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ISOLATION OF A HIGHLY ENRICHED SARCOLEMMA MEMBRANE FRACTION FROM CANINE HEART

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Summary

A highly enriched sarcolemma preparation was isolated by differential centrifugation of a canine ventricular homogenate followed by centrifugation of a membrane fraction layered over 22% (w/v) sucrose. Ouabain binding, ouabain-sensitive potassium phosphatase activity and 5′-nucleotidase activity were enriched 19—27 fold over the homogenate whereas Ca^{2+}-ATPase and succinate dehydrogenase activities were 0.75 and 0.36, respectively, of that for the homogenate. The isolation procedure was relatively rapid and yielded about 2.0 mg protein/100 g of ventricular muscle. The highest salt concentration used in the procedure was 0.6 M KCl and no detergents were employed. Initial characterization studies suggested that the sarcolemma-enriched fraction consists predominantly if not totally of freely permeable membrane vesicles and that the sarcolemma does not manifest a Ca^{2+}-ATPase activity, at least within the limits of the assay procedures employed. This preparation was concluded to be about 1.5- to 4-fold more highly enriched with sarcolemmal markers than preparations obtained by previously published procedures. Accordingly, the preparation provides an improved basis for the probe of calcium movements that occur across the sarcolemma in association with the excitation-contraction-relaxation sequence of the mammalian myocardial cell.

Introduction

The cycle of excitation-contraction-relaxation for the mammalian myocardial cell is now generally agreed to involve the movement of calcium into and out of these cells on a beat-to-beat basis. Since in diastole, the sarcolemma

separates millimolar (extracellular) from submicromolar (intracellular) free calcium, appropriate contractile function must involve the selective activation and inactivation of sarcolemmal pathways for calcium movements. Inferences about the nature of these pathways have been drawn [1—3], generally from studies carried out with isolated organ, tissue or cell preparations, but a molecular probe of the pathways for calcium movement has been limited by the lack of suitably purified sarcolemma preparations from cardiac tissue.

In 1969, Harigaya and Schwartz [4] reported a method by which to obtain sarcoplasmic reticulum-enriched preparations from ventricular tissue and in 1976, Besch et al. [5] showed by use of detergents that this preparation contained enzymatic activities that are ascribed, at least in part, to the sarcolemma. These studies suggested a source from which to obtain sarcolemma-enriched preparations. In 1966, Matsui and Schwartz [6] showed that pellets derived from intermediate centrifugations (e.g. 8000—10 000 $\times g$ for 20—30 min) of cardiac homogenates contained most of the $(Na^+ + K^+)$-ATPase which is presumed to be concentrated, if not exclusively localized, in the sarcolemma. Such a pellet is obtained and discarded at an early point in the Harigaya and Schwartz procedure, thus suggesting a means to increase the yield of the sarcolemma-enriched preparation (i.e. rehomogenization of the pellet). Based on these previous studies, this paper describes a purification procedure by which a highly enriched sarcolemma preparation from canine ventricular tissue can be obtained.

Experimental procedures

Materials. Imidazole, disodium adenosine triphosphate, adenosine 5′-monophosphoric acid, maleic acid, Trizma base, glycine, sodium azide, antimycin A, ouabain, pyruvate kinase, lactate dehydrogenase, cytochrome *c*, reduced from of nicotinamide adenine dinucleotide (NADH), ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), *p*-nitrophenylphosphate (disodium salt), succinic acid and deoxycholic acid were obtained from Sigma Chemical Co., St. Louis, MO. [^{3}H]Ouabain was obtained from New England Nuclear, Boston, MA, and sodium dodecyl sulfate was obtained from Accurate Chemical and Scientific Corp., Hicksville, NY. All other chemicals were of reagent grade.

