medium (5, 6). A similar control of the contraction-relaxation cycle in cardiac and skeletal muscle is indicated by the fact that contractile proteins of heart as well as of skeletal muscle are sensitive to Ca²⁺ (4, 7).

Owing to the great interest in cardiac function in general and in some specific features in disease states and sensitivity to pharmacologic agents, much experimental work has focused on cardiac sarcoplasmic reticulum.
reticulum. Several procedural modifications have been introduced to obtain purer and more active sarcoplasmic reticulum (3, 4). Attempts have been made to relate the activity displayed by cardiac sarcoplasmic reticulum in vitro to the activity required for the control of contraction and relaxation in vivo (6, 8). Finally, pharmacologic agents have been tested on cardiac sarcoplasmic reticulum on the reasonable assumption that certain effects on contractility may be brought about through a primary alteration of its control system (9-15).

In our laboratory we have primarily studied the structure and function of sarcoplasmic reticulum from skeletal muscle because this system is purer, more active, and easier to understand. At the same time we have made comparative studies with that from the heart and obtained useful information concerning this preparation, its structural and functional characterization, and the effect of some pertinent drugs. In addition, we have been able to study separately Ca\(^{2+}\) uptake and Ca\(^{2+}\) release from sarcoplasmic vesicles and to show how these two processes can be differentially altered.

**Methods**

**Preparation of sarcoplasmic reticulum.**—Dog hearts were chilled in ice and perfused with cold isotonic solutions through the coronary vessels. Ventricular muscle was then cut in small pieces and homogenized in buffered medium (1:2, w/v). Several homogenization mediums were compared. Usually, however, 20 mM Tris-HCl (pH 7) with 0.3M sucrose was used. The homogenization was carried out in a Waring Blender for 45 seconds. A hand glass homogenizer was used in some experiments.

After a slow centrifugation at 15,000 \(\times\) g for 20 minutes, the supernatant fluid was centrifuged at 40,000 \(\times\) g for 90 minutes (first cycle of centrifugations). This sediment was resuspended in 20 mM Tris-HCl and 0.3M sucrose. Centrifugations at both 15,000 \(\times\) g and 40,000 \(\times\) g were then repeated, and the final sediment was suspended in 35% sucrose and 5 mM Tris-HCl (pH 7) (second cycle of centrifugations). When no sucrose was added to the homogenization medium, the slow centrifugation was carried out at 5,000 \(\times\) g for 20 minutes and the fast centrifugations at 40,000 \(\times\) g for 30 minutes. In several experiments the material was further purified by centrifugation in a sucrose gradient.

**Ca\(^{2+}\) Accumulation.**—Sarcoplasmic reticulum was incubated with \(^{45}\)Ca\(\cdot\)CaCl\(_2\), ATP, and MgCl\(_2\) in reaction mixtures as required by the experimental schedule (see Results). The amount of radioactive calcium taken up by the reticulum was determined by measuring residual calcium in the medium after removal of the reticulum by rapid filtration (Millipore 0.45\(\mu\)). The uptake was strictly ATP dependent, since control samples contained sarcoplasmic reticulum but no ATP.

**Release of Accumulated Ca\(^{2+}\).**—Vesicles of sarcoplasmic reticulum were loaded with sarcoplasm reticulum vesicles with Ca\(^{2+}\) in the absence of oxalate, 10 mM EDTA was added after 60 seconds of incubation and serial samples were taken thereafter. EDTA chelates Mg\(^{2+}\), a required cofactor for Ca\(^{2+}\) uptake.

**Measurement of ATP\(_{ase}\) Activity.**—Liberation of inorganic phosphate was determined.

**Succinate Oxidation.**—O\(_2\) uptake was measured with an oxygen monitor (YSI model 53) in the presence of 16 mM phosphate buffer (pH 7.2), 84 mM KCl, 15 mM NaCl, 6 mM MgCl\(_2\), 2.5 mM succinate and 0.2 to 1.0 mg membrane protein/ml.

**Cytosochrome C.**—Oxidation was measured on a spectrophotometer, at 550-nm wavelength. Cytoschrome C was reduced with sodium dithionite before initiating the reaction.

**Electron microscopy.**—Droplets of suspensions of sarcoplasmic reticulum containing approximately 0.3 mg protein/ml were placed on 400-mesh grids covered with a carbon-backed Formvar film. Negative staining with 2% phosphotungstic acid (neutralized to pH 7) was done as previously described (14). The preparations were examined with a Siemens Elmiskop 1 A electron microscope with double condenser illumination (400-\(\mu\) apertures), 50-\(\mu\) objective aperture, and accelerating voltage of 80 kv.

