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ISOLATION OF SEALED VESICLES HIGHLY ENRICHED WITH SARCOLEMMA MARKERS FROM CANINE VENTRICLE

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Summary

A highly enriched sarcolemma preparation was isolated by a combination of homogenizations and differential centrifugations of a homogenate of canine ventricular tissue followed by centrifugation of a membrane fraction layered over 24% (w/v) sucrose. The membrane fragments in the preparation were found to reside in a vesicular configuration by electron microscopy. Adenylate cyclase activity, specific ouabain binding sites, ouabain-sensitive potassium phosphatase activity and high-affinity (—)dihydroalprenolol binding sites were enriched from 27- to >40-fold compared to the homogenate. 5′-Nucleotidase and antimycin A-insensitive NADH-cytochrome *c* reductase activities were enriched 10.1- and 3.0—3.9-fold, respectively, compared to the homogenate. Conversely, Ca^{2+}-ATPase and succinic dehydrogenase activities were somewhat less than those expressed by the homogenate. The vesicles, or at least a fraction thereof, in the preparation were osmotically active as monitored by changes in 90° light scatter upon increases in the osmolarity of the extravesicular medium. ($Na^+ + K^+$)-ATPase activity in the preparation was 23.6 and 99.9 μmol/mg per h before and after 15 consecutive freeze-thaw cycles, respectively. This suggests that the preparation contains a large fraction of intact right-side-out vesicles but that about 24% of the vesicles in the preparation may be freely permeable. Conversely, the number of specific ouabain binding sites was increased about 1.4-fold by the freeze-thaw cycles which is consistent with the presence of

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

intact inside-out vesicles in the preparation. The preparation manifested the ability to take up calcium through a sodium-dependent process which is consistent with the presence of a sodium-calcium exchange system. Total process time for 100 g ventricular tissue was about 5.5 h and the yield of the preparation was 10.6 mg protein per 100 g starting tissue. Recovery of putative sarcolemma markers from the initial homogenate, however, was only about 4%. It is concluded that the isolation procedure yields a highly enriched sarcolemma preparation that is suitable for further sarcolemma purification and for the study of ion movements across the sarcolemma that are believed to participate in excitation-contraction coupling in heart.

Introduction

Despite a large number of attempts [1—11], the isolation of highly enriched sarcolemma fractions from heart has not been achieved until recently. The major difficulty seems to be that the presence of myofibrils in cardiac homogenates severely complicates subcellular fractionation. In 1976, Besch et al. [12] reported that a cardiac membrane preparation, isolated by using the procedure of Harigaya and Schwartz [13], contained significant amounts of sarcolemma markers. This finding generated strategies in their laboratory [14] and in ours [15] for the development of isolation schemes to obtain sarcolemma preparations much more highly enriched than those previously reported. The procedure from our laboratory [15] allowed a 19—27-fold purification of putative sarcolemma markers with a decrease in Ca^{2+}-ATPase and succinic dehydrogenase activities relative to those in the homogenate.

There are, however, several problems with this procedure [15]. First, one would expect at least a 30—40-fold purification of plasma membrane markers for highly purified plasma membrane preparations [16]. Second, a considerable fraction of the vesicles in the preparation appeared to be freely permeable. Third, the yield was too low (i.e., about 2 mg protein/100 g ventricular tissue) to allow either substantial studies on sarcolemma function or further attempts at purification. An examination of sarcolemma markers in the various fractions showed that most of the markers were discarded at an early step in the fractionation scheme (i.e., Pellet-2, Fig. 1, Ref. 15). Attempts to separate the markers from this fraction generated a new scheme by which to obtain a sarcolemma preparation from heart. The new preparation is characterized by greater enrichment of sarcolemma markers, a larger fraction of sealed vesicles and about a 5-fold increase in yield.

Sarcolemma enrichment in this preparation seems to be quite similar to that reported by Jones et al. [14] but the procedure does not require the use of calcium (with oxalate) to obtain substantial sarcolemma enrichment at the expense of sarcoplasmic reticulum and the total centrifugation time is considerably less than that required by the procedure of Jones et al. [14]. These differences may provide distinct advantages for studies on the role of the cardiac sarcolemma in excitation-contraction coupling as it pertains to Ca^{2+} and other ion movements across this membrane.

