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# $(Ca^{2+} + Mg^{2+})$-ATPase IN ENRICHED SARCOLEMMA FROM DOG HEART

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## Summary

An enriched fraction of plasma membranes was prepared from canine ventricle by a process which involved thorough disruption of membranes by vigorous homogenization in dilute suspension, sedimentation of contractile proteins and mitochondria at $3000 \times g$ followed by sedimentation of a microsomal fraction at $200\,000 \times g$. The microsomal suspension was then fractionated on a discontinuous sucrose gradient. Particles migrating in the density range 1.0591—1.1083 were characterized by $(Na^+ + K^+)$-ATPase activity and [$^3$H]ouabain binding as being enriched in sarcolemma and were comprised of nonaggregated vesicles of diameter approx. 0.1 $\mu$m. These fractions contained $(Ca^{2+} + Mg^{2+})$-ATPase which appeared endogenous to the sarcolemma. The enzyme was solubilized using Triton X-100 and 1 M KCl and partially purified. Optimal $Ca^{2+}$ concentration for enzyme activity was 5—10 $\mu$M. Both $Na^+$ and $K^+$ stimulated enzyme activity. It is suggested that the enzyme may be involved in the outward pumping of $Ca^{2+}$ from the cardiac cell.

## Introduction

In the heart, contractile force declines as rapidly as $Ca^{2+}$ is cleared from the interstitial space. This suggests that $Ca^{2+}$ involved in the contractile process is rapidly exchangeable and implies a site of origin other than, or in addition to, the sarcoplasmic reticulum. Studies on the effects of lanthanum on cellular $Ca^{2+}$ [1] have indicated (since lanthanum does not penetrate intracellularly [1—3]) that $Ca^{2+}$ displaced from the sarcolemma is the immediate source of this ion for contraction. Thus $Ca^{2+}$ stores in sarcolemma appear to be crucial

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; SDS, sodium dodecyl sulfate.

for cardiac contractility [4] and it has been shown that sarcolemma may be involved in $Ca^{2+}$ transport in myocardial cells [5]. Evidence exists for a $Ca^{2+}$ binding and uptake system in isolated cardiac plasma membranes (sarcolemma) [6—8]. Isolated sarcolemma also appear to contain ($Ca^{2+}$ + $Mg^{2+}$)-ATPase [6,7], an enzyme which exists in high activity in the sarcoplasmic reticulum where it functions in regulating intracellular $Ca^{2+}$. The role of ($Ca^{2+}$ + $Mg^{2+}$)-ATPase in the sarcolemma is not clear; it may mediate the outward pumping of $Ca^{2+}$.

Establishing the presence of ($Ca^{2+}$ + $Mg^{2+}$)-ATPase in sarcolemma requires that membranes be available in a highly purified state completely free of sarcoplasmic reticulum and other intracellular membranes. In our previous studies [6] plasma membranes were prepared from guinea pig ventricle by a procedure which involved extraction of cut fibers with 1.25 M KCl followed by centrifugation on a discontinuous sucrose gradient at pH 8.2; fractions sedimenting between 55 and 65% sucrose were combined. In the present study a different procedure has been developed in which salt extraction is avoided and thoroughly disrupted tissue is subjected to centrifugation on a discontinuous sucrose gradient. In this procedure plasma membranes migrate in the low-density sucrose regions. Such fractions contain an active ($Ca^{2+}$ + $Mg^{2+}$)-ATPase. Some properties of the enzyme are reported.

## Materials and Methods

Dithiothreitol, Tris, EGTA, EDTA and Tris-ATP were purchased from Sigma. $^{45}CaCl_2$ (15—20 Ci/g), [G-$^3$H]ouabain (20 Ci/mmol) and *N*-[*ethyl*-1-$^{14}$C]ethylmaleimide (40 Ci/mol) were obtained from New England Nuclear.

*Enzyme assays and other measurements*

$Mg^{2+}$-ATPase was measured by incubating membrane protein in a medium (final vol. 1.1 ml) containing 5.0 mM Tris-ATP, 5.0 mM $MgCl_2$ and 0.25 mM EGTA in 50 mM Tris-maleate (pH 7.0) for 5 min at 37°C. Controls contained all components except $MgCl_2$. ($Ca^{2+}$ + $Mg^{2+}$)-ATPase was assayed in the same medium with varying amounts, usually 5 $\mu$M of free $CaCl_2$; control tubes contained all components except $CaCl_2$. $Ca^{2+}$-ATPase was similarly measured in a medium containing 4 mM $CaCl_2$ and in which $Na^+$, $K^+$ and $Mg^{2+}$ were absent, and in addition to EGTA the assay contained 0.25 mM EDTA. ($Na^+$ + $K^+$)-ATPase was measured by incubating membrane protein in a medium containing 5.0 mM ATP, 5.0 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 0.25 mM EGTA in 50 mM Tris-maleate (pH 7.0) for 5 min at 37°C. Ouabain-sensitive ATPase was measured by including 0.1 mM glycoside in the assay. All assays, which were conducted in duplicate, were terminated by the addition of trichloroacetic acid to a final concentration of 6%. Precipitated protein was removed by centrifugation and the supernatants were assayed for inorganic phosphate by the method of Martin and Doty [9]. Specific activities are expressed as nmol $P_i \cdot min^{-1} \cdot mg^{-1}$. Protein was determined by the method of Lowry et al. [10].