**Results**

**Methods of Sarcoplasmic Reticulum Preparation.**—The single most important feature of various modifications introduced into the preparation of cardiac sarcoplasmic reticulum was the addition of sucrose. An account of the use of sucrose to carry out gradient centrifugations of cardiac microsomes was given by Carsten (3). In our experiments we have noticed that simple addition of sucrose, independent of gradient centrifugation, preserves the

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1Ethylenediaminetetraacetic acid.
activity of cardiac sarcoplasmic reticulum, which is otherwise very labile. These results are in agreement with those reported by other investigators (15, 16). In the presence of sucrose, addition of antioxidant vitamins (3, 5) is unnecessary.

For the homogenization procedure we found a Waring Blender most convenient. When we used a glass homogenizer in an attempt to avoid formation of mitochondrial fragments, minimal yield and negligible activity (in spite of much manual labor) were obtained. No consistent advantage was offered by any special homogenization medium. Maintenance of neutral pH and addition of sucrose as a preservative were the most important conditions.

Density-gradient centrifugation eliminated some mitochondrial contaminants. However, the final suspensions obtained after density-gradient centrifugation were too dilute and the yield too low to work with conveniently in kinetic experiments.

When two slow and two fast centrifugations (but no density gradient) were performed, the average yield was 45 to 50 mg of sarcoplasmic reticulum protein/100 g of fresh tissue.

Electron Microscopy.—Previous studies on sections of fixed pellets of cardiac microsomes showed uniform vesicles and failed to differentiate various types of membranes (see ref. 5 for pictures of our own preparations). Negative staining, however, allows differentiation of smooth and rough membranes. The electronmicroscopic appearance of negatively stained microsomes obtained from skeletal muscle was previously reported by us and other investigators (17-19).

Cardiac preparations appear more heterogeneous than those of skeletal muscle. Figure 1 is an electronmicroscopic view of cardiac microsomes obtained in our laboratory from dog heart (two cycles of different centrifugations). This view (which cannot be taken to indicate the average amounts of contaminants in the preparation) very distinctly shows fragments of sarcoplasmic reticulum and other contaminants. Vesicles of sarcoplasmic reticulum acquire a peculiar elongated appearance when stained with phosphotungstic acid. This elongated appearance is probably an artifact; it is, however, a convenient feature for recognition of the vesicles in a contaminated preparation. Some of these vesicles have a granular outer surface (Fig. 2) similar to that of sarcoplasmic reticulum from skeletal muscle (17-19). Mitochondrial fragments (rough membranes with 80-A particles attached to the outer surface) are also present. It should be noticed that the size of these fragments is quite similar to that of vesicles of sarcoplasmic reticulum (Figs. 1 and 2). Some fibrous material with 350-A periodicity (similar to the actomyosin arrowheads) found when screening different fields is shown in Figure 1.

Quantitative Evaluation of Mitochondrial Contaminations.—While electronmicroscopic inspection of microsomal suspensions allows a qualitative recognition of heterogeneous material, a quantitative evaluation of the extent of contaminations was better made by measuring succinate or cytochrome C oxidase activity in the various fractions obtained by differential centrifugation. In these experiments, the initial homogenate was centrifuged at 6,000 × g for 20 minutes before the first centrifugation at 15,000 × g. The mitochondrial fraction (6,000 to 15,000 × g) was then collected and used as a reference of oxidative activity. In these experiments, the initial homogenate was centrifuged at 6,000 × g for 20 minutes before the first centrifugation at 15,000 × g. The mitochondrial fraction (6,000 to 15,000 × g) was then collected and used as a reference of oxidative activity. The data on succinate oxidation were easier to calculate, since in our experimental conditions, the activity proceeded linearly, with rates of 15 to 18 μl/min/mg protein for the mitochondrial fraction. It was estimated that the microsomal fraction obtained after the first cycle of centrifugations contained 55% to 67% of mitochondrial contaminants. The contamination was reduced to 25% to 30% after the second cycle of centrifugations, and to 7% to 10% after centrifugation on a sucrose gradient. It should be pointed out that no significant succinate oxidation was catalyzed by microsomal fractions from skeletal muscle.

Due to the low yield of cardiac microsomes after centrifugation on a sucrose gradient we performed most of our kinetic experiments.
FIGURE 1

Microsomal preparation from heart tissue. Negative staining with phosphotungstate. X 41,000.