Experimental Procedures

Materials. Adenosine 5′-monophosphoric acid, adenosine 5′-triphosphate (disodium salt) for adenylate cyclase assays, bovine serum albumin, theophylline, Trizma base, glycine, NaN_3, antimycin A, ouabain, pyruvate kinase, lactate dehydrogenase, phospho*enol*pyruvate (monocyclohexylammonium salt), cytochrome *c*, reduced form of nicotinamide adenine dinucleotide (NADH), EGTA, *p*-nitrophenylphosphate (disodium salt), succinic acid, creatine phosphate, creatine kinase, adenosine-3′,5′-cyclic monophosphoric acid (cyclic AMP) and Lubrol PX were obtained from Sigma Chemical Co., St. Louis, MO. Sodium dodecyl sulfate was obtained from Accurate Chemical and Scientific Corp., Hicksville, NY. Neutral Al_2O_3 for column chromatography was acquired from ICN Nutritional Biochemicals, Cleveland, OH. Resin AG 50W-X4 was obtained from Bio-Rad Laboratories, Richmond, CA. Dithiothreitol was obtained from Calbiochem-Behring Corp., La Jolla, CA. Adenosine 5′-triphosphate (disodium salt) for ATPase assays was acquired from Boehringer Mannheim, Indianapolis, IN, and (±)propranolol was obtained from Ayerst Laboratories, New York, NY. Epon 812 and glutaraldehyde were acquired from Electron Microscopy Sciences, Fort Washington, PA. OsO_4, uranyl acetate and lead citrate were obtained from Polysciences, Warrington, PA. Sodium cacodylate was acquired from Fisher Scientific Co., Atlanta, GA. All radioactively labeled ligands were acquired from New England Nuclear, Boston, MA. All other chemicals were of reagent grade.

Isolation of the sarcolemma preparation. Male and female dogs weighing 10—30 kg were anesthetized with pentobarbital, 30 mg/kg intravenously. Heparin (1.5 ml of a 1 : 1000 solution) was given with the pentobarbital. Upon opening the chest, the heart was rapidly removed and immersed in ice-cold 0.9% saline. The left ventricular free wall, the interventricular septum and the right ventricular free wall were stripped of epicardial and endocardial tissue and fat, weighed and minced with scissors. All of the following procedures were carried out at 2—4°C and centrifugal forces were those at the middle of the tube. The mince was suspended in 4—5 vols. of a medium containing 10 mM $NaHCO_3$ and 5 mM NaN_3, pH 7.0 (medium A), and homogenized with three low-speed (160 W) 15-s pulses (separated by 15-s intervals) with an Ultra Turrax T-45 Tissumizer (Tekmar Instruments, Cincinnati, OH). The homogenate was centrifuged at 8700 × *g* for 20 min to yield supernatant-1, which was discarded, and pellet-1 (Fig. 1). The latter was suspended in the original volume of medium A, homogenized in a large glass homogenizing vessel with one pass of a motor-driven Teflon pestle and centrifuged at 8700 × *g* for 20 min. This yielded supernatant-2, which was discarded, and pellet-2 which was dispersed in 6 vols. of 10 mM Tris-HCl, pH 7.4 (medium B) and subjected to four passes of a moter-driven Teflon pestle as above. Centrifugation at 8700 × *g* for 20 min yielded pellet-3, which was discarded, and supernatant-3. The latter was centrifuged at 35 000 × *g* for 20 min to obtain supernatant-4, which was discarded, and pellet-4. This pellet was resuspended in a small glass homogenizer by five passes of a hand-driven Teflon pestle in 20 ml of medium B. The suspension was then layered over 15 ml of a 24% (w/v) sucrose solution (verified by refractometry; ABBE-31, Bausch and Lomb, Rochester, NY)