Cytochrome *c* oxidase was measured by the method of Cooperstein and Lazarow [11].

$Ca^{2+}$ binding was monitored by incubating membrane protein at 37°C in a

medium (final vol. 1.1 ml) containing 5 $\mu$M free $Ca^{2+}$ ($1.2—1.5 \cdot 10^5$ cpm/nmol), 0.25 mM EGTA, 5.0 mM $MgCl_2$ and 120 mM KCl in the absence and presence of 5 mM ATP in 50 mM Tris-maleate (pH 7.0). ATP-dependent binding was taken as the difference between the amount of cation bound in the absence of ATP and that bound in its presence. $Ca^{2+}$ accumulation was measured in the same system except that 5 mM potassium oxalate was included. Reactions were terminated by filtering 0.5 ml aliquots of the reaction mixtures through microfilters (Millipore HAWP025, 0.45 $\mu$) and bound $Ca^{2+}$ was determined as described previously [6]. Free $Ca^{2+}$ was calculated by the method of Katz et al. [12], except that EGTA was held constant while total $Ca^{2+}$ concentration varied. A binding constant of $2.5 \cdot 10^5$ $M^{-1}$ [13] was used.

[$^3$H]Ouabain binding was quantitated by incubating plasma membrane or sarcoplasmic reticulum (0.5 mg/ml) in a medium containing 0.5 mM ATP, 50 mM NaCl in 50 mM Tris-maleate (pH 7.0) and 2 $\mu$M [$^3$H]ouabain ($1.3 \cdot 10^4$ cpm/pmol) for 10 min at 37°C. Reactions were stopped by diluting 10-fold with 50 mM Tris-maleate at 4°C followed by centrifugation at 200 000 $\times g$ for 20 min. The pellets were suspended in the same volume of cold buffer, centrifuged and washed once more. The pellets were then suspended in 1 ml buffer containing 2% Triton X-100 to solubilize membrane protein and radioactivity was determined by scintillation spectrometry. Binding of the glycoside was calculated as pmol $\cdot$ $mg^{-1}$ membrane protein.

Sarcoplasmic reticulum was labeled with *N*-ethylmaleimide by incubating 200 $\mu$g membranes with 8 $\mu$M *N*-[*ethyl*-1-$^{14}$C]ethylmaleimide (1500 cpm/pmol) in 0.25 M sucrose/10 mM Tris-HCl (pH 8.2) for 1 h at 4°C [14]. Excess label was removed by sedimentation and washing and radioactivity was determined as described for [$^3$H]ouabain above.

For electron microscopy, camples were fixed for 2 h in 5% glutaraldehyde, 0.05% ruthenium red in 0.1 M cacodylate buffer at room temperature followed by the addition of 2% $OsO_4$.

Electrophoresis on 5% polyacrylamide gel slabs (7 $\times$ 10 cm) was performed at pH 7 according to Allen and Moore [15]. The sodium dodecyl sulfate concentration in electrode buffer and gels was 0.1%. Preparations and appropriate standards were incubated at 37°C with 1% sodium dodecyl sulfate and 1% mercaptoethanol for 1 h prior to each run. Approx. 1 mg protein of each sample was applied.

*Preparation of plasma membranes*

Hearts were removed from mongrel dogs anesthetized with sodium pentobarbital and exanguinated through a canulated carotid artery. Ventricles were dissected free of fat, large vessels and chordae tendinae, then washed with oxygenated Krebs-Ringer-bicarbonate, cut into approx. 10 g portions and stored at —80°C until use. Usually 2—4 g tissue was used for each experiment; 10—25 g were used for studies involving solubilization and purification of the ($Ca^{2+}$ + $Mg^{2+}$)-ATPase. Tissue was minced and homogenized in 25 vols. of 0.25 M sucrose/10 mM Tris-HCl/2 mM dithiothreitol (pH 8.2) in a Potter-Elvehjem homogenizer with a loose-fitting (drop time in water 5 s) Teflon pestle (20 passes), then for 20 passes using a tight-fitting pestle (drop time in water 28 s) driven at 500 rev./min. The tissue was further fragmented by

similar homogenization in a sintered glass homogenizer. The resulting homogenate was centrifuged at 3000 × *g* for 15 min. The supernatant fluid was centrifuged at 200 000 × *g* for 1 h in a Beckman Ti60 rotor; the resulting pellet was dispersed in 2 vols. (w/v) of the above buffer and homogenized in an all-glass Potter-Elvehjem homogenizer (30 passes) driven at 500 rev./min. The suspension was then subjected to fractionation on a discontinuous sucrose gradient. 4-ml aliquots were applied to a gradient consisting of 5 ml each of 45, 40, 35, 28, 26 and 24% sucrose in 10 mM Tris-HCl/2 mM dithiothreitol (pH 8.2). Centrifugation was conducted in a Beckman SW27 rotor at 100 000 × *g* (calculated for the middle of the tube) for 2 h. Fractions were harvested with a Psteur pipet as follows: each sucrose layer and the band of particles on top of the next most dense layer were removed together. Frations were diluted 3-fold with 2 mM dithiothreitol/10 mM Tris-HCl (pH 7.5) and centrifuged at 200 000 × *g* for 1 h. The resulting pellets were suspended in the above buffer at a concentration of approx. 2 mg/ml and were used immediately.

