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Structure-function studies of canine cardiac sarcolemmal membranes. I. Estimation of receptor site densities

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A novel method for the estimation of receptor site densities in purified canine cardiac sarolemmal vesicles is described. Canine sarcolemmal vesicles, purified by the method of Jones et al. (Jones, L.R., Maddock, S.W. and Besch, H.R. (1980) J. Biol. Chem. 255, 9971–9980) had high ($Na^+ + K^+$)-ATPase specific activity (127 ± 1.9 μmol P_i/mg per h). Total phospholipid content, estimated by measurements of total phosphorus and total fatty acid contents, was 3.09 μmol/mg. Saturation isotherms for several receptor ligands gave the following values for K_d and B_{max}: ouabain 32.6 ± 2.7 nM, 365 ± 59 pmol/mg; quinuclidinyl benzilate 0.055 ± 0.010 nM, 5.8 ± 0.7 pmol/mg; dihydroalprenolol 4.6 ± 1.0 nM, 2.2 ± 0.2 pmol/mg; and nitrendipine 0.21 ± 0.04 nM, 0.93 ± 1.04 pmol/mg. Membrane phospholipid surface area per ligand-binding sites was estimated from the B_{max} values for each receptor ligand utilizing 3.09 μmol phospholipid/mg and 60 $Å^2$ as the average surface area occupied by each phospholipid molecule. The following receptor site densities per μm^2 phospholipid surface were obtained: ouabain, 400; quinuclidinyl benzilate, 6; dihydroalprenolol, 2; and nitrendipine, 1. As the surface area contributed by protein was estimated to be less than 20% of the lipid surface area, these values must be reduced by approx. 20% to estimate site densities per μm^2 membrane surface. These data demonstrate much lower beta-adrenergic and muscarinic receptor density compared to that of Na^+ pump sites.

Introduction

The cardiac sarcolemma plays a central role in cardiac excitation, conduction, excitation-contraction coupling, and regulation of myocardial contractility. It is now generally agreed that calcium currents in the heart are regulated by autonomic innervation and strong evidence suggests that a second messenger (cAMP) is involved in such regulation [1–6]. However, the exact mechanism of the interaction between the beta-adrenergic or muscarinic receptors and the calcium channel is still unknown.

Several preparations of purified sarcolemmal membrane vesicles from rat [7,8], rabbit [9], dog [10–13] and adult myocytes [14] have been characterized by relatively high specific activities for ($Na^+ + K^+$)-ATPase, Na^+-Ca^+ exchange, adenylate cyclase, and a relatively high density of ligand-binding sites for beta-adrenergic and muscarinic agonists and antagonists, and calcium channel blocking drugs [15]. While certain aspects of each of these preparations have been stressed, an extensive characterization of the functional, compositional and structural aspects of any one preparation is not available. In this and the accompanying paper [16] we define a number of

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

functional and structural parameters that allow us to propose a model for the organization of the purified canine cardiac sarcolemmal membrane. While this characterization is not exhaustive, the available data provide a basis for the calculation of receptor site densities in the purified sarcolemmal membrane which could be compared directly to densities estimated in the intact tissues, and serve as the basis for some inferences about receptors function in the intact myocardium.

Methods

Preparation of purified sarcolemmal membrane vesicles. Sarcolemmal vesicles were prepared as described by Jones et al. [11] without a calcium-loading step. Sarcolemmal membrane vesicles were formed by two 30-s homogenizations with a Brinkmann Polytron at half-maximal setting. The homogenates were centrifuged at $21\,500 \times g_{max}$ for 20 min, the pellets discarded and the supernatant recentrifuged at $45\,000 \times g_{max}$ for 30 min. These pellets were resuspended in 0.25 M sucrose/20 mM Hepes (pH 7.8) (solution A) and purified further as described by Jones et al. [11]. The purified sarcolemmal vesicles were resuspended in 10 vol. 10 mM $NaHCO_3$/20 mM Hepes (pH 7.8) (solution B) and centrifuged in a Beckman SW-27 rotor at 25000 rpm for 30 min. The resulting pellets were resuspended by gentle homogenization in solution A to a protein concentration of 4–8 mg/ml. The sarcolemmal vesicles were frozen in liquid nitrogen and stored frozen at −80°C. All assays were performed within 2–3 weeks of preparation during which time no observable deterioration of the vesicles was observed.