Isolation of the sarcolemma preparation. Male and female mongrel dogs weighing 20—30 kg were anesthetized with pentobarbital, 30 mg/kg intravenously, and artificially respired with room air. The chest was opened, 1.5 ml of 1 : 1000 heparin was injected into the right atrium and after 5 min, the heart was rapidly removed and immersed in ice-cold 0.9% saline. The left ventricular free wall, the interventricular septum and, in some cases, 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 are those at the middle of the tube. The mince was suspended in 4—5 vols. glass-distilled water containing 10 mM sodium bicarbonate and 5 mM sodium azide, pH 7.0, (medium A) and was homogenized with three 10—15-s pulses (separated by 15-s intervals) with an Ultra Turrax T-45 Tissumizer (Tekmar Instruments, Cincinnati, OH) at low speed. The homogenate was centrifuged at 8700 $\times g$ for 20 min to yield pellet 1 and

supernatant 1 (Fig. 1). Pellet 1 was suspended in the original volume of medium A, homogenized in a glass homogenizing vessel with one pass of a motor-driven Teflon pestle and was centrifuged at 8700 × *g* for 20 min. This yielded pellet 2, which was discarded, and supernatant 2 which was combined with supernatant 1 and centrifuged at 35 000 × *g* for 20 min. This step yielded supernatant 3, which was discarded, and pellet 3 which was suspended in approx. 50 ml of 20 mM Tris/maleate, pH 6.8, with 0.6 M KCl (medium B) using a glass homogenizer and hand-driven Teflon pestle. The suspension was centrifuged at 73 400 × *g* for 20 min. The resulting supernatant (supernatant 4) was discarded and pellet 4 was resuspended in 20 ml of 10 mM Tris-HCl, pH 7.4, (medium C) in a glass homogenizer with a motor-driven Teflon pestle. This suspension was layered on top of a 22% (w/v) sucrose solution (verified by refractometer, ABBE-3L, Bausch and Lomb, Rochester, NY) containing 10 mM Tris-HCl, pH 7.4, in a Type 30 Rotor tube. Centrifugation at 73 400 × *g* for 30 min yielded a top layer at the buffer-sucrose interface and pellet 5 at the bottom of the tube. The former was aspirated with a syringe, diluted to about 50 ml with medium C and centrifuged at 73 400 × *g* for 20 min. The pellet was then resuspended in medium C to a final concentration of approximately 0.5—1.0 mg/ml. In some cases, pellet 5 was suspended in medium C, centrifuged at 73 400 × *g* for 20 min and resuspended in a small volume of medium C for subsequent use.

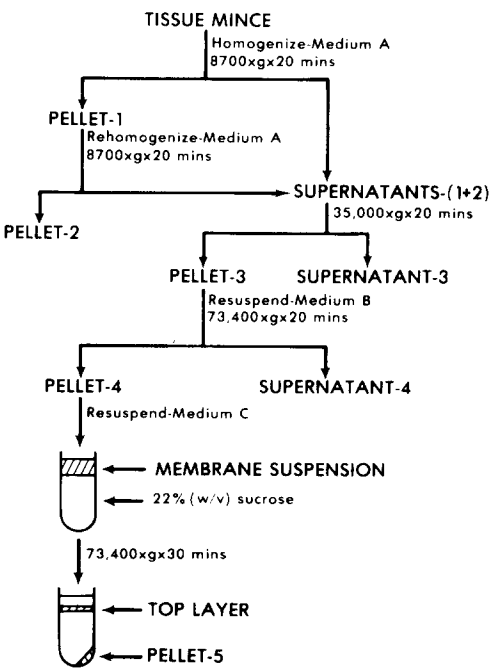

Fig. 1. Flow scheme for isolation of a sarcolemma-enriched fraction from canine myocardial ventricular tissue. Details are presented under Experimental procedures. Medium A: 10 mM sodium bicarbonate, 5 mM sodium azide, pH 7.0. Medium B: 20 mM Tris/maleate, pH 6.8, 0.6 M KCl. Medium C: 10 mM Tris-HCl, pH 7.4. The 22% (w/v) sucrose solution was made up in 10 mM Tris-HCl, pH 7.4. The top layer was diluted with medium C and pellet 5 was suspended in medium C, centrifuged at 73 400 × *g* for 20 min and then resuspended in small volumes of medium C for storage and subsequent use.