## Results

In this preparation initial homogenization was performed at pH 8.2 and with a large excess of buffer (25 vols.) to minimize aggregation of fragmented material; 0.25 M sucrose was included to stabilize enzyme activity. The homogenization procedure used was developed to thoroughly disrupt and fragment the sarcolemma. Connective tissue, contractile protein and some mitochondria were removed by the initial sedimentation at low gravitational forces (3000 × *g*). The supernatant from this homogenization contained 30% of the initial cytochrome *c* oxidase activity, 66% of the initial ($Ca^{2+}$ + $Mg^{2+}$)-ATPase and 50% of the ($Na^+$ + $Mg^+$)-ATPase. Following sedimentation at 200 000 × *g*, the pellet fraction consisted mainly of vesicular structures and mitochondria as observed by electron microscopy. Fractions harvested from the gradient were examined by electron microscopy, marker enzyme activities and [$^3$H]ouabain binding. Fractions from 8—30% sucrose were composed of non-aggregated membrane vesicles (Fig. 1A—D) (28% sucrose fraction not shown) of diameter approx. 0.1 μm. Fig. 1E shows the 35% sucrose fraction; the presence of mitochondria and membrane aggregates is evident. The 40 and 45% sucrose fractions also contained mitochondria as well as some vesicles and membrane aggregates. Fig. 2A shows the distribution of ($Na^+$ + $K^+$)-ATPase on the density gradient. The three lowest density fractions ($d_{20°C}$ 1.0591—1.1083) contained the highest specific activity for this plasma membrane marker as well as a large fraction of the total activity applied. A portion of this activity was also present in the 35—40% sucrose region of the gradient; however this material had low specific activity. The distribution of [$^3$H]ouabain binding is shown in Fig. 2B. Ouabain was first incubated with membranes from the 200 000 × *g* pellet; excess label was removed by washing before fractionation on the gradient. The binding site for ouabain is known to be the ($Na^+$ + $K^+$)-ATPase [16] and is considered a reliable marker for plasma membrane. The distribution of bound [$^3$H]ouabain on the gradient paralleled that of ($Na^+$ + $K^+$)-ATPase, the three lowest density fractions contained the highest specific binding (50—105 pmol ·