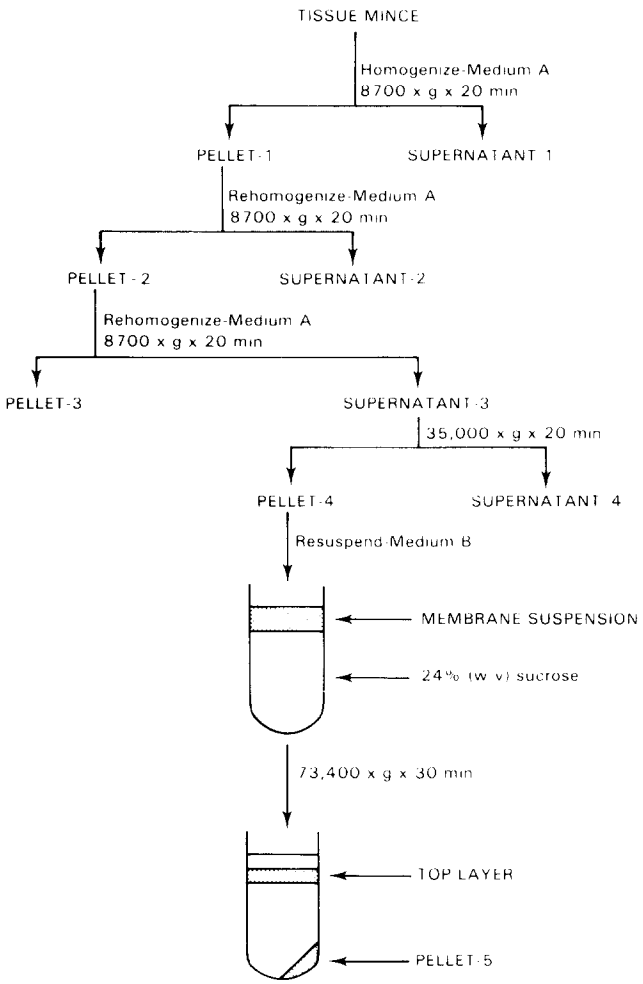

Fig. 1. Flow scheme for isolation of a sarcolemma-enriched fraction from canine myocardial ventricular tissue. Details are presented in Experimental Procedures. Medium A: 10 mM $NaHCO_3$, 5 mM NaN_3, pH 7.0. Medium B: 10 mM Tris-HCl, pH 7.4. The 24% (w/v) sucrose solution was made up in 10 mM Tris-HCl, pH 7.4. The top layer was diluted with medium B, centrifuged at 73 400 × *g* · 20 min and then resuspended in small volumes of medium B for storage (4—5° C) until subsequent use.

containing 10 mM Tris-HCl, pH 7.4, in a Type 30 Rotor tube (Beckman Instruments, Inc., Fullerton, CA). Centrifugation at 73 400 × *g* for 30 min yielded a top layer at the buffer/sucrose interface and pellet-5 which was discarded. The top layer was harvested with a Pasteur pipet, diluted to approx. 50 ml with medium B and centrifuged at 73 400 × *g* for 20 min. The pellet was resuspended in medium B to a final protein concentration of 1—2 mg/ml as determined by using the method of Lowry et al. [17]. The preparation was stored at 4—5°C until use. Total centrifugation time (at the desired force) was 130 min and total process time for 100 g of tissue was about 5.5 h.

Ouabain and (−)dihydroalprenolol binding. All assays were carried out in duplicate or triplicate and were performed within 24 h following completion

of the isolation procedure. Total specific [^{3}H]ouabain binding was measured according to the method of Inagaki et al. [18] in the presence of 5 mM $MgCl_2$, 5 mM Tris-phosphate and 10 mM Tris-HCl, pH 7.4. Assays for the presence of β-adrenergic receptors were carried out in the presence of 0.5—300 nM (−)1-[*propyl*-1,2,3-^{3}H]dihydroalprenolol in an incubation medium containing 10 mM $MgCl_2$ and 50 mM Tris-HCl, pH 7.5. After 20 min at 37°C, the reactions were terminated by addition of 2 ml of ice-cold buffer followed by rapid vacuum filtration through Whatman GF/B glass-fiber filters. The filters were rapidly washed twice by vacuum filtration of 5 ml of ice-cold incubation buffer. After drying, the filters were placed directly into a toluene-based cocktail containing 200 ml Biosolv (Beckman) per l and counted in a liquid scintillation spectrometer. In each experiment, nonspecific binding was determined by measuring radioactivity retained on filters when incubations were performed in the presence of 200 μM (±)propranolol. After correction for nonspecific binding, Scatchard plots were consistent with the presence of two binding components. Dissociation constants and maximal binding sites present for each component were determined by a nonlinear, least-squares curve-fitting procedure.

Enzyme activities. All enzyme reactions were linear within the time periods of the assays and protein concentrations employed. Ca^{2+}-ATPase activities were determined by measuring the appearance of P_i from ATP according to Jones et al. [19] using the colorimetric procedure of Martin and Doty [20]. Specifically, net Ca^{2+}-ATPase activity was measured by subtracting the activity in the basal buffer supplemented with 0.2 mM Tris-EGTA from that in the basal buffer which did not contain EGTA and to which 0.1 mM $CaCl_2$ had been added. K^+-stimulated *p*-nitrophenylphosphatase was measured as described by Skou [21] and the activity reported is that fraction which was ouabain sensitive. Ouabain-sensitive (Na^+ + K^+)-ATPase activity was determined by using the linked enzyme assay previously described by Schwartz et al. [22]. 5′-Nucleotidase activity was assayed according to the method of Heppel and Hilmoe [23], succinic dehydrogenase activity was determined by using the method of Green et al. [24] and antimycin A-insensitive NADH-cytochrome *c* reductase activity was assayed by using the method of Tolbert [25]. Adenylate cyclase activities were measured by determining the production of cyclic AMP from [α-^{32}P]ATP. The assay system contained 0.5 mM Na_2ATP with radioactively labeled ATP, 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM cyclic AMP, 5 mM creatine phosphate, 50 units/ml creatine kinase, 10 mM theophylline and 3 mg/ml bovine serum albumin. The reaction was initiated by addition of 15—20 μg protein of the homogenate or of the preparation derived from the top layer. After 10 min at 30°C, the reactions were terminated by addition of 0.1 ml of a stopping solution containing 2% SDS, 40 mM Na_2ATP and 1.4 mM cyclic AMP. Cyclic[^{3}H]-AMP was added to each sample to monitor cyclic AMP recovery. Cyclic AMP was then isolated and determined by using the method of Salomon et al. [26,27].