Ouabain binding, enzyme assays and protein content. All assays carried out in duplicate or triplicate were performed within 24 h and most were done immediately following completion of the isolation procedure. Total specific [^{3}H]ouabain binding was measured according to the method of Inagaki et al. [7] in the presence of 5 mM $MgCl_2$, 5 mM Tris/phosphate and 10 mM Tris-HCl, pH 7.4. All enzyme reactions were linear within the time periods of the assays and the protein concentrations employed. Ca^{2+}-ATPase activities were determined by measuring the appearance of inorganic phosphate from ATP after Besch et al. [5] using the colorimetric procedure of Martin and Doty [8]. 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. (Ca^{2+} + K^+)-ATPase and (Ca^{2+} + Na^+)-ATPase activities were those in which the calcium-containing buffer was supplemented with either 75 mM KCl or NaCl, respectively, minus the activity of the EGTA-containing buffer [9]. 5′-Nucleotidase (5′-AMPase) was assayed according to the method of Heppel and Hilmoe [10]. K^+-stimulated *p*-nitrophenylphosphatase was measured as described by Skou [11] and the activity reported is that fraction which is ouabain sensitive. NADH-cytochrome *c* reductase was assayed after the method of Tolbert [12] and the fraction reported is that which is antimycin A insensitive. Succinate dehydrogenase was assayed using the linked enzyme method of Green et al. [13]. The protein concentration of the various fractions was measured by the method of Lowry et al. [14].

Activation studies. In some cases, portions of the initial homogenate and top layer were treated with deoxycholate or sodium dodecyl sulfate. Three equal aliquots of each fraction were diluted to equal volumes with solutions containing either (a) sodium dodecyl sulfate (0.3 mg/ml final concentration) plus 10 mM Tris-HCl, pH 7.4, (b) deoxycholate (1.4 mg/ml final concentration) plus 10 mM Tris-HCl, pH 7.4, or (c) 10 mM Tris-HCl, pH 7.4, alone [5]. After a 20 min incubation in ice, the suspensions were sedimented at 140 000 × *g* for 15 min at 4° C, the pellets were resuspended in ice-cold 10 mM Tris-HCl, pH 7.4, and the fractions were assayed immediately for protein concentration, total ouabain sites and 5′-nucleotidase activity.

Results and Discussion

The purification scheme (Fig. 1) down to pellet 4 is a modification of the Harigaya and Schwartz procedure [4] for the isolation of a sarcoplasmic reticulum-enriched preparation from cardiac tissue. A major modification is the rehomogenization of pellet 1, centrifugation of this suspension and combination of the resulting supernatant with the supernatant derived from centrifugation of the original homogenate. This step, suggested by the previous study of Matsui and Schwartz [6], increased the yield of ouabain-binding sites in the sarcolemma-enriched fraction by four to five fold. Centrifugation of a suspension of pellet 4, layered over a buffered sucrose solution, yielded a layer (top layer) which did not penetrate the sucrose and a pellet (pellet 5) at the bottom of the sucrose (Fig. 1).

The top layer was enriched in ouabain-binding sites, ouabain-sensitive

p-nitrophenylphosphatase activity and 5′-nucleotidase activity relative to the other fractions of the purification scheme (Table I). These functions are thought to be manifestations of proteins associated with the plasma membrane of mammalian cells. Other fractions, however, had higher activities of Ca^{2+}-ATPase (a putative sarcoplasmic reticulum marker), antimycin A-insensitive NADH cytochrome *c* reductase (thought to reside in sarcoplasmic reticulum and the outer mitochondrial membrane but perhaps also in the sarcolemma) and succinate dehydrogenase (mitochondrial marker).