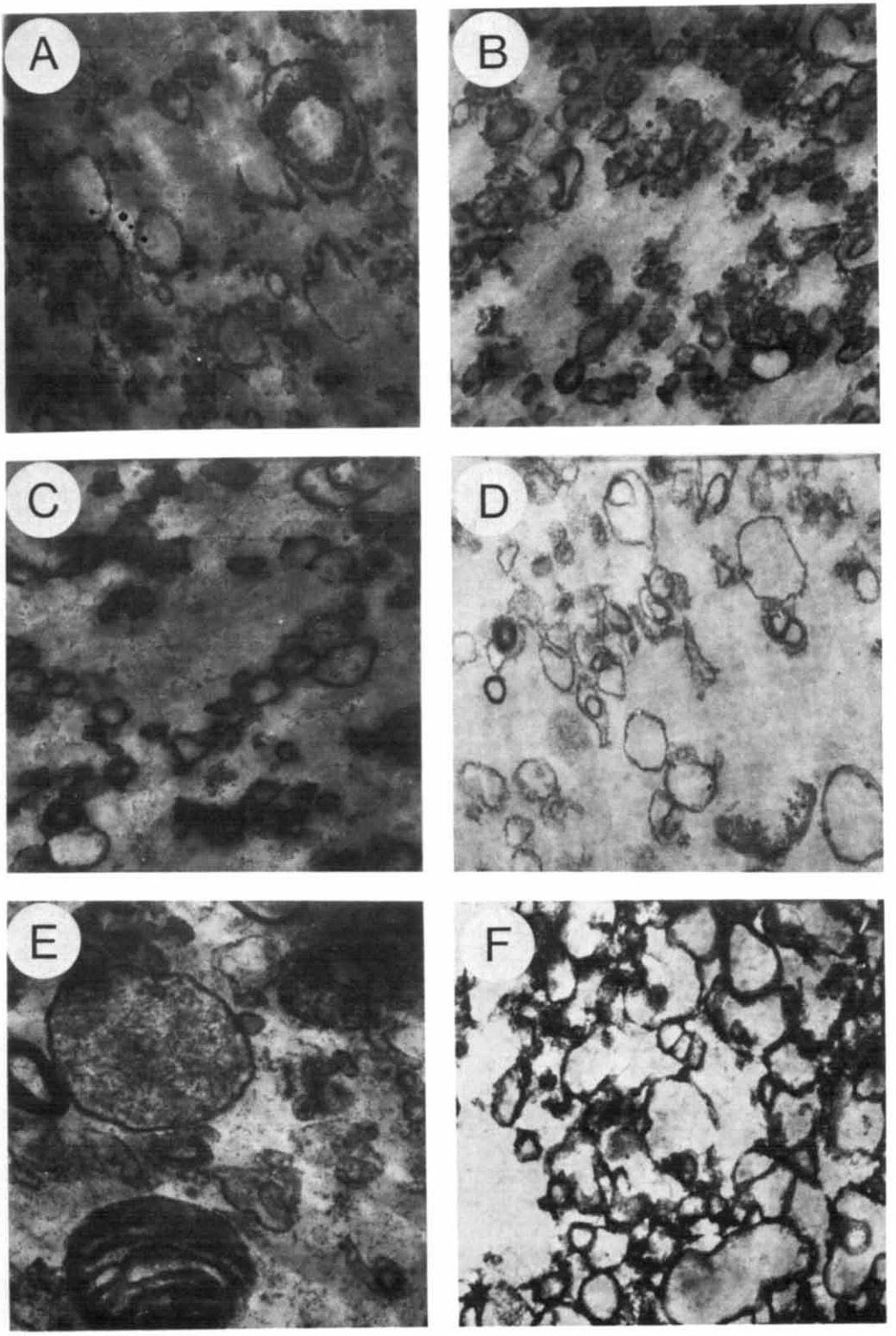



Fig. 1. Electron photomicrographs of various fractions. A, 8% sucrose fraction, $d_{20^\circ C}$ 1.0591; B, 24% sucrose, $d_{20^\circ C}$, 1.0942; C, 26% sucrose, $d_{20^\circ C}$ 1.1083; D, 30% sucrose, $d_{20^\circ C}$ 1.1257; E, 25% sucrose, $d_{20^\circ C}$ 1.1446; F, vesiculated partially purified $(Ca^{2+} + Mg^{2+})$-ATPase preparation. Magnification 50 000×.

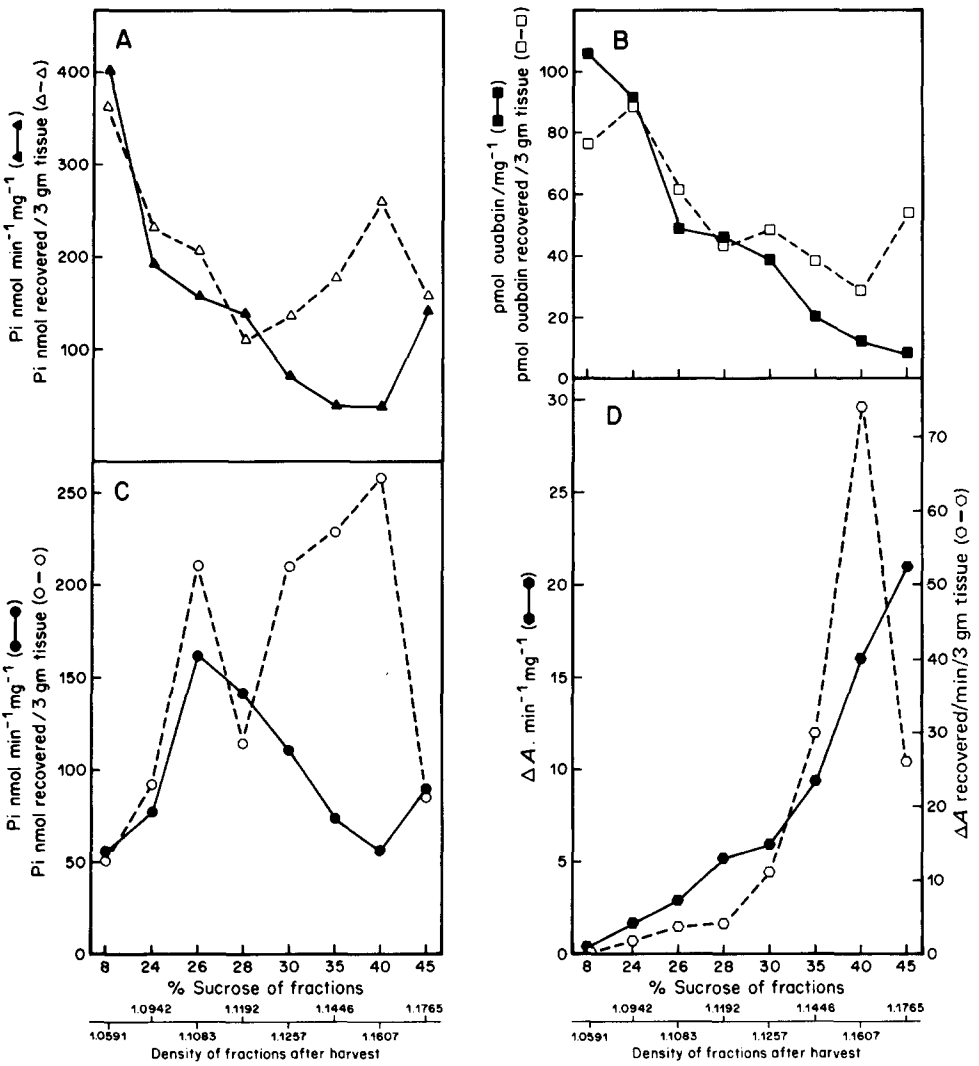



Fig. 2. Distribution of various activities in fractions obtained from density gradient centrifugation. A, $(Na^+ + K^+)$-ATPase; B, [$^3$H]ouabain binding; C, $(Ca^{2+} + Mg^{2+})$-ATPase; D, cytochrome *c* oxidase. Protein in each determinations was 100—200 μg.

