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GEL ELECTROPHORETIC AND DENSITY GRADIENT ANALYSIS OF THE ($K^+ + Ca^{2+}$)-ATPase AND THE ($Na^+ + K^+$)-ATPase ACTIVITIES OF CARDIAC MEMBRANE VESICLES

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Summary

The two major ATPase activities of intact and leaky cardiac membrane vesicles (microsomes) were characterized with respect to ionic activation requirements. The predominant ATPase activity of intact vesicles was ($K^+ + Ca^{2+}$)-ATPase, an enzymic activity localized to sarcoplasmic reticulum, whereas the predominant ATPase activity of leaky, sodium dodecyl sulfate-pretreated vesicles was ($Na^+ + K^+$)-ATPase, an enzymic activity localized to sarcolemma. The ($K^+ + Ca^{2+}$)-ATPase activity was stimulated 4- to 5-fold by 100 mM K^+ in the presence of 50 μM Ca^{2+}. Phosphorylation of the ($K^+ + Ca^{2+}$)-ATPase of intact vesicles with [γ-^{32}P]ATP was Ca^{2+} dependent, and monovalent cations including K^+ increased the level of [^{32}P]phosphoprotein by up to 50% when phosphorylation was measured at 5°C. After the intact vesicles were treated with SDS (0.30 mg/ml), ($K^+ + Ca^{2+}$)-ATPase was inactivated, as was Ca^{2+}-dependent ^{32}P incorporation. The monovalent cation-stimulated ATPase activity of the particulate residue (SDS-extracted membrane vesicles) displayed the usual characteristics of ouabain-sensitive ($Na^+ + K^+$)-ATPase and the activity was increased 9- to 14-fold over the small amount of patent ($Na^+ + K^+$)-ATPase activity of intact membrane vesicles. ^{32}P incorporation by the ($Na^+ + K^+$)-ATPase of SDS-extracted vesicles was Na^+ dependent, and Na^+-stimulated incorporation was increased 7- to 9-fold over that of intact vesicles.

Slab gel polyacrylamide electrophoresis of both intact and SDS-extracted crude vesicle preparations revealed at least 40 distinct Coomassie Blue-positive protein bands and provided evidence for a possible heterogeneous membrane origin of the vesicles. Periodic acid-Schiff staining of the gels revealed at least

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Abbreviation: EGTA, ethyleneglycol bis(β-aminoethylether)-*N*,*N*'-tetraacetic acid.

two major glycoproteins. Simultaneous electrophoresis of the ^{32}P intermediates of the $(K^+ + Ca^{2+})$-ATPase and the $(Na^+ + K^+)$-ATPase in the same gels did not resolve the two enzymes clearly. With sucrose gradient centrifugation of intact membrane vesicles, it was possible to physically resolve the two ATPase activities. Latent $(Na^+ + K^+)$-ATPase activity (unmasked by exposing the various fractions to SDS) was found in the higher regions of the gradient, whereas $(K^+ + Ca^{2+})$-ATPase activity was primarily in the denser regions. A reasonable interpretation of the data is that cardiac microsomes consist of membrane vesicles derived both from sarcolemma and sarcoplasmic reticulum. $(Na^+ + K^+)$-ATPase is localized to intact vesicles of sarcolemma but is mainly latent, whereas $(K^+ + Ca^{2+})$-ATPase is mostly patent and is localized to vesicles of sarcoplasmic reticulum.

Introduction

Microsomal fractions isolated from myocardium contain high levels of Ca^{2+} transport enzyme and associated Ca^{2+}-dependent ATPase activity [1—4]. These preparations have generally been thought to be devoid of significant $(Na^+ + K^+)$-ATPase activity and, therefore, have often been referred to as relatively purified cardiac sarcoplasmic reticulum fractions [2—7]. However, it has recently been shown that cardiac microsomal preparations also contain appreciable quantities of latent $(Na^+ + K^+)$-ATPase activity which can be revealed by carefully treating the membranes with detergents [1,8,9] as originally described for renal microsomes by Jorgensen [10]. Non-destructive unmasking by use of the channel-forming ionophore alamethicin reveals a similar quantity of $(Na^+ + K^+)$-ATPase activity [11].

Because adenylate cyclase and beta-receptor binding activity have also been found in cardiac microsomes [5—7,11], the conclusion has been drawn that cardiac sarcoplasmic reticulum contains a catecholamine-responsive adenylate cyclase system [6,7,12]. The demonstration of probable sarcolemmal contamination in the preparations originally used to identify the activities, however, now questions the original interpretation of the data [13].

The present study was undertaken to ascertain whether the $(K^+ + Ca^{2+})$-ATPase * and the $(Na^+ + K^+)$-ATPase activities of cardiac microsomes (membrane vesicles) are indeed separate enzymes and whether they can be used as valid enzyme markers for cardiac sarcoplasmic reticulum and sarcolemma, respectively.

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* No standard abbreviation exists for one of the major enzymes discussed herein. The name $(K^+ + Ca^{2+})$-ATPase has been used in this and previous communications [1,11,14,15] in recognition that turnover of this enzyme of the sarcoplasmic reticulum is regulated by monovalent cations [1,11,14—21]. This same enzyme has been variously termed by others as Ca^{2+}-ATPase [22], $(Ca^{2+} + Mg^{2+})$-ATPase [23] and even $(K^+ + Ca^{2+} + Mg^{2+})$-ATPase [17]. By analogy to the widely used nomenclature for $(Na^+ + K^+)$-ATPase (Mg^{2+}-dependent, $(Na^+ + K^+)$-activated adenosinetriphosphatase, EC 3.6.1.3), the $(K^+ + Ca^{2+})$-ATPase should probably be formally designated Mg^{2+}-dependent, $(K^+ + Ca^{2+})$-activated adenosinetriphosphatase, EC 3.6.1.3. Inclusion of K^+ in the abbreviated name, $(K^+ + Ca^{2+})$-ATPase, does not demand a unique specificity for this particular monovalent cation; other monovalent cations will substitute for K^+ in activating turnover, just as other monovalent cations such as Rb^+ and NH_4^+ will substitute for K^+ at the K^+ site on $(Na^+ + K^+)$-ATPase.