Sarcolemma markers in the top layer were purified 19—27 fold over the homogenate, the putative sarcoplasmic reticulum marker, Ca^{2+}-ATPase, was purified to 0.75 of the homogenate and the mitochondrial marker, succinate dehydrogenase, was purified to 0.36 of the homogenate. Neville [15], based on plasma membrane preparations from other tissues, stated that this type of purification profile is consistent with about 75% of the preparation being plasma membrane or plasma membrane derived. The one marker which deviates from this profile is the antimycin-insensitive NADH-cytochrome *c* reductase (Table II). Two explanations exist: (a) the top layer contains membrane fragments derived from the outer mitochondrial membrane or from zones of sarcoplasmic reticulum with little Ca^{2+}-ATPase activity; and/or (b) the enzyme is associated, in part, with the sarcolemma. Thus, the percent purity of the top layer is unknown but, in general, the enrichment of sarcolemma markers presented here is about 1.5- to 4-fold greater than that reported by others for sarcolemma preparation from heart [16—25].

The purification procedure was relatively rapid (about 5 h upon removal of the heart) and mild (highest salt concentration was 0.6 M KCl; no detergent employed) and yielded about 2.0 mg protein of the sarcolemma-enriched fraction/100 g of tissue. The recovery of sarcolemma markers in the top layer from the homogenate, however, was quite low (Table III). A considerable amount of the remaining sarcolemma markers was located in pellet 2.

TABLE I

MARKER ACTIVITIES OF SELECTED FRACTIONS FROM THE PURIFICATION SCHEME

Ouabain binding (pmol/mg) and enzymatic activities (μmol/mg per h) were determined as described under Experimental procedures. Fractions are defined in Fig. 1. Abbreviations: K^+-Pase is that component of K^+-stimulated phosphatase that is ouabain sensitive; 5′-AMPase is 5′-nucleotidase; NADH-cyto *c* reductase is the antimycin A-insensitive component of NADH-cytochrome *c* reductase. Values are means ± S.E. *N*, number of experiments.

Fraction	Ouabain binding *	K^+-Pase **	5′-AMPase *	Ca^{2+}-ATPase ***	NADH-cyto *c* reductase *	Succinate dehydrogenase *
Homogenate	7.4 ± 0.9	0.2 ± 0.05	0.6 ± 0.08	2.8 ± 0.3	0.54 ± 0.09	20.2 ± 2.4
Pellet 4	35 ± 3	0.9 ± 0.06	9.7 ± 1.9	4.7 ± 0.9	4.3 ± 0.08	17.8 ± 2.0
Top layer	121 ± 8	3.6 ± 0.2	14 ± 2	2.0 ± 0.6	2.9 ± 0.6	7.1 ± 0.8
Pellet 5	24 ± 3	0.9 ± 0.1	9 ± 2	6.8 ± 0.9	5.4 ± 1.1	17.8 ± 2.0

* $N = 10$.
** $N = 9$.
*** $N = 7$.

TABLE II

PURIFICATION OF MARKER ENZYMES RELATIVE TO INITIAL HOMOGENATES

Values were calculated from specific binding and activities for individual experiments, the mean values of which are presented in Table I. Values represent means ± S.E. *N* number of experiments. For the abbreviations used, see Table I.

Fraction	Ouabain binding *	K^+-Pase **	5'-AMPase *	Ca^{2+}-ATPase ***	NADH-cyto *c* reductase *	Succinate dehydrogenase *
Homogenate	1	1	1	1	1	1
Pellet 4	5.9 ± 1.6	6.3 ± 1.4	16 ± 2	1.6 ± 0.2	7.7 ± 0.5	0.97 ± 0.1
Top layer	19 ± 3	27 ± 5	25 ± 3	0.75 ± 0.3	6.3 ± 1.3	0.36 ± 0.04
Pellet 5	4.4 ± 1.1	6.0 ± 1.1	15 ± 2	2.5 ± 0.3	10.4 ± 1.5	1.0 ± 0.2

* $N = 10$.
** $N = 9$.
*** $N = 7$.