$mg^{-1}$). These fractions also contained a large portion of the total bound glycoside. $(Ca^{2+} + Mg^{2+})$-ATPase (Fig. 2C) seemed to be distributed in two regions on the gradient; a portion appeared in the low-density regions (8—26% sucrose) but the greater amount of this activity appeared in higher density fractions (30—40% sucrose). Most of the cytochrome *c* oxidase activity (Fig. 2D) was found in the higher density fractions (30—45% sucrose); specific activity of this enzyme in these fractions was high reflecting the presence of mitochondrial membranes. The data indicate that the three fractions of lowest density (8, 24 and 26% sucrose) contained largely plasma membranes with little contamination from mitochondria. $(Ca^{2+} + Mg^{2+})$-ATPase was clearly present in these fractions suggesting the presence of this enzyme in plasma membranes. The

8—26% sucrose fractions, representing densities from 1.0591—1.1083, were routinely combined as the plasma membrane enriched fraction.

It was necessary to examine the purity of the plasma membrane fraction to determine whether the $(Ca^{2+} + Mg^{2+})$-ATPase residing there was endogenous to these membranes or resulted from contamination with fragmented sarcoplasmic reticulum which is rich in this activity. One way of providing such evidence would be to add purified sarcoplasmic reticulum to the fraction applied to the gradient and determine whether it altered the enzymatic profile especially of $(Na^+ + K^+)$-ATPase and $(Ca^{2+} + Mg^{2+})$-ATPase. However, it is well known that sarcoplasmic reticulum isolated by conventional procedures is contaminated with fragments of plasma membrane and therefore contains $(Na^+ + K^+)$-ATPase [17—19]. This enzyme can be labeled with ouabain and should be removed by sedimentation on a density gradient. Thus, sarcoplasmic reticulum was isolated by the method of Harigaya and Schwartz [20] and labeled with [$^3$H]ouabain. Excess label was washed away and the preparation was fractionated on the density gradient. Fractions were harvested and analyzed for bound [$^3$H]-ouabain (Fig. 3). A fraction with high specific activity appeared at the top of the gradient. This fraction (8—26% sucrose) was discarded. The fractions located between 28 and 35% sucrose were harvested as sarcoplasmic reticulum. Some binding occurred in these regions; it has been demonstrated previously [21,22] that sarcoplasmic reticulum has some ouabain binding capacity with low specific activity. A sample of sarcoplasmic reticulum recovered from the 28 to 45% sucrose regions of the gradient was next labeled with *N*-[*ethyl*-1-$^{14}$C]ethylmaleimide, combined with a 200 000 × *g* pellet fraction and the mix-

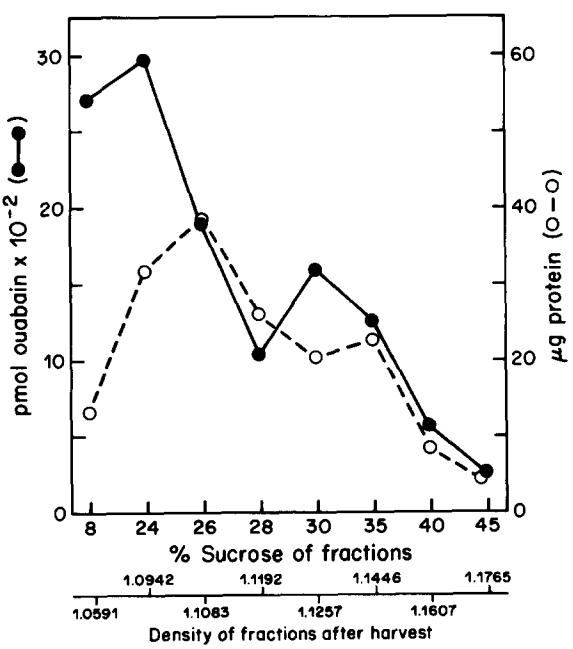



Fig. 3. Binding of [$^3$H]ouabain to sarcoplasmic reticulum fractionated by sucrose density gradient. Preparation of sarcoplasmic reticulum and labeling with [$^3$H]ouabain is described in Materials and Methods.

ture was fractionated on the usual sucrose gradient. Protein in each fraction was dissolved and radioactivity determined by scintillation spectrometry. Over 97% of the radioactivity applied was present in the density regions above 28% sucrose. The low-density fractions (8—26% sucrose) were essentially devoid of label. This suggested that sarcoplasmic reticulum did not get trapped in, or adsorbed to, plasma membranes during the gradient fractionation. The studies provide evidence that sarcoplasmic reticulum has a higher buoyant density than sarcolemma under the conditions used. The density to which sarcoplasmic reticulum sedimented was in the range 1.1192—1.1765 which is in excellent agreement with other authors [21,23]. The density at which the enriched plasma membrane sedimented is in good agreement with that recently found by Jones et al. [24]. The results strongly indicate that the $(Ca^{2+} + Mg^{2+})$-ATPase appearing in the lower density regions along with $(Na^+ + K^+)$-ATPase is a component of the sarcolemma and not due to contamination of these membrane vesicles with fragmented sarcoplasmic reticulum.