Sodium-dependent calcium uptake. Sodium-dependent calcium uptake by vesicles from the top layer was measured by the use of a procedure similar to that of Reeves and Sutko [28]. Aliquots of the sarcolemma-enriched prepa-

ration were exposed overnight at 5°C to a medium containing 10 mM Tris-HCl, pH 7.4, and 160 mM NaCl, LiCl or KCl. Calcium uptake was initiated by adding 0.01 ml of the suspension containing the loaded vesicles to 0.3 ml of a medium containing 160 mM LiCl, 10 mM Tris-HCl, pH 7.4, and 40 μM $CaCl_2$ with ^{45}Ca at 37°C. After 5 min, the reactions were terminated by addition of 5 ml of an ice-cold medium containing 200 mM KCl and 5 mM KH_2PO_4 buffered to pH 7.4. A 5 ml aliquot of this suspension was then filtered (Millipore, 0.45 μm); the filter was washed twice with the stopping solution and then assayed for ^{45}Ca content.

Morphology. Morphology of membrane fragments from the top layer was determined by electron microscopy. Aliquots of the preparation were pelleted by centrifugation at $160\,000 \times g \cdot 20$ min. The membranes were fixed for 60 min in 2% glutaraldehyde and 0.1 M sodium cacodylate buffer, pH 7.2, post-fixed in 2% OsO_4 and 0.1 M cacodylate buffer for 60 min, dehydrated with graded ethanol and embedded in Epon 812. Ultra-thin sections were stained with uranyl acetate followed by lead citrate. The sections were examined with a Hitachi HS-8 electron microscope (Hitachi, Ltd., Tokyo).

Osmotic responsiveness. The ability of vesicles from the top layer to respond to increases in the osmolarity of the extravesicular medium was monitored by 90° light scatter at 500 nm with an Aminco Bowman ratio spectrophotofluorometer (American Instruments Co., Silver Spring, MD) equipped with a xenon source and attached to a strip-chart recorder. Temperature was maintained at 25°C. Aliquots of the sarcolemma-enriched preparation were suspended in 1 ml of 10 mM Tris-HCl, pH 7.4. After obtaining a stable baseline value for 90° transmittance, solute (0.5 ml) in 10 mM Tris-HCl, pH 7.4, was added to a final concentration of 200 mM and the solution was mixed with a Pasteur pipet. The solution was continually stirred with a Teflon-coated magnetic stirring bar before and after mixing with the pipet.

Results and Discussion

The membrane fragments in the top layer (Fig. 1) resided in a vesicular configuration (Fig. 2) with diameters ranging from about 0.05 to about 0.15 μm.

The $(Na^+ + K^+)$-ATPase is a putative sarcolemma marker that is thought to play a major role in myocardial excitation-contraction coupling [29,30]. This system appeared to be enriched between 34- and 45-fold in the sarcolemma-enriched fraction relative to the homogenate (Fig. 1). These estimates were based on the ratios of the number of specific ouabain binding sites and ouabain-sensitive K^+-phosphatase activities in the two fractions (Table I). Enrichment of $(Na^+ + K^+)$-ATPase activity per se was not determined due to the presence of very high ouabain-insensitive ATPase activity in the homogenate which precluded meaningful assessment of $(Na^+ + K^+)$-ATPase activity in that fraction. Nonetheless, $(Na^+ + K^+)$-ATPase activity in the final membrane preparation was quite high and 15 consecutive freeze-thaw cycles increased the activity by 4.2-fold (Table I). The $(Na^+ + K^+)$-ATPase activity in this fraction, after the freeze-thaw cycles, was somewhat higher than the values of 77—79 μmol/mg per h reported by Jones et al. [14] after treatment of a