Experimental procedure

Preparation of intact cardiac membrane vesicles. Intact vesicles were prepared from dog ventricle homogenized in dilute sodium bicarbonate buffer as previously described [1,8,11]. Briefly, two low speed centrifugations at 11 600 $\times g_{max}$ and 15 300 $\times g_{max}$ were employed to remove mitochondria and other large particles. The membrane vesicles were then sedimented from the supernatant fraction by centrifugation at 43 600 $\times g_{max}$. The sedimental material was resuspended in 0.6 M KCl/20 mM imidazole maleate, pH 6.8, to extract contractile proteins, and recentrifuged at 43 600 $\times g_{max}$. The sedimented vesicles were resuspended in 0.25 M sucrose, 30 mM histidine (pH 7.4) and stored frozen at —20°C in small aliquots. The frozen samples are termed intact membrane vesicles. In all cases reported here, enzymic activities of the intact vesicles remained stable after freezing (up to one month).

Preparation of SDS-extracted membrane vesicles. Freshly prepared or thawed intact membrane vesicles were diluted 1 : 10 in a solution consisting of 30 mM imidazole (pH 7.1) and 0.3 mg SDS/ml. The final protein concentration was 0.6—0.9 mg/ml. After incubation for 30 min at room temperature, the suspension was centrifuged at 105 700 $\times g_{max}$ for 60 min. The pellet was resuspended in ice-cold H_2O, recentrifuged, and then resuspended in 0.25 M sucrose/30 mM histidine (pH 7.1) and stored frozen at —20°C. This fraction is termed SDS-extracted membrane vesicles.

Sucrose gradient centrifugation of intact membrane vesicles. Discontinuous sucrose gradients were formed by successively layering 10 ml each of 1.0 M, 0.8 M and 0.6 M sucrose into cellulose nitrate tubes. 5 ml intact vesicles suspended in 0.25 M sucrose (6—9 mg/ml) was then layered over the 0.6 M sucrose. Centrifugation was in a Beckman SW 27 rotor at 27 000 rev./min for 1 h. Fraction A was collected at 0.25 M/0.6 M interface, fraction C at the 0.6 M/0.8 M interface and fraction E at the 0.8 M/1.0 M interface. Fraction B was the material which entered the 0.6 M sucrose but did not reach the 0.8 M sucrose interface, and fraction D was the material which entered the 0.8 M sucrose but did not reach the 1.0 M sucrose interface. The various fractions were diluted with ice-cold H_2O and recentrifuged at 105 700 $\times g_{max}$ in a Beckman type 30 fixed angle rotor. The pellets were resuspended in 0.25 M sucrose and stored frozen at —20°C.

Assay of ATPase activities. All ATPase assays were conducted at 37°C in a medium containing 15—20 μg of membrane protein/ml, 50 mM histidine (pH 7.4), 3 mM $MgCl_2$, 3 mM Tris · ATP (basal medium). When only one monovalent cation was added to the basal medium the final concentration was 0.1 M (as the chloride salt) in all cases. ($K^+ + Ca^{2+}$)-ATPase activity was taken as the difference between ATP hydrolysis measured in the presence of basal medium containing 50 μM added Ca^{2+} and that in basal medium containing 0.8—1.0 mM Tris · ethyleneglycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA). Various monovalent cations were also added to the assay tubes when this enzyme was measured (as indicated in the legends to the figures). ($Na^+ + K^+$)-ATPase activity is defined as the activity inhibitable by 10^{-3} M ouabain, measured in the presence of 100 mM Na^+ plus 10 mM K^+. When this activity was measured the basal medium usually contained either 50 μM Ca^{2+} or 0.8—

1.0 mM EGTA. ATP hydrolysis was measured by following the rate of inorganic phosphorus release from ATP with a modified Fiske-SubbaRow assay [1,8,11].

Phosphorylation of membrane vesicles. When membrane vesicles were phosphorylated for autoradiography, the reaction medium contained 50 mM histidine (pH 7.4), 3 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP, 600 μg of membrane protein, and various concentrations of cations as indicated in the text. Phosphorylation was conducted at 5°C for 15 s in 1.0 ml reaction medium. Sample tubes containing intact and SDS-extracted vesicles received $5 \cdot 10^7$ and $3 \cdot 10^8$ dpm of [γ-^{32}P]ATP, respectively, so that maximal incorporation of radioactivity was comparable in both cases. Phosphorylation was terminated by adding 0.2 ml ice-cold 50% trichloroacetic acid to the reaction tubes. Precipitated protein was then sedimented at $5000 \times g_{max}$ for 20 min at 5°C. The pellets were washed three times with 2.0 ml ice-cold 5% trichloroacetic acid/1 mM ATP/10 mM Na_2PO_4 and once with 2.0 ml ice-cold H_2O. The pellets were then resuspended in 500 μl electrophoresis-dissociation medium and aliquots applied to polyacrylamide gels. Other aliquots were assayed for radioactivity by liquid scintillation spectrometry.

For routine assay of phosphoprotein levels ^{32}P-labeled phosphoprotein was separated from unreacted [γ-^{32}P]ATP and $^{32}P_i$ by filtration. The trichloroacetic acid-precipitated membrane vesicle suspension was applied to Whatman GF-C filters and subsequently rinsed 8 times with 5 ml ice-cold 5% trichloroacetic acid/1 mM ATP/10 mM NaH_2PO_4. After a final rinse with 5 ml ice-cold H_2O, the radioactivity remaining on the filters was measured with liquid scintillation spectrometry.

SDS-polyacrylamide gel electrophoresis and autoradiography. Membrane vesicle proteins were solubilzed at room temperature in dissociation medium which contained 0.0625 M Tris-HCl, 2.5% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue as the tracking dye. Inclusion of 10 M urea in the dissociation medium or boiling the samples prior to electrophoresis did not improve the resolution on the gels. Electrophoresis was performed with a slab gel apparatus (Bio-Rad), on slabs of 1.5 mm thickness and 10 cm length. The greatest resolution of membrane proteins was obtained with electrophoresis carried out according to the method described by Laemmli [24]. Gels containing 3% (stacking) and 7.0—8.5% (separating) acrylamide were used. The ratio of acrylamide to *N,N'*-methylenebisacrylamide was 30 : 1.5. Electrophoresis was also performed with 5.6% acrylamide gels by the method of Fairbanks et al. [25]. After electrophoresis with either system the gels were fixed overnight in methanol/water/acetic acid (5 : 4 : 1, v/v) and subsequently stained either in 0.2% Coomassie Blue dissolved in the fixing solution or with periodic acid-Schiff stain [25]. The molecular weights of the vesicle proteins were estimated comparing their mobility to the mobility of protein standards of known M_r. The protein standards used were RNA polymerase from *Escherichia coli* (α-subunit 39 000; β-subunit, 155 000; β'-subunit, 165 000); β-galactosidase (130 000), phosphorylase a (94 000), transferrin (76 000), bovine serum albumin (68 000), ovalbumin (43 000) and chymotrypsinogen (26 000).