Marker enzyme assays. ATPase activity was measured as inorganic phosphate (P_i) liberation in three 0.25-ml aliquots of reaction mixtures (10 μg/ml protein) that were added to 0.1 ml 20% ice-cold trichloroacetic acid at 1-min intervals for a total of 3 min and P_i analyzed by the method of Chen et al. [17]. ATPase activities were linear over these time periods.

$(Na^+ + K^+)$-ATPase activity was defined as the difference in ATP hydrolysis rate measured in 120 mM NaCl/30 mM KCl/0.1mM EGTA/1 mM MgATP/20 mM Tris-HCl (pH 7.4) at 37°C, and that measured under the same conditions except that KCl was replaced with NaCl. Estimates of $(Na^+ + K^+)$-ATPase obtained by measuring the decrease in ATPase activity (measured in 120 mM NaCl/30 mM KCl) after addition of $1 \cdot 10^{-4}$ M ouabain gave similar values. Valinomycin (Calbiochem, La Jolla, CA) and alamethicin, dissolved in 95% ethanol (redistilled, constant boiling, 77°C), were added to ATPase assay mixtures so as to give a final ethanol concentration of 0.5% (v/v). This ethanol concentration had no effect on control $(Na^+ + K^+)$-ATPase activities.

Ca^{2+}-ATPase activity was measured as the difference between ATP hydrolysis rates in 100 mM KCl/100 μM $CaCl_2$/20 mM Tris-HCl (pH 7.4) at 37°C, 1 mM MgATP and that measured in 100 mM KCl/0.1 mM EGTA/20 mM Tris-HCl (pH 7.4)/1 mM MgATP ('zero Ca^{2+}').

Cytochrome *c* oxidase activity was assayed by the method of Sottocasa et al. [18] using Sigma Type III cytochrome *c*. Monoamine oxidase activity was assayed by the method of Wurtman and Axelrod [19] as modified by Seiler and Fleischer [20] using [^{14}C]tryptamine bissuccinate as a substrate. Angiotensin-II-converting enzyme was assayed by the method of Cushman and Cheung [21] using Hip-His-Leu (Sigma, St. Louis, MO) as a substrate. A unit of enzyme activity was the amount required to catalyze formation of 1 μmol hippuric acid/min at 37°C under their standard conditions.

Compositional assays. Membrane phosphorus content was measured in samples that had been resuspended 1:100 (v/v) in solution B at 4°C and then centrifuged at 25000 rpm for 30 min in a Beckman SW-27 rotor. Resuspended samples (2–4 mg/ml in solution B) (5 μl) were added to 2 ml nitric acid (5 mg $CaCO_3$/100 ml conc. HNO_3) and the samples were carefully boiled to dryness over a flame. Phosphate in the residue was assayed by the method of Chen et al. [17] using K_2HPO_4 as a standard.

To prepare extracted sarcolemmal lipids (see below), 1 ml membrane suspension (0.2–0.4 mg/ml in solution B) was added to 22 ml of 2:1 chloroform/methanol (v/v). An additional 2 ml of 2:1 chloroform/methanol was added to wash the sides of the tube after which the samples were incubated for 1 h at 25°C. 5 ml 0.9% NaCl was added, the tubes were inverted several times and were centrifuged at 1000 rpm for 10 min to separate the

phases. The upper layer was removed and the lower layer stored under N_2 at −20°C. 5 ml extract were evaporated under N_2 at 55°C and the residue was assayed for total cholesterol [22]. Sigma grade (99 + %) cholesterol was used as a standard.

Fatty acyl contents of 2–3 ml extracted sarcolemmal lipids were analyzed by the method of Jordan and Schenkman [23] utilizing a mass spectrometer/gas chromatrography apparatus (Hewlett Packard Model 5992B) with a 6 ft. column packed with 3% silar 10-C on Gas-chrom Q (100–120 mesh). Heptadecanoic acid (NuCheck Prep, Inc., Elysian, MN) was added to each sample as an internal standard. Calculations of fatty acid concentrations were based on the ratio of the recovery of each fatty acid to the recovery of the internal standard. which were between 60–70%.