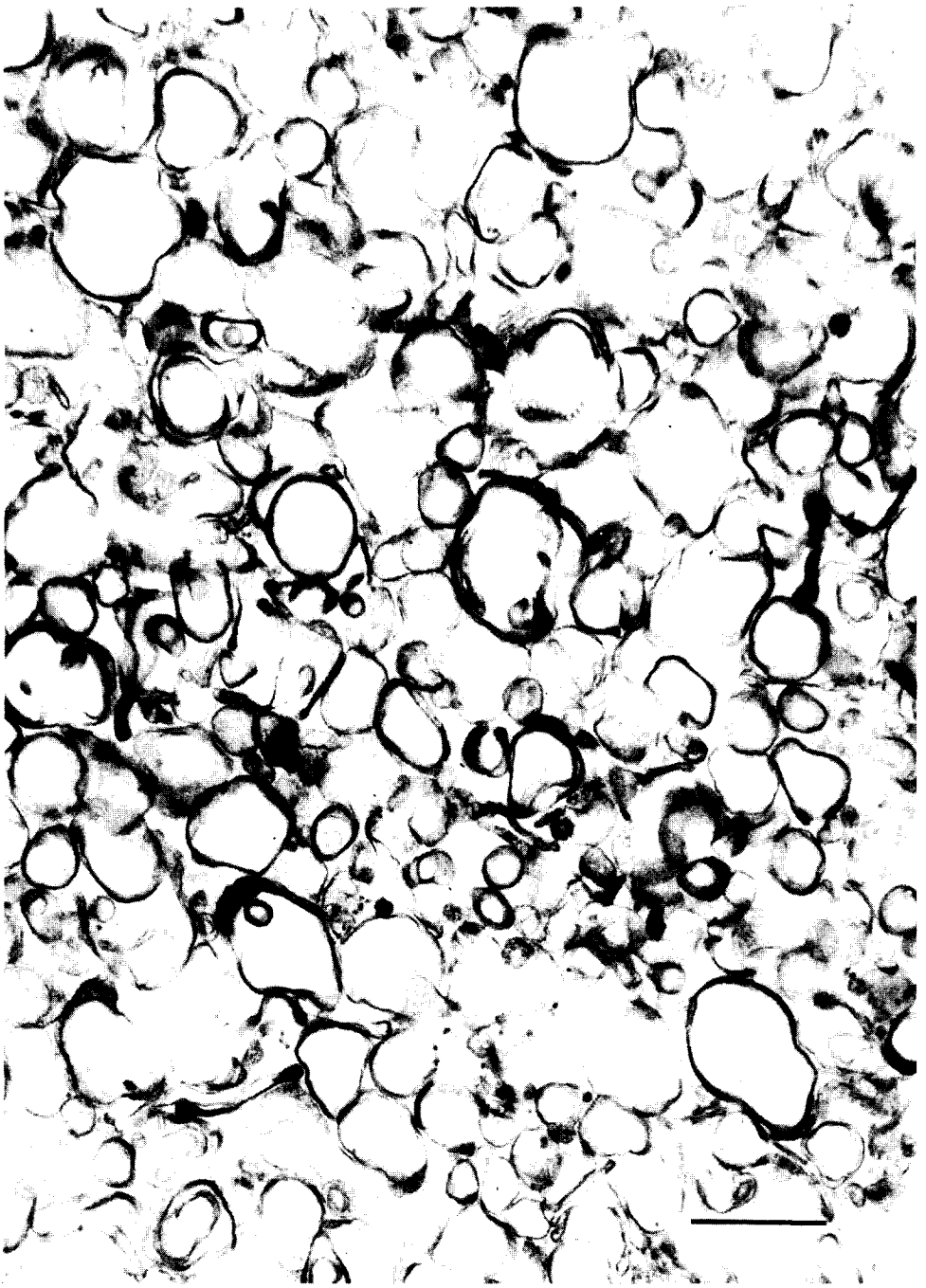

Fig. 2. Morphology of membrane fragments from the top layer at a magnification of 37 500. Bar = 0.2 μm.

TABLE I

ACTIVITIES ASSOCIATED WITH THE ($Na^+ + K^+$)-ATPase AND OUABAIN BINDING

Treated: 15 consecutive freeze-thaw cycles. Ouabain binding: pmol/mg protein; n= 6; means ± S.E. K^+-phosphatase: μmol/mg per h that were ouabain sensitive; n = 6; means ± S.E. ($Na^+ + K^+$)-ATPase: μmol/mg per h that was ouabain sensitive; n = 4; means ± S.E.