For autoradiography, the Coomassie Blue-stained gel slabs which contained the ^{32}P-labeled proteins were dried under vacuum for 3 h at 80—82°C. The

dried gels were then placed in contact with DuPont Cronex 4 medical X-ray film for 5—50 days and the film was then developed.

Gel scanning of Coomassie Blue-stained gels was performed at 660 nm with an Isco UA-5 Absorbance Monitor equipped with a linear transport gel scanner.

Materials. Tris · ATP, ouabain, histidine, β-galactosidase (grade IV), bovine serum albumin (fraction V) and transferrin were purchased from the Sigma Chemical Company, St. Louis, Mo. RNA-polymerase from *E. coli* was purchased from Boehringer Mannheim, Indianapolis, Ind. Phosphorylase *a* was obtained from Worthington, Freehold, N.J. Ovalbumin and chymotrypsinogen were obtained from Pharmacia, Piscataway, N.J. SDS was purchased from Bio-Rad, Richmond, Va. [γ-^{32}P]ATP tetra (triethylammonium) salt (>10 Ci/mmol) was obtained from New England Nuclear, Boston, Mass.

Results

Cation-stimulated ATPase activity of intact cardiac membrane vesicles. The principal patent ATPase activity of intact cardiac membreane vesicles (intactness being defined as the ability to actively sequester Ca^{2+}) required both K^+ and Ca^{2+} for optimal activity (Fig. 1a). This ($K^+ + Ca^{2+}$)-ATPase activity directly parallels the extent of Ca^{2+}-uptake by the preparation [1]. In the presence of 50 μM Ca^{2+}, the ATPase activity of intact vesicles was approximately doubled by addition of 100 mM K^+, whereas in the presence of excess EGTA, only a negligible increase in ATPase activity occurred upon addition of K^+ (Fig. 1a). As has been previously shown [1,14—17], stimulation of ($K^+ + Ca^{2+}$)-ATPase activity by any of the various combinations of monovalent cations (approx. 100 mM) was approximately 4-fold, as indicated in the numbers above the brackets in Fig. 1a. The major portion of the patent ATPase activity was ouabain insensitive, even in the presence of both Na^+ and K^+ (Fig. 1a). Only a low level of patent, ouabain-sensitive ($Na^+ + K^+$)-ATPase activity was present in intact membreane vesicles (2—3 μmol P_i/mg protein per h in the preparation of Fig. 1a). 3 mM Ca^{2+} completely inhibited stimulation of ouabain-sensitive as well as EGTA-sensitive ATPase activity by monovalent cations in intact vesicles (Fig. 1a).

In order to produce substantial phosphorylation of intact membrane vesicles by [γ-^{32}P]ATP, it was necessary to have at least micromolar amounts of ionized Ca present, suggesting that the majority of the phosphorylated moiety was a Ca^{2+}-ATPase (Fig. 1b). In the presence of monovalent cations the concentration of phosphorylated intermediate was increased 20- to 30-fold by 50 μM Ca^{2+}. The steady-state level of phosphorylated intermediate measured at 5°C was also greatly increased by 50 μM Ca^{2+} in the presence of Mg^{2+} alone, although the level achieved was somewhat lower (approx. 30%) than that measured in the presence of Mg^{2+} plus monovalent cations (Fig. 1b). These data (Fig. 1, a and b) suggest that the bulk of the monovalent cation sensitive ATPase activity of intact cardiac membrane vesicles was contributed by the Ca^{2+} pump of cardiac sarcoplasmic reticulum [1,9,15].

Cation-stimulated ATPase activity of SDS-extracted cardiac membrane vesicles. After cardiac membrane vesicles were exposed to optimal concentrations of the detergent SDS, ouabain-sensitive ($Na^+ + K^+$)-ATPase activity was

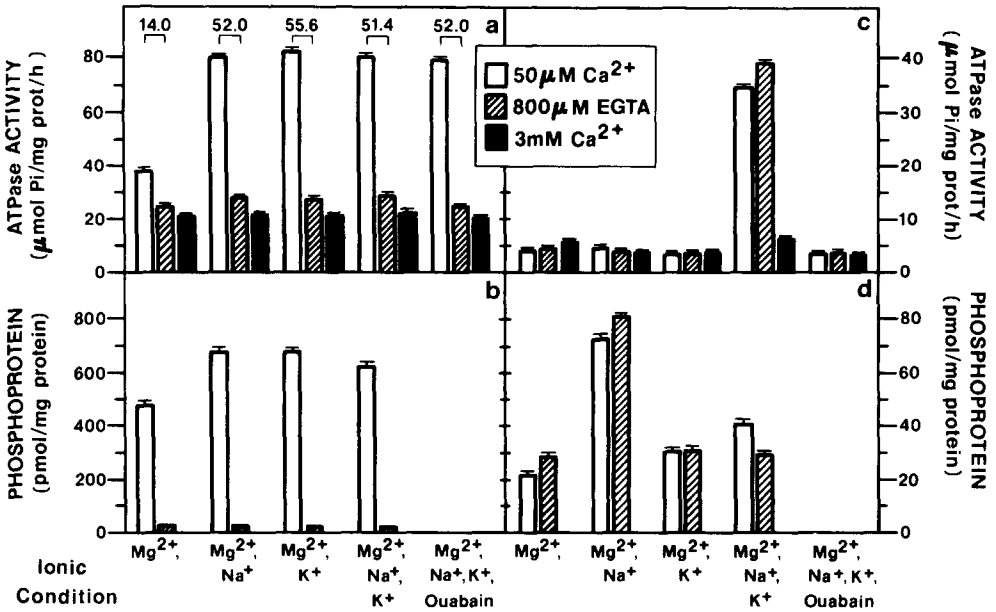

Fig. 1. ATP hydrolysis and ^{32}P incorporation by intact and SDS-extracted membrane vesicles. ATP hydrolysis was measured at 37°C and ^{32}P incorporation at 5°C. All media contained 50 mM histidine (pH 7.4), 3 mM $MgCl_2$ and, in addition, 50 μM added $CaCl_2$ (clear bars), 800 μM EGTA (hatched bars) or 3 mM $CaCl_2$ (solid bars). Monovalent cations (100 mM NaCl; 100 mM KCl; or 100 mM NaCl plus 10 mM KCl) and 10^{-3} M ouabain were also included as indicated on the abscissa. ATPase activity and ^{32}P incorporation were measured in the presence of 3 mM non-radioactive ATP and 100 μM [γ-^{32}P]ATP, respectively. The data of panels a and b were obtained with intact membrane vesicles, whereas the data of panels c and d were obtained with SDS-extracted membrane vesicles. Values are expressed as the mean ± S.D. from triplicate determinations. Calculated ($K^+ + Ca^{2+}$)-ATPase activities (the difference between the ATPase activities of the clear and hatched bars in panel (a) measured in the presence or absence of monovalent cations are indicated by the values above the brackets at the top of the bars in panel a. Phosphoprotein levels were not determined in the presence of ouabain in panels b and d.

