CAPRINE CARDIAC SARCOPLASMIC RETICULUM ISOLATION AND BIOCHEMICAL CHARACTERISATION WITH EMPHASIS ON Ca²⁺-ADENOSINE TRIPHOSPHATASE

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ABSTRACT

This study was aimed at isolating, in its pure form, and characterizing the sarcoplasmic reticulum from caprine (Capra hircus) heart. The sarcoplasmic reticulum from thirty caprine heart ventricular homogenates was isolated and purified. It was characterized on the basis of both, its protein and lipid composition. The protein content was 142±10 mg/g of tissue. Ca²⁺-ATPase activity equaled 3.75 ± 1.06mmol Pi/mg protein/min while the uptake rate was 24 ± 1.14 nmol/mg protein/min. 205kD, 110kD, 90kD, 84kD, 66kD, 55kD and 29kD molecular weight proteins were seen on an SDS polyacrylamide gel. Triglyceride, Cholesterol and Phospholipids (phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, sphingomyelin and phosphatidylserine) were present in increasing order of their concentration. Long chain fatty acids predominated over the unsaturated ones. The ryanodine receptor displayed two binding sites for ryanodine. Characterisation encompassing the above biochemical aspects of normal caprine cardiac sarcoplasmic reticulum was thus achieved after isolating it in the pure form.

KEY WORDS

Sarcoplasmic reticulum, Ca²⁺-ATPase, Calcium, Calcium homeostasis, Excitation-contraction coupling

INTRODUCTION

Cardiac muscle consists of myocardial cells containing many bundles of protein strands (myofibrils) surrounded by an extensive network of tubules - the Sarcoplasmic Reticulum (SR). The location and regional specialisation of the SR reflects its role in the excitation-contraction coupling (1). The SR comprises four major functional units: i) The Ca²⁺-ATPase / SERCA2a (or the SR Ca²⁺ pump) responsible for the active uptake of calcium into the SR lumen, transporting two calcium ions from the cytoplasm into the lumen of the SR membrane (in each cycle of the pump) at the expense of one ATP molecule in order to bring about relaxation of a contracted muscle (2) ii) the Ca²⁺-release channels (SRCCs), the most important being the Ryanodine Receptors (2), so called

because they can bind the plant alkaloid ryanodine with nanomolar affinity and high specificity (3), are responsible for the contraction phase of the excitation-contraction cycle, iii) a number of calcium-binding proteins (the most important of which is Calsequestrin), and iv) SR regulatory proteins (e.g. Phospholamban) (2). This membrane thus has all the requirements to substantiate its active role in the excitation-contraction coupling (in the heart) which is dependent on calcium (4).

The SR forms the chief calcium store of the cardiomyocytes, plays a key role in the maintenance of intracellular calcium homeostasis and is actively involved in the excitation-contraction coupling within the heart (5). In the resting state of muscle, the SR is a reservoir of Ca²⁺ ions, the concentration being >10⁻³ M inside the SR sacs as against <10⁻⁶ M in the cytoplasm (6). Depolarisation of the muscle cell is transmitted to the interior by the T tubules and results in a sudden release of calcium from the ryanodine receptors of the SR. The released calcium stimulates muscle contraction. Uptake and sequestering of the calcium by the sarcotubules leads to muscle relaxation. Briefly, studies carried out so far have focused on *in vivo* reversible ischemia and its subsequent

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effects on the heart. Researchers have, in the past, isolated and characterised the canine SR (7). The preparation exhibited efficient, high affinity, ATP-dependent, oxalate-facilitated Ca^{2+} accumulation. The goal of the present study is to establish a correlation between ischemia and the degree of impairment of the functional units of the SR, in particular the effects of ischemia on the Ca^{2+} -ATPase *in vitro*. Knowledge of the levels of proteins involved in SR function is important to understand the pathophysiology of ischemia and to develop new therapeutic strategies. It is also of equal importance to evaluate these proteins in myocardium from the same hearts to appreciate relative changes between the different components of SR function.

This study was undertaken to understand in greater depth the importance of the sarcoplasmic reticulum as a fundamental functional unit of the heart and its major functional units carrying out the heart's fundamental function - excitation contraction coupling. The study was therefore aimed at isolating the SR from caprine heart in the ultrapure form and biochemically studying the functional units under normal conditions with a greater emphasis on Ca^{2+} -ATPase.