	Homogenate		Top layer		Purification (-fold)	
	Native	Treated	Native	Treated	Native	Treated
Ouabain binding	7.4 ± 1.5	6.1 ± 0.8	132 ± 21	187 ± 26	19.9 ± 3.0	33.7 ± 7.4
K^+-phosphatase	0.15 ± 0.01	0.21 ± 0.01	4.7 ± 0.4	9.3 ± 0.9	32.6 ± 3.9	44.6 ± 3.7
($Na^+ + K^+$)-ATPase	—	—	23.6 ± 3.6	99.9 ± 3.2	—	—

sarcolemma-enriched preparation from heart with SDS. Activation by either intervention appears to reflect, at least in part, the disruption of intact right-side-out membrane vesicles such that a more complete expression of ($Na^+ + K^+$)-ATPase activity in the preparation can be achieved (Ref. 14; see below). Conversely, the increase in the number of specific ouabain binding sites after the freeze-thaw cycles (Table I) is consistent with the disruption of intact inside-out vesicles, since the receptor site is thought to be located on the external in situ surface of the sarcolemma [29].

A second system postulated to exist in the sarcolemma and to participate in excitation-contraction coupling is a sodium-calcium exchange system which couples the movement of calcium to the movement of sodium in the opposite direction across the sarcolemma [31,32]. Reeves and Sutko [28] and, subsequently, Pitts [33] reported evidence for the existence of such a system in myocardial membrane preparations. We employed a protocol similar to those used previously (Refs. 28 and 33; Methods) to test for sodium-dependent calcium uptake by sodium-loaded membrane vesicles from the top layer. After a 5 min exposure to calcium at 37°C, these vesicles had taken up 49.3 ± 2.5 nmol calcium/mg protein which is 1.5—2.2 times higher than that previously reported for other sarcolemma-enriched preparations from cardiac tissue [28,33]. On the other hand, lithium-loaded vesicles and potassium-loaded vesicles had taken up only 2.0 ± 0.3 and 2.3 ± 0.2 nmol of calcium/mg protein, respectively, after 5 min at 37°C.

The β-adrenergic receptor and the adenylate cyclase enzyme constitute two additional putative sarcolemma systems that are believed to be intimately involved in the regulation of events associated with excitation-contraction coupling by the myocardial cell [34,35]. Scatchard analyses of (−)dihydroalprenolol binding to the membranes from the top layer were consistent with the presence of two independent binding components with dissociation constants of $9.7 \pm 2.6 \cdot 10^{-9}$ and $592 \pm 45 \cdot 10^{-9}$ M. The former, which has an affinity similar to that of the β-adrenergic receptor for (−)dihydroalprenolol (e.g., Ref. 36), is assumed to reflect binding of the ligand to the β-adrenergic receptor. The density of this receptor in membranes from the top layer was similar (Table II) to the value for maximal (−)dihydroalprenolol binding of 2.0 pmol/mg protein previously reported by Jones et al. [14] for their sarcolemma-enriched preparation. A 75-fold enrichment of this receptor in the top

TABLE II

ADENYLATE CYCLASE ACTIVITIES AND DIHYDROALPRENOLOL BINDING

DHA binding: high affinity dihydroalprenolol binding: pmol/mg protein; n = 4 for homogenate and 8 for top layer; means ± S.E. Adenylate cyclase: pmol/mg protein per min; n = 8 for homogenate and top layer comparisons; 6—7 for all other comparisons; Lubrol PX = 40 μg; 2:1 detergent/protein; NaF = 5 mM; GTP = 50 μM; isoproterenol = 10 μM; means ± S.E.

	Homogenate	Top layer	Purification (-fold)
DHA binding	0.032 ± 0.017	2.4 ± 0.5	75
Adenylate cyclase			
(basal)	57 ± 2	1535 ± 171	26.7 ± 2.8
basal		1224 ± 171	
basal + Lubrol		2214 ± 377	
basal		1121 ± 62	
NaF		2616 ± 258	
GTP		1207 ± 108	
GTP + isoproterenol		1819 ± 208	

layer relative to homogenate was calculated although the variance of the values for receptor density in the homogenate was high (Table II). Tentatively, however, it seems reasonable to conclude that enrichment of the β-adrenergic receptor was at least similar to, if not greater than, that of the $(Na^+ + K^+)$-ATPase (Table I). Adenylate cyclase activity was enriched about 27-fold in membranes from the top layer relative to homogenate (Table II), and the basal activities were within the range of those for the preparation described by Jones et al. (i.e., 790 to about 2000 pmol/mg per min [14]). It should be noted that activities reported by Jones et al. [14] were measured in the presence of the channel-forming ionophore, alamethicin, to unmask adenylate cyclase activity in intact membrane vesicles whereas our assays did not include this agent. On the other hand, Lubrol PX did cause activation (Table II) which is consistent with disruption of intact membrane vesicles. The adenylate cyclase activity of membranes from the top layer was stimulated by NaF and, in the presence of GTP, by isoproterenol (Table II).