increased 4- to 6-fold while at the same time all Ca^{2+}-dependent, monovalent cation-stimulated ATPase activity was inactivated [1,8]. After centrifugation to separate the membrane-bound proteins from those solubilized by SDS, the SDS-extracted membrane vesicle pellet retained the ouabain-sensitive ($Na^+ + K^+$)-ATPase activity, the specific activity of which was 9—14 times that exhibited by the intact membrane vesicles (Fig. 1, a and c). The combination of Na^+ plus K^+ was required to stimulate the ($Na^+ + K^+$)-ATPase activity of SDS-extracted vesicles and all stimulation by Na^+ plus K^+ was completely inhibited by the cardiac glycoside, ouabain (Fig. 1c). Ouabain-sensitive activity was maximal in the virtual absence of free Ca^{2+}; 50 μM added Ca^{2+} inhibited ouabain-sensitive activity slightly (14%), and 3 mM added Ca^{2+} inhibited ouabain-sensitive activity almost completely (88%) (Fig. 1c).

When SDS-extracted vesicles were incubated with [γ-^{32}P]ATP, the cation requirements for phosphoprotein formation were different from those required to phosphorylate the ($K^+ + Ca^{2+}$)-ATPase of intact vesicles. Maximal formation of phosphorylated intermediate occurred only in the presence of Na^+, and EGTA did not decrease the level of phosphorylated intermediate (Fig. 1d). K^+

TABLE I

$(Na^+ + K^+)$-ATPase ACTIVITIES AND PHOSPHOPROTEIN LEVELS IN INTACT AND SDS-EXTRACTED MEMBRANE VESICLES

ATPase activities were measured at 37°C in a medium containing 50 mM histidine (pH 7.4), 3 mM $MgCl_2$, 3 mM Tris · ATP, 100 mM NaCl and 10 mM KCl in the presence or absence of 10^{-3} M ouabain. The ouabain-inhibitable activity was the $(Na^+ + K^+)$-ATPase activity. Phosphoprotein was measured in a medium containing 50 mM histidine (pH 7.4), 3 mM $MgCl_2$, 800 μM EGTA, 100 μM [γ-^{32}P]ATP and either 100 mM NaCl or 100 mM KCl. The Na^+-stimulated incorporation was the phosphoprotein level. Turnover numbers were calculated by dividing the $(Na^+ + K^+)$-ATPase activities (expressed per min) by the phosphoprotein levels. The percent recovery of the SDS-extracted vesicles (particulate proteins) plus the proteins solubilized by SDS was always greater than 90% of the original intact membrane vesicle protein content. Results are the mean ± S.E. from three separate preparations each of intact and SDS-extracted membrane vesicles.

Membrane preparation	% Protein	ATPase activity (μmol P_i/mg protein per h)			Phosphoprotein (pmol/mg protein)			Apparent turnover number (min^{-1})
		Mg^{2+} + Na^+ + K^+	(Mg^{2+} + Na^+ + K^+) + ouabain	Δ	Mg^{2+} + Na^+	Mg^{2+} + K^+	Δ	
Intact membrane vesicles	100	45.8 ±4.0	41.3 ±4.1	4.5 ±0.8	25.1 ±5.7	15.1 ±5.2	10.0 ±1.4	7,490 ±320
SDS-extracted membrane vesicles	31 ±1.9	52.0 ±5.9	4.16 ±0.17	47.6 ±5.8	94.0 ±9.0	18.0 ±4.8	76.1 ±11.4	10,500 ±390

did not stimulate phosphorylation above that measured in the presence of Mg^{2+} alone, and the combination of Na^+ plus K^+ gave only a slight increment in the level of phosphoprotein above that which was measured in the presence of Mg^{2+} alone (Fig. 1d). Cationic activation of ATP-dependent phosphoprotein formation by SDS-extracted membrane vesicles was identical to that found in partially purified $(Na^+ + K^+)$-ATPase isolated from cardiac or other tissue [26,27]. The data of Fig. 1c and d therefore suggest that the monovalent cation-sensitive ATPase activity of SDS-extracted membrane vesicles was contributed by ouabain-sensitive $(Na^+ + K^+)$-ATPase, which is commonly regarded as an enzyme marker for cardiac sarcolemma [11,26].

Apparent turnover of cardiac $(Na^+ + K^+)$-ATPase and $(K^+ + Ca^{2+})$-ATPase activities. Table I lists the average ouabain-sensitive $(Na^+ + K^+)$-ATPase activities and the levels of phosphorylated enzyme intermediate (measured in the presence of EGTA) in intact and in SDS-extracted membrane vesicles from three preparations. After the vesicles were extracted with SDS, both the Na^+-sensitive increment of membrane phosphorylation and the ouabain-sensitive ATPase activity increased 8- to 11-fold. The apparent turnover numbers for the ouabain-sensitive enzyme active at 37°C, calculated from the Na^+-dependent levels of membrane phosphorylation assuming that these latter values reflect the content of $(Na^+ + K^+)$-ATPase molecules [26—28] were 7500 min^{-1} and 10 500 min^{-1} for the $(Na^+ + K^+)$-ATPase of intact and SDS-extracted membrane vesicles, respectively. Thus, the increased turnover after SDS extraction could only partially account for the substantial augmentation of $(Na^+ + K^+)$-

TABLE II

$(K^+ + Ca^{2+})$-ATPase ACTIVITIES AND PHOSPHOPROTEIN LEVELS IN INTACT MEMBRANE VESICLES

$(K^+ + Ca^{2+})$-ATPase activities were measured at 37°C in the presence or absence of 100 mM KCl in a medium containing 50 mM histidine (pH 7.4), 3 mM $MgCl_2$, 3 mM Tris · ATP and either 50 μM $CaCl_2$ or 0.8—1.0 mM EGTA. The difference between the activity measured in the presence of Ca^{2+} and the activity measured in the presence of EGTA was the $(K^+ + Ca^{2+})$-ATPase activity. Phosphoprotein levels were determined in the same medium which was used to measure the ATPase activities except that 20 μM [γ-^{32}P]ATP was substituted for 3 mM non-radioactive ATP. This concentration of radioactive ATP was sufficient to maximally label the phosphoprotein [15]. Ca^{2+}-dependent phosphoprotein was the difference between phosphoprotein measured in the presence of 50 μM $CaCl_2$ and phosphoprotein measured in the presence of 1.0 mM EGTA. When phosphoprotein was measured at 5°C the incubation time was 5 s and when phosphoprotein was measured at 22°C the incubation time was 1—3 s with no decrease in phosphoprotein levels noted over this time period. Turnover numbers were calculated by dividing the respective $(K^+ + Ca^{2+})$-ATPase activities (expressed per min) by the respective phosphoprotein levels. Paired Student's *t*-tests were used to compare the various phosphoprotein levels. Results are the mean ± S.E. from seven separate preparations.