Protein was determined by the method of Lowry et al. [24].

Equilibrium binding assays. Equilibrium binding assays for [^{3}H]ouabain (18 Ci/mmol), [^{3}H] quinuclidinyl benzilate (33 Ci/mmol) and [^{3}H]nitrendipine (85 Ci/mmol) were carried out at 25°C in 5 ml volumes. For [^{3}H]dihydroalprenolol (38.5 Ci/mmol) binding, 100 μl volumes were used. Ouabain binding was assayed after incubation of vesicles in 120 mM NaCl and 1 mM MgATP for 30 min. [^{3}H]Ouabain was diluted to 0.45 Ci/mmol with nonradioactive ouabain (Sigma) before use. No specific [^{3}H]ouabain binding was observed in the absence of MgATP. [^{3}H]quinuclidinyl benzilate and [^{3}H]dihydroalprenolol binding were assayed after incubation in 5 mM $MgCl_2$/20 mM Tris-HCl (pH 7.4) for 90 and 30 min, respectively. [^{3}H]Nitrendipine binding was assayed in 50 mM Tris-HCl (pH 7.4) after incubation for 90 min. Specific binding of [^{3}H]ouabain, [^{3}H]quinuclidinyl benzilate, [^{3}H]dihydroalprenolol and [^{3}H]nitrendipine was the amount of binding displaced by $2 \cdot 10^{-4}$ M ouabain, $1 \cdot 10^{-6}$ M atropine sulfate (Sigma), $1 \cdot 10^{5}$ M alprenolol (Sigma) and $2 \cdot 10^{-8}$ M nitrendipine (Miles Laboratories, New Haven, CT), respectively. For Scatchard analysis, concentration ranges for [^{3}H]ouabain, [^{3}H]quinuclidinyl benzilate, [^{3}H]dihydroalprenolol and [^{3}H]nitrendipine were 20–500, 0.01–1.0, 2–100 and 0.028–0.84 nM, respectively. Protein present in each assay tube was 20 μg. Samples were rapidly filtered by vacuum through Whatman GF/B filters on a Brandel Cell Harvester (Gaithersburg, MD). The filters in all assays were washed twice with 5 ml 50 mM Tris-HCl (pH 7.4), placed in 4 ml Biofluor and radioactivity determined in a liquid scintillation counter.

All radioisotopes were from New England Nuclear (Boston, MA).

Results

All results described in this paper, unless otherwise noted, are the mean ±S.E. of values obtained from three different preparations. The sarcolemmal marker $(Na^+ + K^+)$-ATPase had a specific activity of 127 ± 1.9 (μmol/mg per h) when measured in 12.5 μg/ml alamethicin (Upjohn Co., Kalamazoo, MI, courtesy of Dr. Joseph Grady) to unmask latent $(Na^+ + K^+)$-ATPase activity in sealed vesicles. As reported by Jones et al. [11], maximal stimulation of $(Na^+ + K^+)$-ATPase activity by alamethicin was 70.6% observed at a ratio of 1 μg alamethicin/μg sarcolemmal protein. Sarcolemmal vesicles exhibited a much smaller stimulation after addition of 1 μg/ml valinomycin. Valinomycin (a K^+-selective ionophore), by allowing K^+ to enter the vesicle, would expose latent activity only in sealed inside-out vesicles. The observed stimulation by valinomycin was less than 10% of the stimulation of $(Na^+ + K^+)$-ATPase activity by alamethicin; therefore, this preparation was composed of predominantly sealed right-side-out vesicles (Table I). Contaminating membranes were estimated to be less than 10% from marker enzume assays [$(Ca^{2+} + Mg^{2-})$-ATPase (sarcoplasmic reticulum), less than 1 μmol/mg per h; cytochrome *c* oxidase

TABLE I

ATPase ACTIVITIES USED TO DETERMINE SIDEDNESS IN SARCOLEMMAL VESICLES

See text for details.