The enrichment of ouabain binding sites and ouabain-sensitive K^+-phosphatase activity in vesicles from the top layer relative to homogenate (Table I) is 1.77- and 1.78-fold greater, respectively, than that of the sarcolemma-enriched preparation previously reported from this laboratory (i.e., Table II in Ref. 15). It is of interest, therefore, that the enrichment of another putative sarcolemma marker, 5′-nucleotidase, was 10.1-fold (Table III) which is only 40% of the enrichment obtained for the previous preparation. Similarly, antimycin A-insensitive NADH-cytochrome *c* reductase was enriched 3—4-fold (Table III) which is only 47—62% of that obtained for the previous preparation. It is possible that these two enzymes are intimately associated with the same vesicles that contain high densities of the $(Na^+ + K^+)$-ATPase, sodium-calcium exchange, β-adrenergic receptors and adenylate cyclase. If so, the two enzymes are presumably also present at higher densities in other regions of the myocardial cell, since their enrichment was relatively low. Alternatively, their presence in the top layer may reflect contamination by vesicles from non-sarcolemma membranes. In this regard, the enrichment of the mitochondrial marker, succinic

TABLE III

OTHER MARKER ACTIVITIES IN THE HOMOGENATE AND TOP LAYER

Treated: 15 consecutive freeze-thaw cycles. Activities expressed as μmol/mg protein per h; $n = 6$ for all enzymes except for Ca^{2+}-ATPase where $n = 5$; means ± S.E.

	Homogenate		Top layer		Purification (-fold)	
	Native	Treated	Native	Treated	Native	Treated
5′-Nucleotidase	0.37 ± 0.07	0.33 ± 0.07	3.4 ± 0.4	3.0 ± 0.3	10.1 ± 1.2	10.1 ± 1.0
NADH-cytochrome *c* reductase	0.53 ± 0.10	0.25 ± 0.02	2.34 ± 1.02	0.70 ± 0.12	3.91 ± 0.92	2.96 ± 0.55
Ca^{2+}-ATPase	5.1 ± 0.9	4.6 ± 1.1	3.4 ± 1.1	2.7 ± 1.0	0.69 ± 0.15	0.81 ± 0.33
Succinic dehydrogenase	6.0 ± 0.9	14.7 ± 2.3	2.9 ± 0.2	13.9 ± 2.8	0.52 ± 0.07	0.94 ± 0.14

dehydrogenase, and of the putative sarcoplasmic reticulum marker, Ca^{2+}-ATPase, in the top layer relative to homogenate averaged slightly less than unity (Table III) which suggests sarcolemma enrichment at the expense of mitochondria and sarcoplasmic reticulum. Thus, the 27- to 40-fold or greater enrichment of adenylate cyclase, ouabain binding sites, ouabain-sensitive K^+-phosphatase and β-adrenergic receptors is consistent with the conclusion that the top layer is highly enriched with sarcolemma [16]. The presence of some succinic dehydrogenase and Ca^{2+}-ATPase activity, however, clearly demonstrates that complete purification of the sarcolemma was not achieved.

We wish to emphasize that the lack of enrichment of Ca^{2+}-ATPase activity does not eliminate the possibility that a Ca^{2+}-ATPase is associated with the sarcolemma. Assay conditions other than those used herein might reveal the existence of such a system.

The sarcolemma preparation, previously equilibrated with 10 mM buffer, responded very rapidly to increases in the osmolarity of the suspension medium as indicated by an increase in 90° light scatter (Fig. 3). After the increase, light scatter slowly returned towards its initial value. The interpretation of this profile is that the immediate increase in scatter reflected a loss of water from the vesicles with a concomitant change in the configuration of the vesicles (e.g., Ref. 37). The return towards the original baseline then reflected a net movement of water back into the vesicles in association with the movement of solute into the vesicles. Based on this interpretation, vesicles in the top layer manifested certain permeability characteristics that are qualitatively similar to those expected for the in situ case of the sarcolemma, viz., low permeability to a salt, NaCl, and even lower permeability to sucrose (Fig. 3). These characteristics complement the observation that calcium uptake by the vesicles is low in the absence of intravesicular sodium (see above). The ability