*Some properties and solubilization of the $(Ca^{2+} + Mg^{2+})$-ATPase*

Activity of $(Ca^{2+} + Mg^{2+})$-ATPase in the enriched plasma membrane fraction was markedly stimulated by micromolar concentrations of $Ca^{2+}$ (Fig. 4); concentrations above 100 $\mu$M were inhibitory. Both $Na^+$ and $K^+$ stimulated enzyme activity at $Ca^{2+}$ concentrations of 5—10 $\mu$M.

The $(Ca^{2+} + Mg^{2+})$-ATPase was solubilized by extraction of sarcolemmal membranes with Triton X-100 in a ratio of 0.1 mg detergent/mg protein in 0.25 M sucrose, 1 M KCl, 2 mM dithiothreitol (pH 8.2). Insoluble material was sedimented at 200 000 × *g* for 30 min. Usually 50—70% of the activity was solubilized. The presence of 1 M KCl was essential for an adequate yield and for retention of activity in the soluble fraction. The specific activity of the solubilized enzyme was 12-fold greater than that of the initial homogenate and approx. 6-fold greater than that of the membranes recovered from the density gradient (Table I). The sarcolemmal $(Ca^{2+} + Mg^{2+})$-ATPase, unlike the sarco-

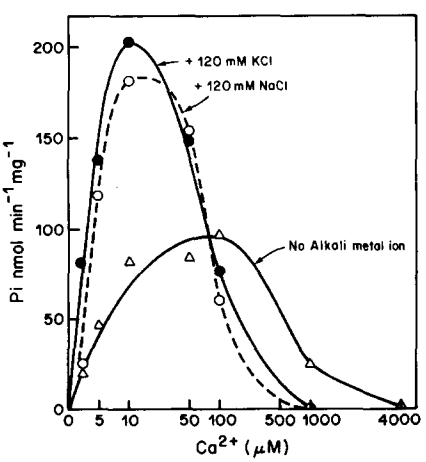



**Fig. 4. Effect of $Na^+$ and $K^+$ on sarcolemma $(Ca^{2+} + Mg^{2+})$-ATPase in the enriched sarcolemmal fraction at various $Ca^{2+}$ concentrations. Basal $Mg^{2+}$-ATPase under each condition has been subtracted.**

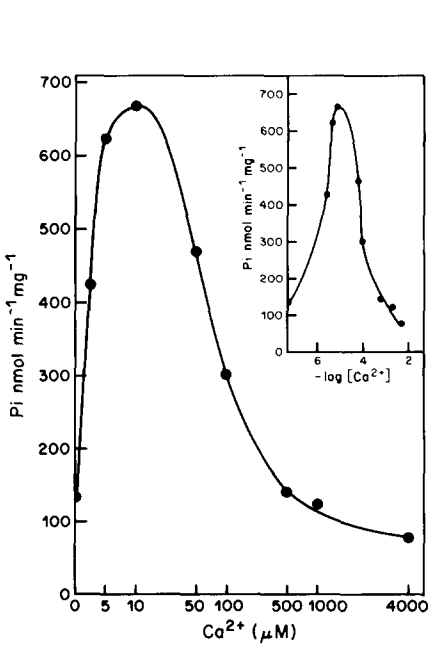
TABLE I

DISTRIBUTION OF ATPase ACTIVITY AND ATP-DEPENDENT $Ca^{2+}$ BINDING AND ACCUMULATION IN VARIOUS FRACTIONS

| Fraction | Protein (mg/g tissue) | $(Na^+ + K^+)$-ATPase | | | $(Ca^{2+} + Mg^{2+})$-ATPase | | | $Ca^{2+}$-ATPase | | | $Mg^{2+}$-ATPase | | | $Ca^{2+}$-binding (nmol · $min^{-1}$ · $mg^{-1}$) | $Ca^{2+}$-accumulation (nmol · $min^{-1}$ · $mg^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (nmol · $min^{-1}$ · $mg^{-1}$) | -fold purification | Yield (%) | (nmol · $min^{-1}$ · $mg^{-1}$) | -fold purification | Yield (%) | (nmol · $min^{-1}$ · $mg^{-1}$) | -fold purification | Yield (%) | (nmol · $min^{-1}$ · $mg^{-1}$) | -fold purification | Yield (%) | | |
| Crude homogenate | 160 | 36.7 | 1 | 100 | 24.5 | 1 | 100 | 113.3 | 1 | 100 | 36.7 | 1 | 100 | 0.22 | 0.72 |
| 200 000 × *g* pellet | 15 | 42 | 1.7 | 16 | 53.5 | 2.0 | 20 | — | — | — | — | — | — | — | — |
| Purified plasma membranes | 3.1 | 233.4 | 6.3 | 12.3 | 163.3 | 6.7 | 12.9 | 75.0 | 0.7 | 1.3 | 277 | 23 | 23 | 1.6 | 7.4 |
| Solubilized fraction in Triton X-100 | 1.3 | — | — | — | 297 | 12.8 | 9.8 | — | — | — | — | — | — | — | — |
| Partially purified vesiculated preparation | 0.8 | 244.3 | 6.6 | 3.3 | 488 | 20 | 10 | 48.3 | 0.4 | 0.2 | 137 | 3.7 | 2.9 | 0.2 | 0.34 |