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with microsomal fractions obtained after two cycles of differential centrifugations.

Calcium Accumulation.—As previously reported by our and other laboratories (3-6, 8), cardiac sarcoplasmic reticulum accumulated Ca\(^{2+}\) both in the absence and in the presence of oxalate. Figure 3 shows a time curve of Ca\(^{2+}\) accumulation by sarcoplasmic reticulum from heart muscle (A) and from skeletal muscle (B).

The dependence of sarcoplasmic reticulum activity on concentration is shown in Figure 4. Considering that 0.1 mM EGTA\(^2\) (a Ca\(^{2+}\)-

\(\text{EGTA}^2\) Ethyleneglycol-bis (beta-amino-ethyl-ether) N, N\'-tetraacetic acid.
chelating agent) is present in the reaction mixture, half maximal activation is produced by a concentration of Ca\(^{2+}\) ion of approximately 1 \(\times 10^{-7}\)M. It is important to notice that the apparent Ca\(^{2+}\) affinities of cardiac and skeletal muscle sarcoplasmic reticulum are the same.

Maximal Ca\(^{2+}\) accumulation by sarcoplasmic reticulum from heart in the absence of oxalate is 15 to 20 \(\mu\)moles/g protein, and by that from skeletal muscle it is 120 to 140 \(\mu\)moles/g protein. In the presence of oxalate, large amounts of Ca\(^{2+}\) can be accumulated because of precipitation of calcium oxalate inside the vesicles.

There was no significant difference when only KCl, NaCl, or a mixture of the two was added to the reaction mixture.

**ATPase Activity.**—Figure 5 shows the activity of ATPase in the absence and in the presence of oxalate in the sarcoplasmic reticulum of heart muscle (A) and of skeletal muscle (B) sarcoplasmic reticulum. Reaction mixture: 16 mM Tris-maleate (pH 6.8), 80 mM KCl, 5 mM MgCl\(_2\), 5 mM ATP, 0.1 mM EGTA, 0.68 to 0.35 mg membrane protein/ml, 25°C. Ammonium oxalate, when indicated, was 5 mM.
ATP hydrolysis catalyzed by cardiac (A) and skeletal (B) sarcoplasmic reticulum. Reaction mixture: 16 mM Tris-maleate (pH 6.8), 80 mM KCl, 5 mM MgCl₂, 5 mM ATP, 0.34 to 0.18 mg membrane protein/ml. CaCl₂ added to a final concentration of 0.1 mM, 25°C. Ammonium oxalate, when indicated, was 5 mM.

The ratios of Ca²⁺ accumulated to ATP hydrolyzed were 1.5 to 2.0 when the initial rate of Ca²⁺ accumulation (tangent to the initial part of the time curve) and ATP hydrolysis were compared in experiments with muscle (B). Ca²⁺-independent ATP hydrolysis proceeds at a similar rate in both preparations. On addition of Ca²⁺, activation is registered. However, Ca²⁺ activation is much lower in cardiac sarcoplasmic reticulum, an indication of low specific activity. In the experiment illustrated in Figure 5, the rates of Ca²⁺-dependent ATPase (total minus Ca²⁺-independent) were 0.05 μmoles/min/mg protein in the absence of oxalate and 0.10 in the presence of oxalate; these values for skeletal muscle were 0.22 and 1.96. It should be pointed out that in the absence of oxalate it is very difficult to measure a true ATPase rate at 25°C because of the very rapid Ca²⁺ uptake and consequent inhibition of further activity by the high concentration of intravesicular Ca²⁺. We could only obtain the first sample 15 seconds after the addition of CaCl₂; at that time the burst of activity was already over. In the presence of oxalate, Ca²⁺ uptake continued longer and the concomitant ATP hydrolysis was easier to measure (Fig. 5A). In the experiment recorded in Figure 5B, the activity stopped after 15 seconds because of the exhaustion of Ca²⁺ in the reaction mixture; yet there was enough activity to measure because of the large amount of Ca²⁺ accumulated. A semiquantitative evaluation made on the basis of Ca²⁺ activation of ATPase in the presence of oxalate indicates 10 to 20 times lower specific activity in cardiac than in skeletal muscle sarcoplasmic reticulum. ATPase activation of sarcoplasmic reticulum was also obtained in the presence of diethyl ether (20). A comparison of ether activation in cardiac and skeletal muscle again shows much lower specific activity in cardiac preparations (Fig. 6).
skeletal muscle sarcoplasmic reticulum in the presence of oxalate. Somewhat lower values (1.0 to 1.5) were obtained with cardiac sarcoplasmic reticulum. In the absence of oxalate, the ratios of calcium to inorganic phosphate were higher. These values, however, may not be reliable because of the difficulty in measuring initial ATPase at 25°C in the absence of oxalate.