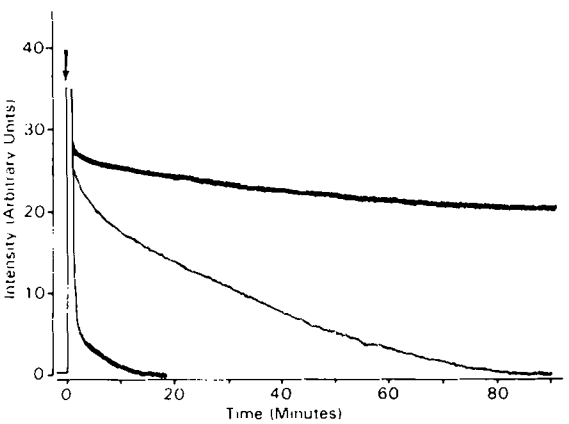

Fig. 3. Change in intensity of light scattered by the sarcolemma-enriched fraction upon exposure to a hyperosmotic medium. Aliquots of the preparation were suspended in 1.0 ml of 10 mM Tris-HCl, pH 7.4, at 25°C. Solute (0.5 ml) in 10 mM Tris-HCl, pH 7.4, was added at the arrow. The suspension was mixed with a Pasteur pipet during the time indicated by the blank space in the recordings and the intensity of 90°-angle scattered light was then monitored at 500 nm. Solutes are 200 mM sucrose (upper trace), 100 mM NaCl (middle trace), and 100 mM NaCl in the presence of the sarcolemma-enriched fraction previously treated with freeze-thaw cycles (lower trace).

of the vesicles to respond to an osmotic intervention was severely compromised by five consecutive freeze-thaw cycles (Fig. 3). This effect suggests that the freeze-thaw cycles substantially increase the permeability of the vesicles (or at least of some of the vesicles). Such a conclusion is consistent with activation of the $(Na^+ + K^+)$-ATPase and increase in number of ouabain binding sites by freeze-thaw cycles (see above).

The data presented in Table I, however, also suggest that some of the vesicles from the top layer are freely permeable to small ligands. $(Na^+ + K^+)$-ATPase activity, prior to the freeze-thaw cycles, was 23.6% of that observed after the cycles. We had originally thought that such activity might be the manifestation of intact inside-out vesicles in the preparation. Thus, ATP, magnesium and sodium in the extravesicular medium would be bathing the appropriate surface of these vesicles for $(Na^+ + K^+)$-ATPase activity [29] and it was possible that potassium entered the vesicles to an extent sufficient for such activity. Other experiments did not support these expectations. Light-scatter experiments, similar to those of Fig. 3, showed that KCl entered the osmotically responding vesicles at a rate similar to that of NaCl. Furthermore, we could not detect any time-dependent increase in ATPase activity (i.e., in the presence of ATP, magnesium and sodium) upon the addition of potassium in the middle of the assay. Rather, the increase in activity appeared immediately (data not shown). Accordingly, it seems reasonable to conclude that about 24% of the vesicles in the top layer are freely permeable.

Total protein recovered from the top layer was 10.6 ± 0.5 mg/100 g ventricular tissue (n = 10) which is 5.1-fold greater than the recovery of the sarcolemma preparation previously described from this laboratory [15]. The protein content of the homogenate was 13 025 ± 564 mg/100 g ventricular tissue. Thus, the recovery in the top layer of specific ouabain binding sites, ouabain-sensitive K^+-phosphatase activity, high-affinity (−)dihydroalprenolol binding sites and adenylate cyclase activity was 2.5, 5.1, 6.1 and 3.2%, respectively, which yields a mean estimate of 4.2% for the recovery of these putative sarcolemma markers.

The isolation procedure described herein provides the highest enrichment ratios for a group of putative sarcolemma markers from heart thus far reported. The product of the procedure, a highly enriched sarcolemma preparation, requires a relatively short process time and is obtained in amounts suitable for further sarcolemma purification and for the study of ion movements across the sarcolemma that are thought to participate in excitation-contraction coupling in heart. Appropriate caution for the latter type of study must be employed, however, because of the following considerations. (a) Some of the vesicles in the preparation appear to be freely permeable; (b) a mixture of right-side-out and inside-out vesicles appears to result from the isolation procedure; (c) complete sarcolemma purification is not achieved; and (d) a considerable amount of sarcolemma, perhaps with unique structural and functional features, is not recovered. Conversely, the isolation procedure was relatively mild in the sense that neither high salt concentrations nor detergents were employed. This strategy should favor retention of the in situ structural and functional characteristics of the sarcolemma in the isolated membrane preparation.

Acknowledgements

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