Addition	ATPase activity (μmol/mg per h)		
	Total	Mg^{2+}-ATPase	$(Na^+ + K^+)$-ATPase
Control	62.9 ± 3.4	25.6 ± 3.9	37.3
Alamethicin	155 ± 1.9	28.5 ± 3.2	127
Valinomycin	68.1 ± 4.6	23.3 ± 3.3	44.8

TABLE II

LIPID COMPOSITION OF PURIFIED SARCOLEMMAL VESICLES

Fatty acids were quantified by gas chromatography of fatty acid methyl esters and comparison with an internal standard 17:0 (heptadecanoic acid), see Methods.

	μmol/mg
Total fatty acyl composition	6.17 ± 0.66
Total membrane phosphorus	3.09 ± 0.29
Total cholesterol	0.29 ± 0.03

(mitochondrial inner membrane), 0.32 ± 0.032 μmol/mg per min; monoamine oxidase (mitochondrial outer membrane), 217 ± 19 pmol/mg per min; and angiotensin-II-converting enzyme (endothelial plasma membrane) 1.3 ± 0.3 munit/mg].

An estimate of receptor site density can be obtained from a knowledge of the lipid-to-protein ratio of sarcolemmal vesicles along with the maximum binding capacity of a 'site-specific' compound obtained from the same membrane preparation. The average phospholipid-to-protein ratio of sarcolemmal vesicles was determined by gas chromatography of fatty acid methyl esters and determination of total membrane phosphorus.

Table II shows that the total amount of fatty acid (Table II; 6.17 μmol/mg) was approximately twice the total membrane phosphorus content (3.09 ± 0.29 μmol/mg, Table II) as would be expected if most of the phospholipids contained 2 mol fatty acid/mol phosphate.

Comparison of cholesterol (0.286 ± 0.03 μmol/mg) content with total membrane phosphorus (3.09 ± 0.29 μmol/ml, Table II) indicated that the sarcolemmal membrane contained approx. 10 mol% cholesterol.

For each receptor ligand, maximum binding capacity was determined by Scatchard analysis of saturation isotherms (see Table III). The receptor sites studied were the sodium pump ([^{3}H]ouabain), the muscarinic receptor ([^{3}H]quinuclidinyl benzilate), the beta-adrenergic receptor ([^{3}H]dihydroalprenolol), and the calcium channel ([^{3}H]nitrendipine). The maximum binding capacities obtained for each ligand demonstrate the high degree of purity of this preparation. Further, a comparison of the values suggests that if each [^{3}H]nitrendipine-binding site represents a single Ca^{2+} channel, there are more beta-adrenergic and muscarinic receptor sites than calcium channels.

Utilizing the average phospholipid-to-protein ratio for sarcolemmal vesicles of 3.09 μmol/mg protein (Table II, assuming each mol phospholipid contains 2 mol fatty acid) and the B_{max} data of Table III, the molar ratio of ligand-binding sites to phospholipid could be estimated (see Table IV). The molar ratios obtained ranged from as low as 8000 lipids per ouabain-binding site to as high as 4 000 000 lipids per nitrendipine-binding site. To estimate lipid surface area per site, receptor sites were assumed to be distributed randomly on the sarcolemmal surface and to be located on only one surface of the sarcolemmal membrane. The latter assumption meant that only one-half the total membrane lipids would be used to calculate site density. As thin-section electron microscopy showed this preparation to be composed predominantly of unilamellar vesicles and X-ray diffraction showed that the membrane had a lipid bilayer

TABLE III

RECEPTOR LIGAND BINDING TO PURIFIED SARCOLEMMAL VESICLES

B_{max} (pmol/mg) and K_d (nM) values were obtained from Scatchard analyses. [^{3}H]Ouabain binding was assayed in the presence of 12.5 μg/ml alamethicin to unmask latent binding sites in sealed vesicles, as described in Results.

Ligand	Receptor	K_d	B_{max}
Ouabain (+12.5 μg/ml alamethicin)	sodium pump	32.6 ±2.7	365 ±59
Quinuclidinyl benzilate	muscarinic	0.055 ± 0.009	5.8 ± 0.7
Dihydroalprenolol	beta-adrenergic	4.6 ±1.0	2.2 ± 0.2
Nitrendipine	calcium channel	0.21 ±0.04	0.73 ± 0.09

TABLE IV

ESTIMATION OF RECEPTOR DENSITY IN THE PURIFIED SARCOLEMMAL MEMBRANE

Receptor densities were estimated by comparison of B_{max} values obtained from Scatchard anaysis of binding isotherms and lipid content of purified sarcolemmal vesicles, as described in Results.