Efflux of Accumulated Ca²⁺.—As shown in Figure 7, efflux of accumulated Ca²⁺ proceeded similarly in the sarcoplasmic reticulum of cardiac and of skeletal muscle. The efflux was relatively slow, indicating low membrane permeability to Ca²⁺. The rate of efflux of accumulated Ca²⁺ can be markedly increased by appropriate experimental procedures such as temperature denaturation, or by the effect of pharmacologic agents (21). An interesting example of sudden Ca²⁺ release can be observed simply by adding cold CaCl₂ to cardiac or skeletal muscle sarcoplasmic reticulum loaded with ⁴⁵Ca²⁺ (Fig. 8). This is consistent with the experiments on Ca²⁺ exchange reported by Weber et al. (22).

Effects of Some Pharmacologic Agents.—Several reports have been published concerning the effect of digitalis glycosides, anesthetic agents, barbiturates, and antiarrhythmic and sympatholytic drugs on cardiac sarcoplasmic reticulum (9-15). The following results were obtained in our laboratory.

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Caffeine in concentrations used to produce contracture in skeletal muscle (23, 24) decreased net Ca$^{2+}$ accumulation by sarcoplasmic reticulum of skeletal muscle and heart (Fig. 9A and ref. 21, 25). An increased efflux of accumulated Ca$^{2+}$ can be demonstrated to cause this decrease (Fig. 10). However, accumulation cannot be totally inhibited even with high concentrations of caffeine (Fig. 9A). Tetracaine, a local anesthetic, sharply decreases net Ca$^{2+}$ accumulation (Fig. 9B) and increases efflux (Fig. 10) at concentrations above 1 mM. These effects can be demonstrated in the sarcoplasmic reticulum.
Effect of propranolol on Ca$^{2+}$ accumulation (•) and efflux (○ and △) in skeletal (left ordinate) and cardiac (right ordinate) sarcoplasmic reticulum. Values on the ordinate express the amounts of Ca$^{2+}$ accumulated in 1 minute, the amount of accumulated Ca$^{2+}$ remaining in the vesicles 1 minute after the addition of EDTA. Propranolol was present at the beginning of the incubation for the experiments on Ca$^{2+}$ accumulation; it was added with EDTA in the experiments on efflux. Reaction mixtures as described in the legends to Figure 3 and 7. Each point is the average of three experiments.

Discussion

Electron microscopy of positively stained preparations of cardiac sarcoplasmic reticulum of both cardiac and skeletal muscle. On the other hand, tetracaine at low concentrations (<1 mM) reduces passive Ca$^{2+}$ fluxes in skeletal muscle sarcoplasmic reticulum and prevents the effect of caffeine (21). On cardiac sarcoplasmic reticulum this effect of low concentrations of tetracaine cannot be consistently shown, probably because of variability of the much smaller experimental values that can be measured with cardiac preparations.

Sympathomimetic amines (epinephrine and isoproterenol) produced no significant effect at concentrations greater than 1 mM. Propranolol,4 a beta-receptor blocking agent, markedly reduced Ca$^{2+}$ accumulation (Fig. 11), as reported by Scales and McIntosh (10) and Hess et al. (13). The effect is produced by an increase in membrane permeability and Ca$^{2+}$ efflux (Fig. 11). Isopropylmethoxamine6 has no effect on Ca$^{2+}$ accumulation by the sarcoplasmic reticulum of either skeletal muscle or heart. Ouabain, a cardioactive glycoside, produced no significant effect in a wide range of concentrations, in either fresh or aged sarcoplasmic reticulum. It should be pointed out, however, that special experimental conditions, as during electrical stimulation of suspensions of sarcoplasmic reticulum (9) or when it is impaired by barbiturates (11), cardioactive glycosides have been found by other investigators to modify the activity of the sarcoplasmic reticulum.

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4Propranolol was supplied by Ayerst Laboratories.

6Isopropylmethoxamine was supplied by Burroughs Wellcome & Co.
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cannot distinguish between vesicles of sarcotubular and mitochondrial origin (5), but heterogeneous contaminations can be easily recognized on negatively stained material, as shown in Figures 1 and 2. The amount of contamination is impressive when compared to that of preparations of sarcoplasmic reticulum from skeletal muscle, in which mitochondrial fragments are rare (16). This is not surprising when the very different ratios between mitochondria and sarcoplasmic reticulum are considered in the two types of intact tissue.