	$(K^+ + Ca^{2+})$-ATPase activity (μmol P_i/mg protein per h) at 37°C	Ca^{2+}-dependent phosphoprotein (pmol/mg protein)		Apparent turnover number (min^{-1})	
		5°C	22°C	E-^{32}P at 5°C	E-^{32}P at 22°C
$-K^+$	13.7 ± 0.9	502 ± 32	569 ± 31	461 ± 29	402 ± 16
$+K^+$	66.6 ± 3.7	763 ± 52	549 ± 46	1480 ± 82	2050 ± 71

ATPase activity by SDS, suggesting that SDS acts by rendering a sub-population of intact vesicles (probably sarcolemmal) permeable to substrate and cations, with resultant activation of latent $(Na^+ + K^+)$-ATPase activity [1,8—11,13].

After intact membrane vesicles were extracted with SDS, approximately 30% of the original protein content remained particulate, as judged from the results of centrifugation (Table I). The solubilized proteins contained no measurable ATPase activity [8].

Apparent turnover numbers were also calculated for the $(K^+ + Ca^{2+})$-ATPase activities of intact membrane vesicles (Table II). At 37°C $(K^+ + Ca^{2+})$-ATPase activity was increased approximately 5-fold by addition of K^+, in good agreement with the 4-fold stimulation shown in Fig. 1a. At 5°C, K^+ increased the steady-state level of phosphorylated enzyme approximately 50% ($P < 0.001$), whereas at 22°C, K^+ exerted no significant effect on the level of phosphoprotein. Irrespective of temperature, K^+ increased the calculated apparent turnover number of the enzyme 3- to 5-fold (Table II) (see ref. 15 for a more detailed analysis of the effects of temperature on enzyme phosphorylation and turnover). The apparent turnover of the $(K^+ + Ca^{2+})$-ATPase (even with K^+ present) remained only 20—25% of the apparent turnover of the $(Na^+ + K^+)$-ATPase (Tables I and III).

Protein composition of intact and SDS-extracted cardiac membrane vesicles. That two distinct ATPase activities were detected in cardiac membrane vesicles suggested that this preparation was more heterogeneous than similar preparations isolated from skeletal muscle [23,29]. This was supported by results obtained with polyacrylamide gel electrophoresis (Figs. 2 and 3). When the gels

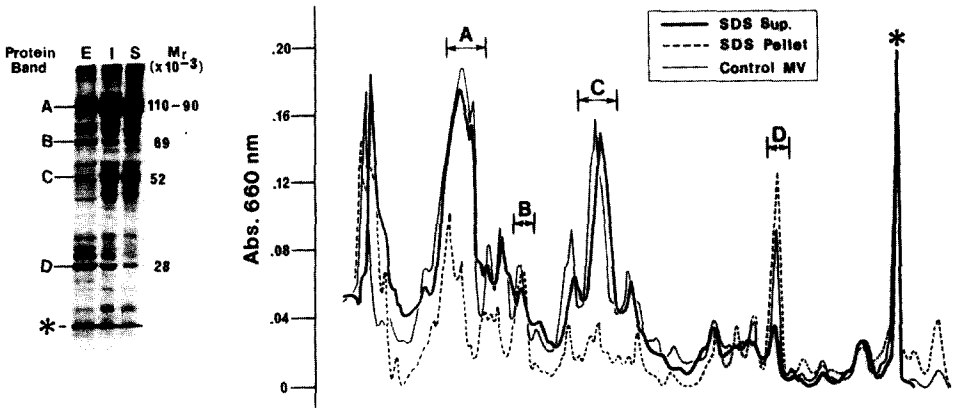

Fig. 2. Polyacrylamide gel electrophoresis of intact (I) and SDS-extracted membrane vesicles (E) and of the proteins solubilized (S) by SDS. For clarity, in the inset, I is termed control MV (membrane vesicles), E is termed SDS pellet and S is termed SDS supernatant (Sup.). SDS extraction was performed as described in Experimental procedure. The proteins solubilized by SDS were concentrated for electrophoresis by precipitation with 5% trichloroacetic acid. Gel electrophoresis was performed according to Laemmli [24]. The concentration of acrylamide in the separating gel was 8.5%. The left-hand portion of the figure depicts the gel slab after it was stained with Coomassie Blue and the right hand portion depicts densitometry of the three fractions after each was separately cut from the whole gel and scanned, 25 μg protein from each fraction was applied to each of the gel wells.

containing the proteins of intact membrane vesicles were stained with Coomassie Blue, at least 40 distinct protein bands were routinely detected (Fig. 2). A large diffuse band (band A) of M_r = 90 000—110 000 accounted for approximately 30—35% of the total staining of the gel as determined by

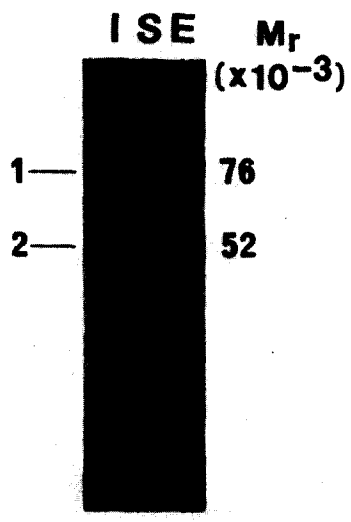

Fig. 3. Glycoproteins of intact (I) and SDS-extracted (E) membrane vesicles, and of the proteins solubilized by SDS (S). Half of the slab gel of Fig. 3 was periodic acid-Schiff-stained as described in Experimental procedure. 80 μg of protein from each fraction was aplied to each of the gel wells.

densitometry (Fig. 2). Band C of $M_r \approx 52\,000$ was the second most prominent staining region and, together, bands A and C accounted for approximately 50% of the total staining. Both bands A and C were frequently resolved into doublets, especially when electrophoresis was performed at 5°C according to Laemmli [24], which gave the greatest resolution of proteins.