While it is well recognized that the sarcoplasmic reticulum manifests a Ca^{2+}-ATPase activity, it is possible that a Ca^{2+}-ATPase is also associated with the sarcolemma. Such may be required to effect calcium efflux from the cell during diastole. The results of this study, however, suggest that the sarcolemma does not manifest Ca^{2+}-ATPase activity. As stated above the specific activity of Ca^{2+}-ATPase in the top layer was slightly less than that of the homogenate and was considerably less than the activity found for pellet 5. In addition, Jones et al. [9] reported that the Ca^{2+}-ATPase of sarcoplasmic reticulum is stimulated by monovalent cations, particularly potassium. The Ca^{2+}-ATPase in the top layer and in pellet 5 was found to be stimulated by potassium to a slightly greater extent than by sodium but the extent of stimulation was similar in the two fractions (data not shown). Accordingly, it would appear that the the Ca^{2+}-ATPase activity observed in the top layer reflects contamination by sarcoplasmic reticulum as opposed to association of this activity with the sarcolemma. This does not eliminate the possibility that a Ca^{2+}-ATPase resides in/on the sarcolemma since assay conditions other than those employed might be required for the enzymatic expression of the system.

Besch et al. [5] originally found that the Harigaya and Schwartz [4] preparation contained significant amounts of sarcolemma markers by the use of detergents and concluded that detergent activation reflected the presence of intact (i.e. selectively impermeable) sarcolemma vesicles. The top layer, however, appeared to contain freely permeable vesicles. First, the vesicles did not penetrate 22% sucrose upon centrifugation. Steck [26] suggested that intact vesicles, being osmotically active, tend to shrink upon contact with a solution of higher osmolarity which increases their density and, thereby, favors penetration into a denser medium upon centrifugation. Permeable vesicles, being osmotically inactive, would retain their original density and tend to remain at the interface between media of two densities. Second, treatment with deoxycholate or sodium dodecyl sulfate, to the point that 1/3—1/2 of the membrane protein was solubilized, did not cause activation of 5'-nucleotidase. On the other hand, there was some increase in total ouabain-binding sites upon

TABLE III

PERCENT RECOVERY OF MARKER ACTIVITIES RELATIVE TO INITIAL HOMOGENATE

Values were calculated from specific binding and activities for individual experiments, the mean values of which are presented in Table I, and from total protein recovered in each fraction. The latter equalled 10 170 ± 1 080, 70 ± 13, 2.0 ± 0.3 and 57 ± 12 mg protein/100 g tissue for homogenate, Pellet 4, top layer, and pellet 5, respectively. Values represent means ± S.E. N, number of experiments. For the abbreviations used see Table I.

Fraction	Ouabain binding *	K^+-Pase **	5'-AMPase *	Ca^{2+}-ATPace ***	NADH-cyto c reductase *	Succinate dehydrogenase *
Homogenate	100	100	100	100	100	100
Pellet 4	4.7 ± 2.1	4.4 ± 1.0	10.2 ± 0.6	1.2 ± 0.3	5.6 ± 0.8	0.72 ± 0.2
Top layer	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.014 ± 0.004	0.10 ± 0.03	0.007 ± 0.001
Pellet 5	2.4 ± 0.9	3.2 ± 0.4	7.6 ± 0.6	1.4 ± 0.2	5.5 ± 0.7	0.64 ± 0.2

* N = 10.
** N = 9.
*** N = 7.

treatment with sodium dodecyl sulfate (data not shown). The discrepancy between these two putative sarcolemma markers may reflect (a) the presence of a detergent-sensitive component which suppresses ouabain binding, (b) solubilization/destruction of 5'-nucleotidase by the detergent, or (c) the presence of impermeable vesicles in the top layer that are enriched in ouabain-binding sites but have low 5'-nucleotidase activity. While the last possibility seems questionable, it is reasonable to suppose that different zones of the sarcolemma in situ vary with respect to function (e.g. transverse tubules versus surface per se) and, therefore, probably do have varying ratios of sarcolemmal proteins and lipids.

The isolation procedure described herein yielded, to our knowledge, a much more highly enriched sarcolemmal preparation than is currently available. As such, the preparation provides an improved means to test existing hypotheses and to develop new hypotheses concerned with the pathways and regulation of components of calcium movement that are involved in myocardial excitation-contraction coupling and calcium efflux.

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