MATERIALS AND METHODS :

The SR was isolated from the heart of *Capra hircus* by a modified method of Feher and Davis (8). Briefly, the heart was homogenized in Imidazole Buffer pH 7.0. The homogenate was subjected to ultracentrifugation at 9,000 rpm, 7,000 rpm and 14,000 rpm in a 70Ti Beckman fixed angle rotor to remove the myofibrils, cell debris and lighter fractions including mitochondria. Purification of the crude SR preparation was achieved in sucrose density gradients of 25% and 35% at a centrifugation speed of 20,000rpm in a Beckman SW20 rotor.

Enzyme Assays : The protein content of the SR samples was measured using the method described by Lowry et al (9), with bovine serum albumin as the standard. Both ATPase activities i.e. Ca^{2+} -ATPase (SR marker enzyme) and Na^+/K^+ - ATPase (Sarcolemmal marker enzyme) were determined at 37°C by measuring P_i production (10). Cytochrome c Oxidase, the mitochondrial marker enzyme was assayed (11). Ca^{2+} -uptake activity of the SR vesicles was determined by employing $^{45}\text{Ca}^{2+}$ and the Millipore filtration technique (3).

SDS-PAGE and Western Blot : Gels containing 10% and 4% acrylamide in the separating and stacking gels respectively were run at 200V for approximately 3 hours. 50mg SR was loaded per well. Wide range molecular weight marker from Sigma and BSA - Fraction V (Sigma) were used as standards.

7.5% and 5% gels were also run to visualise the higher molecular proteins, if any. The presence of Ca^{2+} -ATPase (SERCA2a) in the isolated SR preparations was confirmed by subjecting the SDS-PAGE separated SR samples to Western Blot Analysis on nitrocellulose transfer membrane (12).

[^3H] Ryanodine Binding Assay (13) : Briefly, SR membranes (0.1-0.2 mg) were incubated at 37°C for 1 hour in a total volume of 300µl containing 25mM imidazole (pH 7.4), 1MKCl, 1-40nM [^3H]ryanodine (Amersham), 0.95mM EDTA and 1.013mM CaCl_2 . The reaction was terminated by filtering the entire volume of reaction mixture through 0.45mm filter paper (type HA, Millipore), presoaked in the washing buffer (25mM imidazole and 1MKCl), and then washed twice with 5ml of washing buffer. The filter paper was dried at 60°C in an oven, placed in 8ml of scintillation fluid and counted in a Beckman liquid scintillation counter for 5 minutes. The non-specific binding was determined by calculating the binding in the presence of both, varying concentrations of labeled and a fixed concentration of unlabelled ryanodine (10mM). Maximal binding activity (B_{max}) and rate constants were calculated from Scatchard plots of the data.

Assays of Lipids : The content of cholesterol and triglycerides in the SR membrane was determined by routine diagnostic enzymatic methods while that of membrane phosphorus (phospholipids) was determined by estimating P_i (10), all spectrophotometrically. Cholesterol and triglycerides were measured at an OD of 500nm while phospholipids at 820nm.

HPTLC and GLC : Phospholipids were extracted and analysed by unidimensional high performance thin layer chromatography on commercially available silica gel G plates. The solvent system used was chloroform:methanol:water (5.9:2:0.3). The bands were detected by charring after spraying with the developer copper sulphate in phosphoric acid and water. Phospholipid standards (Sigma) were used for comparison. The post-chromatographic derivatisation agent, ninhydrin was used to confirm the presence of the amino-phospholipids phosphatidylethanolamine and phosphatidylserine (14). Total fatty acid composition was qualitatively analysed by gas-liquid chromatography (GLC). SR lipids were extracted as mentioned earlier and converted to their methyl esters by treatment with 50ml absolute methanol and 1.5ml concentrated H_2SO_4 for 4hours. The methylation and extraction of methyl esters was complete with addition of solvent ether. The methyl esters were concentrated in acetone and injected into a Gas Chromatograph having a stainless steel column (2m in length), packed with 10% Free Fatty Acid

Phase (FFAP) and a flame ionisation detector. The flow rate of nitrogen was 30ml/min and the oven temperature maintained at 150-270°C.