plasmic reticulum enzyme [25], seemed particularly sensitive to deoxycholate even at concentrations as low as 0.1 mg/mg protein and this detergent could not be used for solubilization. Enzyme activity in the detergent supernatant was precipitated between 13 and 30% ammonium acetate. Precipitated protein was recovered as a floating layer following centrifugation at 200 000 × *g* for 15 min. Usually 90—95% of the solubilized activity was recovered in this fraction representing an overall 20-fold purification of the enzyme (Table I). When the ammonium acetate precipitated material was suspended in 0.25 M sucrose/10 mM Tris-HCl/2 mM dithiothreitol (pH 8.2) in the absence of Triton X-100, enzyme activity sedimented at 200 000 × *g*, thus detergent was required to keep the enzyme in solution. When the KCl concentration of solubilized preparations was reduced (either by dialysis or dilution) protein came out of solution and the preparation assumed a vesicular structure as evidenced by electron microscopy (Fig. 1E); the size of the vesicles was approx. 0.1 μM. Routinely the enzyme was stored as a suspension in 0.25 M sucrose, 10 mM Tris-HCl, 2 mM dithiothreitol (pH 7.5) at 25 mg/ml protein, conditions under which it was stable for 24 h at 4°C and for at least one week at —80°C. Enzyme activity in such preparations could be readily solubilized by addition of the above concentrations of Triton X-100 and KCl.

Fig. 5 shows the activity of the partially purified enzyme as a function of $Ca^{2+}$ concentration. Maximal activity occurred at 10 μM $Ca^{2+}$; maximal activity



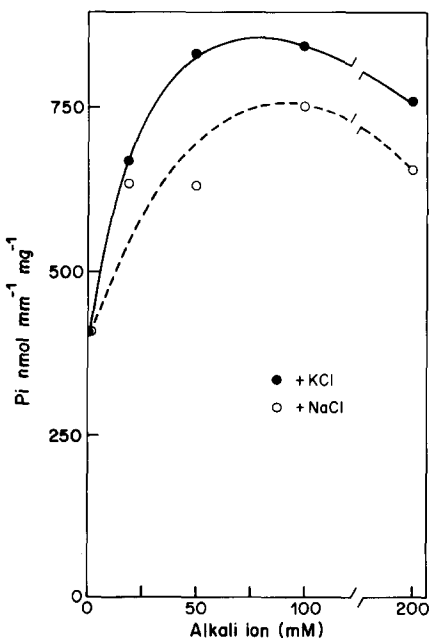




Fig. 5. Effect of $Ca^{2+}$ on purified sarcolemmal ($Ca^{2+}$ + $Mg^{2+}$)-ATPase (solubilized revesiculated preparation). The assay was conducted in the presence of 120 mM KCl. Activity at zero $Ca^{2+}$ was 125 nmol · $min^{-1}$ · $mg^{-1}$.

Fig. 6. Effect of $Na^+$ and $K^+$ on partially purified ($Ca^{2+}$ + $Mg^{2+}$)-ATPase (solubilized revesiculated preparation). $Ca^{2+}$ concentration was 10 μM.

was 3—4 times greater than that of the sarcolemmal fraction from the gradient. Concentrations of $Ca^{2+}$ above 10 $\mu$M were inhibitory. Fig. 6 shows that both $Na^+$ and $K^+$ stimulated enzyme activity.

When subjected to SDS polyacrylamide gel electrophoresis, the partially purified preparation contained three main components with particle mass of about 100 000, 55 000 and 38 000 daltons. When gel scans of the starting homogenate and membrane fraction were compared with the purified fraction it was clear that it was the 100 000 dalton fraction that was enriched during the purification relative to the other components. Tentatively, it might be suggested that the ($Ca^{2+}$ + $Mg^{2+}$)-ATPase may have a molecular weight of approx. 100 000.