Receptor ligand	mol lipid per mol site	Sites/μm^2	
		lipid surface	protein-corrected
Ouabain	$8.5 \cdot 10^3$	400	330
Quinuclidinyl-benzilate	$5.3 \cdot 10^5$	6	5
Dihydroalprenolol	$1.4 \cdot 10^6$	2	1.6
Nitrendipine	$4.2 \cdot 10^6$	0.8	0.6

structure [16], the number of ligand-binding sites per μm^2 of pure phospholipid membrane surface area (Table IV) was calculated using an average surface area of 60 $Å^2$ per phospholipid molecule in the liquid-crystalline state [25], with the assumption that one-half the phospholipid molecules contribute to the surface area of each monolayer of the membrane bilayer. The upper limit of surface area contributed by membrane protein was estimated assuming that all of the protein mass was embedded within the lipid bilayer and that the average molecular weight of the membrane proteins was 91 000 (the molecular weight of the major protein peak found in Coomassie blue-stained gels, Jones et al. [10]). The sarcoplasmic reticulum Ca^{2+} pump protein, estimated to have a molecular volume of 150 000 $Å^3$ [26–28] and a molecular weight of 119 000, was used as a standard to relate molecular weight to molecular volume. Assuming the sarcolemmal protein mass to be a cylinder imbedded in the bilayer equal in length to the width of the sarcolemmal membrane (60 Å, as determined by X-ray diffraction [16]), with a molecular volume of 115 000 $Å^3$ (based on comparison with the sarcoplasmic reticulum Ca^{2+} pump), the protein surface area exposed in the outer membrane monolayer would be 1900 $Å^2$. A molar ratio of 280 mol lipid/mol protein was calculated utilizing 3.09 μmol phospholipid/mg protein (Table II) and assuming an average molecular weight of 91 000 for sarcolemmal protein. The fraction contributed by sarcolemmal protein to membrane surface area would be the surface area of the average sarcolemmal protein divided by the amount of surface area contributed by one-half the amount of phospholipid molecules per protein (i.e., only one-half the phospholipid molecules make up the outer monolayer) plus the area of the protein. Utilizing these calculations, the maximum contribution of protein to the membrane surface area was 18%. The protein-corrected values (shown in the final column of Table IV) and those obtained for a pure phospholipid membrane define the magnitude of site densities for each receptor ligand. The contribution of cholesterol to the surface area of each leaflet of the bilayer, approx. 5% (i.e., one-half the cholesterol content of 10 mol%), was not included in Table IV.

TABLE V

COMPARISON OF RECEPTOR SITE DENSITIES IN SARCOLEMMAL VESICLES WITH THOSE IN INTACT TISSUES

Receptor site	Site densities (sites/μm^2)		Tissue source	Ref.
	This study	Studies in intact sarcolemma		
Sodium pump	330	430–700	guinea-pig left atria	46
		720	cultured rat myocardial cells	45
Calcium channel	0.6	4–5	cultured rat myocardial cells	44
		2–10	guinea-pig ileum	48
β-Adrenergic	1.6	20–200	cultured rat myocardial cells	49
Muscarinic	5	130	frog heart	41

The estimates of site densities obtained are a much more powerful number than maximum binding capacities, since the former can be directly compared to functional site densities in intact muscle preparations and allow meaningful cross-comparisons between different tissues and cell types to be made directly. Such comparisons are illustrated in Table V (see Discussion).

Discussion

The values of lipid composition and binding capacity utilized in this study to calculate site densities are well within the range of values available in the literature. The total cholesterol content of sarcolemmal vesicles (286 ± 30 nmol/mg) is similar to values ranging from 200 to 1000 nmol cholesterol/mg reported in previous studies of rat [7] and rabbit cardiac sarcolemma [29], and rabbit skeletal muscle transverse tubules [30] and sarcolemma [14]. The phospholipid/protein ratio of sarcolemmal vesicles (3.09 μmol/mg) is nearly 3-times that of rabbit skeletal muscle sarcoplasmic reticulum [31,32]. Other studies of skeletal muscle transverse tubule [30] and surface membrane [20], as well as cardiac sarcolemma [14,33] and rat skeletal muscle sarcolemma [31], have also shown phospholipid/protein ratios to be greater than in the sarcoplasmic reticulum membrane.