Electron Microscopy - SR samples for thin sections were fixed in cold 3% glutaraldehyde solution for 2 hours and treated with osmium tetroxide. After dehydration and embedding, thick sections were cut on an LKB Ultratome V, dried on a hot plate (60°C) and stained with 1% toluidine blue and observed under the light microscope. The prepared blocks were trimmed, ultrasections cut and placed on 300 mesh size copper grids. Contrast was enhanced by a double staining technique - alcoholic uranyl acetate and lead citrate. Grids were observed under a JEOL -1010 electron microscope at 80kV accelerating voltage.

RESULTS

We have isolated and characterised the SR from thirty normal male caprine hearts of average ventricular weight 42 ± 4 g.

Characterisation of the sarcoplasmic reticulum : The protein content of the isolated SR preparations averaged 142 ± 10 µg/g of tissue. The SR marker Ca^{2+} -ATPase shows appreciable activity while both mitochondrial and sarcolemmal markers show relatively low activities as indicated in Table 1. Table 2 shows the Ca^{2+} -uptake activity of the SR. SDS-PAGE shows approximately fifteen clearly visible bands after subjecting the pre-treated SR samples to electrophoresis on 10% (figure 1) and 5% (not included) SDS-Polyacrylamide gels. Bands were seen at molecular weights 205kD, 110kD, 90kD, 84kD, 66kD, 55kD, 29kD. Several bands of fairly high intensity were also visible in the range of 45-55kD as well as on the front indicating that the SR contains a number of low molecular weight proteins too. A Western Blot (figure 2) of the SR samples when probed with monoclonal antibody to Ca^{2+} -ATPase

Table 2 : Ca^{2+} -Uptake Rate of the SR

Ca^{2+} -UPTAKE RATE (nmol Ca^{2+} /mg protein/min)			
Caprine ventricles (n=30)	Rabbit ⁽¹⁸⁾	Rat ventricles ⁽¹⁸⁾	Canine ⁽¹⁸⁾
24 ± 1.14	38.6 ± 2.3	40.2 ± 1.6	59.4 ± 0.24

showed a deep bluish gray band in the region between 116kD and 97kD. A Scatchard plot A of total ryanodine binding revealed a non-linear curve which could be resolved into two different affinity sites with differing B_{max} and K_d values given in Table 3.

The lipid composition of the SR membranes was also studied in addition to the major functional components, the proteins

Figure 1. Coomassie Brilliant Blue Stained SDS-PAGE (10%) pattern of 6 caprine cardiac sarcoplasmic reticulum samples. Lanes 1 and 2 are standards. Lane 1: BSA (60kD) Lane 2: Wide range molecular weight marker from Sigma. Thirteen components - Myosin (205kD), β -Galactosidase (116kD), Phosphorylase b (97kD), Fructose-6-Phosphate kinase (84kD), BSA (66kD), Glutamic Dehydrogenase (55kD), Ovalbumin (45kD), Glyceraldehyde-3-Phosphate Dehydrogenase (36kD), Carbonic Anhydrase (29kD), Trypsinogen (24kD), Trypsin Inhibitor (20kD), α -Lactalbumin (14.2kD) and Aprotinin (6.5kD). Lanes 3-8: Caprine Cardiac Sarcoplasmic Reticulum Samples of six individual caprine hearts (50mg each), freshly isolated and heated for 5 minutes in a boiling water bath after addition of a specific volume of sample buffer containing β -mercaptoethanol, Bromophenol Blue, SDS, Glycerol and Stacking Gel Buffer pH 6.8.

Table 1 : Marker enzyme activity

Enzyme	Value of Marker in membrane of origin	Specific activity in normal caprine cardiac SR
$\text{Mg}^{2+}/\text{Ca}^{2+}$-ATPase µmol Pi/mg protein/min	$3.09 \pm 0.11^{(8)}$	3.75 ± 1.06
Cytochrome c Oxidase mmoles/min/mg protein	$0.454 \pm 0.18^{(27)}$	0.00029 ± 0.000015
Na^+/K^+ - ATPase µmol Pi/mg protein/min	$2.12 \pm 0.03^{(16)}$	0.45 ± 0.033