The activity of various ATPases in the starting homogenate, the enriched plasma membrane fraction and the revesiculated (KCl-depleted) preparation of ($Ca^{2+}$ + $Mg^{2+}$)-ATPase is summarized in Table I. Purified membranes contained approx. 13% of the initial ($Ca^{2+}$ + $Mg^{2+}$)-ATPase with a 6.7-fold enrichment. This fraction contained 12% of the initial ($Na^+$ + $K^+$)-ATPase activity with a 6.3-fold enrichment. $Mg^{2+}$-ATPase was also purified to the same extent with a yield of 23%. The membranes did not possess significant $Ca^{2+}$-ATPase; only 1.3% of this activity was recovered. The membranes also possessed $Ca^{2+}$-binding and -accumulating activity which were enhanced 7- and 10-fold, respectively, over the starting homogenate. 10% of the original ($Ca^{2+}$ + $Mg^{2+}$)-ATPase was recovered in the final purified (revesiculated) fraction with a 20-fold purification. In this fraction ($Na^+$ + $K^+$)-ATPase, $Mg^{2+}$-ATPase and $Ca^{2+}$-ATPase were greatly reduced; the recoveries being 3.3, 2.9 and 0.2%, respectively. The partially purified enzyme preparation in revesiculated form was capable of binding and accumulating $Ca^{2+}$ (Table I). These activities, however. were lower than those of the membranes before solubilization.

## Discussion

Isolation of plasma membranes from cardiac muscle in a high degree of purity and free of minimal contamination of fragmented membranes of the sarcoplasmic reticulum and mitochondria has been fraught with many difficulties and uncertainties. Of the numerous methods described in the literature, several depend on the use of salt (LiBr, KBr, KCl, etc.) in high concentration to extract contractile proteins. Complications from prolonged salt extraction can arise such as removal of extrinsic membrane proteins and inactivation of intrinsic proteins. More recently centrifugation through density gradients has been employed; for example Katz et al. [12] and Kidwai et al. [26] used density gradient ultracentrifugation to prepare a microsomal fraction enriched in ($Na^+$ + $K^+$)-ATPase activity. Recently Jones et al. [24] have attempted to improve the resolution of plasma membrane from sarcoplasmic reticulum by 'loading' microsomal preparations with calcium oxalate before density gradient centrifugation, a technique used by Levitsky et al. [25] for isolating sarcoplasmic reticulum. This technique which would increase the density of sarcoplasmic reticulum would presumably give improved resolution of these membranes from sarcolemma. This procedure, however, could change the density of sarcolemma if these vesicles also accumulated $Ca^{2+}$ in the presence of oxalate,

and evidence exists that they do [6,7]. In the present study we have avoided the use of salt extraction and employed initial homogenization procedures that thoroughly disrupted the fibers and extensively disrupted membrane structures. Insoluble contractile proteins were then largely removed by sedimentation at $3000 \times g$. Following sedimentation at high gravitational forces, the microsomal fraction was subjected directly to density gradient ultracentrifugation. Following sedimentation, plasma membranes, as evidenced by $(Na^+ + K^+)$-ATPase and [$^3$H]ouabain binding, were located in the lower density regions ($d_{20°C}$ 1.0591—1.1083) while sarcoplasmic reticulum appeared in higher density regions ($d_{20°C}$ 1.1192—1.1765). In general plasma membranes contain 40—60% lipid and 30—50% protein while sarcoplasmic reticulum consists of approx. 30% lipid and 70% protein [27,28]. It would be expected therefore that the buoyant density of sarcolemma would be lower than that of sarcoplasmic reticulum.

These studies provide additional evidence that $(Ca^{2+} + Mg^{2+})$-ATPase resides within the sarcolemma as well as the sarcoplasmic reticulum. It is too early yet to say what differences will characterize the enzyme from the two membrane sources. The sensitivity of the sarcolemmal enzyme to low concentrations of $Ca^{2+}$ was dependent on $Mg^{2+}$ and activity was stimulated by $K^+$ and by $Na^+$. Only recently [29,30] has $K^+$ stimulation of the enzyme in sarcoplasmic reticulum been demonstrated. The enzyme was effectively solubilized using Triton X-100, but deoxycholate could not be used because of destruction of the enzyme although it proved useful for solubilization of the sarcoplasmic reticulum enzyme from canine heart [25].

In this study we measured $Ca^{2+}$-ATPase as the activity stimulated by $Ca^{2+}$ at concentrations above 50 $\mu$M and in the absence of $Mg^{2+}$. The term $Ca^{2+}$-ATPase has been used interchangeably in the literature to describe $Ca^{2+}$-stimulated activity in the absence or presence of $Mg^{2+}$. During the fractionation procedure $Ca^{2+}$-ATPase was almost completely resolved from $(Ca^{2+} + Mg^{2+})$-ATPase, so the two activities are distinct and separable entities. The loss of $Ca^{2+}$-ATPase from the preparation would suggest that this enzyme is not involved in $Ca^{2+}$ binding and transport. The partially purified preparation of $(Ca^{2+} + Mg^{2+})$-ATPase assumed a vesicular structure when KCl was removed and the vesicles has some ability to bind and accumulate $Ca^{2+}$ (oxalate present) in an ATP-dependent manner. Although these activities were much below those of the plasma membrane fraction, this finding might suggest a role for the $(Ca^{2+} + Mg^{2+})$-ATPase as a $Ca^{2+}$ pump which may function in the pumping of this cation to the exterior of the cell.

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