The protein profiles were quite different for the SDS-extracted membrane vesicles (Fig. 2). Band C was noticeably absent from the extracted membranes and bands B and D were relatively enriched. Furthermore, region A was much less diffuse and was clearly resolved into at least two bands, which together accounted for 17—20% of the total staining. Band C was preferentially extracted by SDS as were some high molecular weight proteins from region A of the gel.

Gels of intact vesicles stained for carbohydrate with periodic acid-Schiff stain reagent revealed two major glycoproteins, bands 1 and 2, of apparent molecular weights of 76 000 and 52 000, respectively (Fig. 3). Two other more faintly staining proteins of apparent molecular weights greater than 90 000 were also routinely present. Band 1 was retained preferentially in the SDS-extracted membranes, whereas band 2 was released preferentially to the soluble fraction. The large area of Schiff-staining material which traveled with the buffer front in both intact membrane vesicles and the SDS-extracted sediment was most probably of glycolipid origin [25]. This material, as well as band 1, was relatively enriched in membranes after they were extracted with SDS.

TABLE III

PHOSPHOPROTEIN LEVELS AND ATPase ACTIVITIES OF INTACT AND SDS-EXTRACTED MEMBRANE VESICLES SUBJECTED TO POLYACRYLAMIDE GEL ELECTROPHORESIS AND AUTORADIOGRAPHY

Intact and SDS-extracted vesicles were phosphorylated for 15 s at 5°C in a medium containing 50 mM histidine (pH 7.4), 3 mM $MgCl_2$, and 100 μM [γ-^{32}P]ATP. $+Ca^{2+}$ samples contained in addition 50 μM added Ca^{2+}, and $-Ca^{2+}$ samples contained in addition 800 μM EGTA. NaCl and KCl (100 mM) were also included in the assay media as indicated. ($K^+ + Ca^{2+}$)-ATPase and ouabain-sensitive ($Na^+ + K^+$)-ATPase activities of intact and SDS-extracted vesicles were determined as described in Tables I and II. The samples in various reaction tubes were prepared for polyacrylamide gel electrophoresis as described in Experimental procedure. The numbers in parentheses refer to the sample numbers as they were applied to the polyacrylamide slab gels. To control for possible non-specific incorporation an intact vesicle sample and an SDS-extracted vesicle sample were exposed to trichloroacetic acid before addition of the 100 μM [γ-^{32}P]ATP and then carried through the remainder of the procedure. These samples correspond to samples 13 and 14, respectively, in Fig. 4.

	Intact membrane vesicles				SDS-extracted membrane vesicles		
	Mg^{2+}	$+Na^+$	$+K^+$		Mg^{2+}	$+Na^+$	$+K^+$
	(pmol E-^{32}P/mg protein)				(pmol E-^{32}P/mg protein)		
$+Ca^{2+}$	554 (1)	839 (2)	862 (3)	$+Ca^{2+}$	21.9 (7)	112 (8)	22.1 (9)
$-Ca^{2+}$	17.2 (4)	32.3 (5)	19.5 (6)	$-Ca^{2+}$	20.0 (10)	112 (11)	16.2 (12)

($K^+ + Ca^{2+}$)-ATPase (μmol P_i/mg protein per h)		($K^+ + Ca^{2+}$)-ATPase (μmol P_i/mg protein per h)	
−KCl	20.2	−KCl	0.0
+KCl	83.3	+KCl	0.0
($Na^+ + K^+$)-ATPase	6.0	($Na^+ + K^+$)-ATPase	56.0

Identification of the phosphorylated intermediates of the ($K^+ + Ca^{2+}$)-ATPase and the ($Na^+ + K^+$)-ATPase by autoradiography. Autoradiography of the phosphoprotein intermediates of the ($K^+ + Ca^{2+}$)-ATPase and the ($Na^+ + K^+$)-ATPase activities confirmed their separate patterns of ionic activation. Both intact and SDS-extracted vesicles were phosphorylated with [γ-^{32}P]ATP in order to optimally label their ($Na^+ + K^+$)-ATPase and ($K^+ + Ca^{2+}$)-ATPase activities

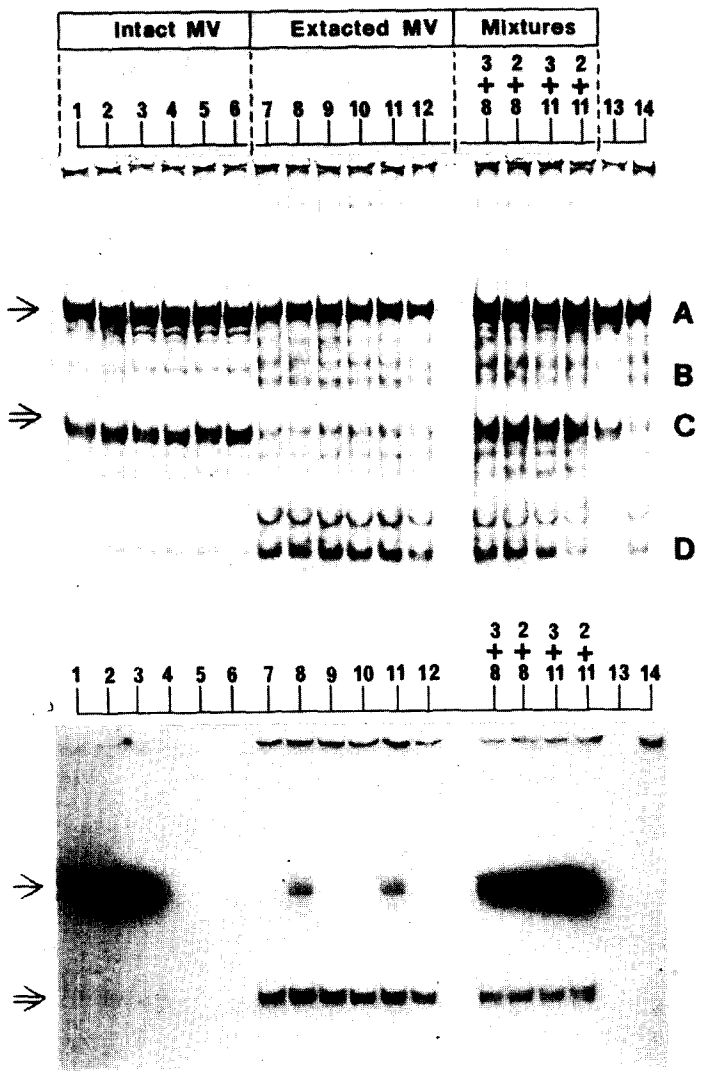

Fig. 4. Autoradiography of intact and SDS-extracted membrane vesicle proteins after electrophoresis according to Fairbanks et al. [25]. The film was developed after an exposure time of 5 days. Electrophoresis of the samples of Table III was performed in 5.6% gels. The numbers in the parentheses in Table III identify the samples as they were applied to the gel. Samples 13 and 14 contained, respectively, intact and SDS-extracted vesicle protein which had been exposed to trichloroacetic acid prior to the addition of [γ-^{32}P]ATP. The region of the gel in which cation-sensitive radioactivity was detected is noted by a single arrow. The region of the gel which contained cation intensitive incorporation is noted by the double arrow.