Cardiac sarcoplasmic reticulum can now be obtained in a sufficiently stable form to exclude a marked loss of activity during the isolation procedure. In addition, the kinetics of Ca$^{2+}$ uptake and release (Figs. 3-7) is closely similar to that in skeletal muscle. It is thus reasonable to assume that the presence of inactive contaminants may contribute to a lowering of the specific activity of the cardiac preparations, when this is expressed per gram of protein. The enhanced accumulation of Ca$^{2+}$ in the presence of oxalate indicates that active vesicles effectively concentrate Ca$^{2+}$ to the degree that the solubility product of calcium oxalate is reached, as do vesicles in preparations from skeletal muscle.

On the basis of the kinetics of Ca$^{2+}$ uptake and the effect of oxalate and of specific inhibitors (8), it can be assumed that the mitochondrial fragments present in the cardiac preparations do not appreciably contribute to the overall Ca$^{2+}$ accumulation.

As shown in Figure 4, the sarcoplasmic reticulum of heart and that of skeletal muscle display the same affinity for Ca$^{2+}$. Half maximal activity is obtained at a Ca$^{2+}$ concentration of approximately $1 \times 10^{-7}$M (the calculation is based on the presence of 0.1 mM EGTA in the reaction mixture). This value is consistent with the affinity required to lower the intracellular Ca$^{2+}$ concentrations below the level required for contraction.

Whether the amount of Ca$^{2+}$ accumulated by cardiac sarcoplasmic reticulum in vitro accounts for the amount to be subtracted from the intracellular environment in vivo to obtain relaxation has been considered by Weber et al. (6) and Katz and Repke (8). The values obtained in vitro in the absence of oxalate were used in these evaluations on the reasonable assumption that no enhancement of activity comparable to the oxalate effect can be assumed in vivo. It appears that the total amount of Ca$^{2+}$ accumulated in vitro barely matches the amount to be taken up in vivo (8).

If, based on our experiments, a simple comparison is made between the maximal amounts of Ca$^{2+}$ accumulated by the sarcoplasmic reticulum isolated from the same weight of cardiac muscle (dog) and skeletal muscle (rabbit), an approximate ratio of 1:25 is obtained. On the other hand, the ratio between the amounts of native actomyosin obtained from the same weight of the two muscles is 1:2. Therefore, it must be concluded that sarcoplasmic reticulum cannot function as effectively in cardiac muscle as in skeletal muscle. This is consistent with the microscopic evidence for a limited development of sarcoplasmic reticulum in cardiac muscle (26). It also indicates that diffusion of Ca$^{2+}$ between the well-developed tubular sarcolemmic invaginations and the contractile elements may play a greater role in the relatively slow cardiac muscle than in skeletal muscle.

A reduction of net Ca$^{2+}$ accumulation and an increased efflux are produced by caffeine (Fig. 9) and, more markedly, by propranolol and tetracaine (Figs. 9 and 11) in sarcoplasmic reticulum of both cardiac and skeletal muscle. How these effects relate to the action of the drugs on cardiac contractility is difficult to assess. The Ca$^{2+}$ release induced by caffeine has been related to the occurrence of contraction in skeletal muscle (25). This relation could be reasonably extended to cardiac muscle. On the other hand, whether the Ca$^{2+}$ release induced by propranolol and tetracaine can account for their depressive effect on cardiac contractility remains to be demonstrated. An interesting hypothesis put forth by Fuchs et al. (15) suggests that some drugs (as quinidine and volatile anesthetics) may de-
press cardiac contractility by depleting the sarcoplasmic reticulum of Ca\(^{2+}\), which is then distributed to other Ca\(^{2+}\)-pumping systems, thus depressing the process of excitation-contraction coupling.

Another interesting question is whether the effect of propranolol on sarcoplasmic reticulum is related to beta-receptor blocking. Interestingly enough, isopropylmethoxamine has no effect on sarcoplasmic reticulum. This compound is a much weaker blocker of sympathomimetic effects on contraction, but it effectively blocks the metabolic effect of sympathomimetic amines.

We would like to emphasize that in our experiments we were able to study Ca\(^{2+}\) accumulation and release separately and demonstrate the effect of some pharmacologic agents on these variables. However, the relation of these effects to pharmacologic actions in vivo remains to be evaluated by extensive studies on intact organs.

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References


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