The present estimates of binding affinity (K_d) and capacity (B_{max}) for [^{3}H]quinuclidinyl benzilate, [^{3}H]dihydroalprenolol and [^{3}H]ouabain are similar to values obtained previously [10,11,15, 34–37]. The [^{3}H]nitrendipine-binding capacity of sarcolemmal vesicles in the present study is greater than previously reported for other canine sarcolemmal preparations [15,35], although estimates of K_d do not differ significantly from those obtained by others. A previous study has shown that [^{3}H]nitrendipine-binding sites are located in the cardiac sarcolemma [37], but others [35,36] have shown [^{3}H]nitrendipine binds to terminal cisternae-enriched subfractions of cardiac sarcoplasmic reticulum with a K_d identical to that found in cardiac sarcolemma. However, no specific nitrendipine binding was found in a 'light' cardiac sarcoplasmic reticulum fraction prepared so as to minimize contamination with sarcolemma and subsarcolemmal cisternae [37]. In skeletal muscle [^{3}H]nitrendipine has been found to bind primarily to a heavy microsomal fraction enriched in t-tubules [38–40], whereas skeletal muscle sarcoplasmic reticulum [37,40] and sarcolemmal membranes [40] being nearly devoid of [^{3}H]nitrendipine binding. These studies can be reconciled if nitrendipine-binding sites are concentrated in regions of the sarcolemma and t-tubules adjacent to subsarcolemmal cisternae, which remain attached to junctional sarcoplasmic reticulum in preparations of heavy, or calcium-loaded microsomes.

The present estimates of receptor site density in sarcolemmal vesicles can be used to estimate receptor site density in the intact sarcolemma, although it should be noted that these estimates would be subject to serious error if the receptor sites are not randomly distributed. Muscarinic receptors have been shown to be randomly distributed in the amphibian ventricle [41]; however, comparison of the present data with those of others [35–37] suggest a nonrandom localization of nitrendipine-binding sites on the sarcolemma and its extensions, the t-tubules (see above). Furthermore, available evidence now suggests that the physiological state of the myocardial tissue can regulate the beta-adrenergic receptor site density [42,43] and the number of functioning calcium channels [44]. Therefore, site densities obtained in this study may represent the unregulated, basal site densities. It is recognized that changes in estimated site density would be expected with changes in physiological state.

The present estimates of receptor site density in sarcolemmal vesicles can be directly compared to estimates of site densities in the intact sarcolemma as illustrated in Table V. The functional site density of the sodium pump has been extensively studied in a variety of mammalian tissues and falls within a narrow range of 400–1000 sites/μm^2. The sodium pump density in the tissue-cultured rat heart [45] and guinea-pig left atria [46], was estimated from [^{3}H]ouabain binding to intact cells and tissues, assuming one ouabain molecule bound to one functioning sodium pump. The values estimated for sodium pump density (Table IV) in sarcolemmal vesicles are comparable to these estimates in intact sarcolemma. The similarities between estimates of sodium pump density in sarcolemmal vesicles and intact tissues also vali-

dates the approach used in this study to estimate site densities in a vesicular preparation. The functional site density of calcium channels obtained from studies of calcium currents by patch clamp analysis of tissue cultured rat heart [47] is within an order of magnitude of the estimated receptor site density for [^{3}H]nitrendipine calculated in this study and in the guinea-pig ileum [48]. This observation suggests that each [^{3}H]nitrendipine-binding site may be associated with only one calcium channel.

Estimates of beta-adrenergic receptor site density in this study are also within one to two orders of magnitude of beta-adrenergic receptor site density obtained in tissue-cultured rat heart [49] and muscarinic receptor site density in frog heart [41]. A comparison of estimated beta-adrenergic and muscarinic receptor site densities with functional calcium channel density demonstrates that receptors site density was greater than functional calcium channel density.

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