(Table III). In intact membrane vesicles most of the phosphate incorporation was Ca^{2+}-dependent, and either Na^+ or K^+ stimulated the level of phosphorylation obtained (Table III). On the other hand, in SDS-extracted membrane vesicles, phosphorylation was specifically Na^+-dependent, with Ca^{2+} having no effect on the level of phosphoprotein obtained. The only monovalent cation-stimulated ATPase activity detectable in SDS-extracted vesicles was ouabain-sensitive ($Na^+ + K^+$)-ATPase activity.

Knauf et al. [30] separated the phosphorylated intermediates of the ($Na^+ + K^+$)-ATPase and Ca^{2+}-ATPase of red blood cells using the gel electrophoresis system of Fairbanks et al. [25]. The membrane vesicle samples of Table III were subjected to electrophoresis with these conditions in gels of 5.6% acrylamide and autoradiograms were prepared (Fig. 4). The pattern of staining with Coomassie Blue was similar to that obtained with electrophoresis according to Laemmli [24]; however, there was resolution of fewer bands with the system of Fairbanks et al. [25]. The pattern of phosphorylation of proteins of M_r = 100 000 was consistent with the distinct patterns of ionic activation of ATPase activities (single arrow). Intact vesicles produced phosphorylated bands only in the presence of Ca^{2+}, and SDS-extracted vesicles produced phosphorylated bands only in the presence of Na^+. Combined samples did not exhibit discrete separation of the ($Na^+ + K^+$)-ATPase and ($K^+ + Ca^{2+}$)-ATPase activities, however. A protein band of apparent molecular weight of 54 000 was also highly labeled in the extracted membrane vesicles (double arrow). However, phosphorylation of this protein appeared to be independent of ionic conditions. The phosphorylated intermediate of the ($K^+ + Ca^{2+}$)-ATPase was also not resolved from that of the ($Na^+ + K^+$)-ATPase by the electrophoresis method of Laemmli [24] (data not shown).

Sucrose gradient centrifugation of cardiac membrane vesicles. Intact membrane vesicles were subfractionated by sucrose gradient centrifugation

TABLE IV

ATPase ACTIVITIES OF MEMBRANE VESICLE FRACTIONS ISOLATED BY SUCROSE GRADIENT CENTRIFUGATION

Intact crude vesicles were subjected to sucrose gradient centrifugation as described in Experimental procedure. ($Na^+ + K^+$)-ATPase activity was measured in the various fractions before and after they were exposed to 0.30 mg per ml SDS in 30 mM imidazole (pH 7.1) for 30 min at room temperature. This activity was measured as ouabain-inhibitable activity as described in Table I; ($K^+ + Ca^{2+}$)-ATPase activity was measured in the presence of 100 mM KCl as described in Table II. The activities of SDS-pretreated vesicles are expressed per mg original intact vesicle protein, i.e., the proteins solubilized by SDS were not removed by centrifugation.

Fraction	Percent of recovered protein	ATPase activity (μmol P_i/mg protein per h)			Activity ratio	
		($Na^+ + K^+$)-ATPase		($K^+ + Ca^{2+}$)-ATPase	(*b*/*a*)	(*b*/*c*)
		−SDS (*a*)	+SDS (*b*)	(*c*)		
A	5	6.4	36.8	2.7	5.8	14
B	18	5.4	33.2	30.9	6.1	1.1
C	35	6.5	19.5	46.8	3.0	0.42
D	21	3.0	8.4	24.3	2.8	0.35
E	21	4.6	13.2	9.7	2.9	1.4

(Table IV). Ouabain-sensitive ($Na^+ + K^+$)-ATPase activity of separated fractions was highest in fraction A, but only when total activity (patent plus latent) was measured. Before the vesicles were exposed to SDS, the ($Na^+ + K^+$)-ATPase activity in all the fractions was low, and the activity measured in fraction A was not appreciably different from that exhibited by the other fractions. The fold-activation of ($Na^+ + K^+$)-ATPase activity by SDS (b/a in Table IV) was highest in fractions A and B, which suggested that most of the vesicles in this region of the gradient had been intact before the SDS treatment. ($K^+ + Ca^{2+}$)-ATPase activity did not distribute on the sucrose gradient like ($Na^+ + K^+$)-ATPase. The former was lowest in fraction A and highest in fraction C. When activity ratios were calculated from the various cation-sensitive ATPase activities, it was apparent that the ($Na^+ + K^+$)-ATPase and ($K^+ + Ca^{2+}$)-ATPase activities were localized in different membrane populations (Table IV). For example, in fraction A the ($Na^+ + K^+$)-ATPase activity was 14 times greater than the ($K^+ + Ca^{2+}$)-ATPase activity, whereas in fractions C and D, the former activity was only 35—40% of the ($K^+ + Ca^{2+}$)-ATPase activity. The disparate ratios between ($Na^+ + K^+$)-ATPase activity and ($K^+ + Ca^{2+}$)-ATPase activity thus suggested that different populations of membranes accounted for the two activities.

Discussion

The principal conclusion of this study is that the two major monovalent cation-regulated ATPases of a cardiac membrane vesicle preparation widely assumed to contain mostly sarcoplasmic reticulum are derived from two different sub-populations of membranes in the preparation. The patent ATPase activity measured directly in intact vesicles was primarily ($K^+ + Ca^{2+}$)-ATPase activity, which is an enzyme marker for sarcoplasmic reticulum [23,29]. Latent ATPase activity, which was measurable after the vesicles have been exposed to SDS, was expressed as ($Na^+ + K^+$)-ATPase activity, an enzyme marker for sarcolemma [11,26]. That separate populations of membrane vesicles were present in the crude vesicle preparation was supported by the results obtained with density gradient centrifugation, in which the membrane source of the ($Na^+ + K^+$)-ATPase activity was partially separated from the membrane source of the ($K^+ + Ca^{2+}$)-ATPase activity (Table IV). The data therefore suggest that these two membrane-localized activities may be used as valid enzymic markers for cardiac sarcolemma and sarcoplasmic reticulum, respectively, provided that care is taken to unmask the latent ($Na^+ + K^+$)-ATPase activity of sarcolemma when activities are assessed [8—11]. (It should be pointed out that activation of latent ($K^+ + Ca^{2+}$)-ATPase activity is not necessary because the quantity of latent ($K^+ + Ca^{2+}$)-ATPase activity is small [1,9]. Moreover, activation of ($K^+ + Ca^{2+}$)-ATPase by the pore-forming ionophore alamethicin is similar for all fractions of the gradient, data not shown). In other experiments, we have utilized these two ATPase activities to document a more complete separation of sarcolemma from sarcoplasmic reticulum, and we have shown that virtually all of the adenylate cyclase activity present in the crude vesicle preparation resides exclusively in sarcolemma [44].

The different enzymic entities, ($Na^+ + K^+$)-ATPase, ($K^+ + Ca^{2+}$)-ATPase, and the more recently characterized ($H^+ + K^+$)-ATPase of gastric mucosa are all

members of a class of integral transmembrane enzymes which can translocate cations [10,15,26,29,31,32]. They all hydrolyse ATP in the presence of Mg^{2+}, and form an acyl-phosphate intermediate of $M_r \approx 100\,000$. The critical or selective cation for activity is the one which is required for phosphoprotein formation: Na^+, Ca^{2+} and H^+, respectively, for $(Na^+ + K^+)$-ATPase (Fig. 4), $(K^+ + Ca^{2+})$-ATPase (Fig. 4) and $(H^+ + K^+)$-ATPase. In all cases K^+ stimulates phosphoprotein hydrolysis and, thus, turnover of the three enzymes with weak selectivity, Rb^+ or other monovalent cations being nearly as effective as K^+. In the case of $(Na^+ + K^+)$-ATPase (Fig. 1c) and $(H^+ + K^+)$-ATPase, Na^+, even at high concentrations, is not as effective as K^+. A specific enzyme inhibitor, ouabain (or other cardiac glycosides), has been identified for only one of the three activities, the $(Na^+ + K^+)$-ATPase (Fig. 1, a and c).

Use of the specific inhibitor, ouabain, the Ca^{2+} chelator, EGTA, and the detergent, SDS, facilitated distinction of the $(Na^+ + K^+)$-ATPase and $(K^+ + Ca^{2+})$-ATPase activities of cardiac membrane vesicles. The majority of the activity of intact vesicles was the ouabain-insensitive $(K^+ + Ca^{2+})$-ATPase of sarcoplasmic reticulum which implies that most of the vesicles of sarcoplasmic reticulum were oriented such that their ATP hydrolytic sites were on the outer surface of the vesicles, the same surface which contains the high affinity Ca^{2+} site [23]. These conclusions are supported by the recent observations of Hidalgo and Ikemoto [33] who, with impermeant labelling probes, estimated that approximately 90% of sarcoplasmic reticulum vesicles were of this right-side-out orientation. SDS selectively inactivated $(K^+ + Ca^{2+})$-ATPase activity, and at the same time greatly increased ouabain-sensitive $(Na^+ + K^+)$-ATPase activity (Fig. 1, a and c). Activation of $(Na^+ + K^+)$-ATPase can be attributed to disruption of intact sarcolemmal vesicles by SDS, which allowed formerly latent enzymic sites to become accessible to substrate and cations [1,8—11]. This conclusion was further supported in the present study by the parallel increase in the phosphoprotein intermediate of $(Na^+ + K^+)$-ATPase, which accompanied the increase in ouabain-sensitive ATP hydrolysis (Table I). Thus SDS apparently did not increase $(Na^+ + K^+)$-ATPase activity by a mechanism other than by obviating latency, e.g. by increasing the turnover of already accessible enzymic sites in intact vesicles. Although use of the detergent SDS greatly facilitated resolution of the two ATPases by selectively inactivating one of them, the destruction of $(K^+ + Ca^{2+})$-ATPase is not necessary for $(Na^+ + K^+)$-ATPase activation. With Lubrol PX or the channel-forming ionophore alamethicin, it was similarly possible to activate $(Na^+ + K^+)$-ATPase in the crude preparation and at the same time preserve all $(K^+ + Ca^{2+})$-ATPase activity [11]. This observation provides strong confirmatory evidence that SDS did not increase $(Na^+ + K^+)$-ATPase activity through the unlikely mechanism of interconversion of the activation properties of $(K^+ + Ca^{2+})$-ATPase, though others [34—36] have suggested that detergents can sometimes alter the cation activation properties of both $(Na^+ + K^+)$-ATPase and $(K^+ + Ca^{2+})$-ATPase. Solubilization by SDS of a large amount of the $M_r = 100\,000$ protein (Fig. 2) which contained the phosphoprotein intermediate of $(K^+ + Ca^{2+})$-ATPase (Fig. 2) suggests that the low SDS concentration employed may have selectively solubilized the $(K^+ + Ca^{2+})$-ATPase, and in this process rendered it inactive.

The major band of $(K^+ + Ca^{2+})$-ATPase and $(Na^+ + K^+)$-ATPase activities of

the crude cardiac vesicles was not resolvable by polyacrylamide gel electrophoresis and subsequent autoradiography. This finding was not completely unexpected because it is well documented that the two enzymes have similar molecular weights (100 000) [37]. In contrast, Knauf et al. [30] separated the phosphoprotein intermediates of the $(Na^+ + K^+)$-ATPase and Ca^{2+}-ATPase activities of red blood cells with electrophoresis; however, in red blood cells the difference between the apparent molecular weights of the two enzymes is greater (50 000). In analogy to the $(K^+ + Ca^{2+})$-ATPase of sarcoplasmic reticulum, red blood cells also have a Ca^{2+}-dependent ATPase activity which is stimulated by Na^+ or K^+ [38—42]. Whether this activity was analogous to that examined by Knauf et al. [30] is not presently known.

A band of cation-insensitive radioactive phosphate incorporation was also detected on the autoradiograms which ran with an apparent molecular weight of 54 000. Dowd and Schwartz [43] have noted a similar activity in crude $(Na^+ + K^+)$-ATPase preparations isolated from bovine heart and have suggested that the phosphoprotein formed may represent autophosphorylated endogenous protein kinase activity. If this is so, the presence of this phosphoprotein in high activity in the SDS-extracted vesicles suggests that at least one type of protein kinase activity in myocardium may be an intrinsic membrane protein of sarcolemma.

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