Figure 2. Western Blot of the Ca^{2+} -ATPase/SERCA 2a of the caprine cardiac sarcoplasmic reticulum samples run on a 10% SDS polyacrylamide gel. SR samples 6, 7, 8 and 9 were run in duplicates as indicated on the photograph above. One set of samples was probed with primary antibody i.e. anti-SERCA2a antibody and later treated with biotinylated secondary antibody, Streptavidin-ALP and developer (PLUS PRIMARY ANTIBODY). The other set was only subject to biotinylated secondary antibody, Streptavidin-ALP and developer (MINUS PRIMARY ANTIBODY). The markers are: (1) wide range molecular weight marker (SIGMA) labelled 'M'. It contains thirteen components - Myosin (205kD), β -Galactosidase (116kD), Phosphorylase b (97kD), Fructose-6-Phosphate kinase (84kD), BSA (66kD), Glutamic Dehydrogenase (55kD), Ovalbumin (45kD), Glyceraldehyde-3-Phosphate Dehydrogenase (36kD), Carbonic Anhydrase (29kD), Trypsinogen (24kD), Trypsin Inhibitor (20kD), α -Lactalbumin (14.2kD) and Aprotinin (6.5kD) and (2)BSA (60kD)

Figure 3. 3-D Scan of High Performance Thin Layer Chromatographic Plate of SR Samples after derivatisation with cupric sulphate

Lanes 1, 2 and 3 (starting from the baseline): standards

Lane 1: Standard Mix (Phosphatidylethanolamine [PE], Phosphatidylinositol [PI], Phosphatidylcholine [PC] and Lysophosphatidylcholine [LPC]); Lane 2: Phosphatidylserine [PS]; Lane 3: Sphingomyelin [Sph]; Lanes 4 to 9: SR samples

Table 3 : Ryanodine Receptor Binding Studies

SAMPLE SIZE	RYANODINE BINDING SITES	$B_{\max}$ (pM/mg)	K_d (nM)
3	High affinity site	0.2	0.143
	Low affinity site	10666.7	1666.7

to establish the identity of the phospholipids contributing to the fluidity of the membrane, an essential factor for the maintenance of membrane function; Cholesterol, Triglycerides and Phospholipids being the major lipid components. Phospholipids were comparatively present in higher quantities as shown in Table 4. Caprine cardiac SR contained a higher concentration of phosphatidylethanolamine (PE) followed by phosphatidylinositol (PI) and phosphatidylcholine (PC) with sphingomyelin (Sph) being comparatively lower as seen in figure 3. Phosphatidylserine (PS) was present in insignificant amounts that were detected only by post-chromatographic derivatisation with ninhydrin (figure 4) to selectively derivatise phospholipids containing amino group. A pilot GLC study was carried out to assess the fatty acid composition of three SR samples. The SR was found to contain octanoate, decanoate, laurate, myristate, palmitate, stearate, cis-oleate, linolenate, linoleate and arachidate. Linoleate, stearate, palmitate and cis-9-oleate were found to be present in higher concentrations

(Table 5 and figure 6).

Electron Microscopy - As shown in figure 7, Electron Microscopy studies of the isolated SR samples revealed an irregularly shaped double-membrane structure without any membrane disruption.

DISCUSSION

SR from thirty caprine hearts were successfully isolated and characterised (on the basis of both its protein and lipid composition). The low activities of the sarcolemmal (15) and mitochondrial (16) marker enzymes and the electron micrographs (figure 6) indicate that the isolated SR is in a fairly purified form.

The presence of the SR in the resulting preparation was confirmed within a week by estimating its Ca^{2+} -ATPase (marker enzyme of the SR) activity. As seen in Table

Table 4 : Lipid profile of caprine cardiac SR

SAMPLE SIZE	TRIGLYCERIDE CONTENT (μ moles/mg SR protein)	CHOLESTEROL CONTENT (μ moles/ mg SR protein)	PHOSPHOLIPID CONTENT (μ moles of Pi / mg protein)
30	0.142 ± 0.02	0.321 ± 0.065	2.48 ± 0.224

1, the activity was found to be comparable to other animal systems (7, 8). Loss of enzyme during the isolation procedure was found to be minimal.

The main function of the SR Ca^{2+} -ATPase is the uptake of calcium into the SR lumen leading to relaxation. The Ca^{2+} -uptake rate of the SR is a function of the enzyme activity itself. The samples showed a rather low uptake rate when compared to both male Sprague Dawley rats and canine systems (Table 2). This difference is attributed to both, the differing animal systems and the effect of freezing on the SR vesicles (17). Also contributing to this observation is the possibility that the calcium pumping activity is less concentrated in the caprine cardiac SR *in situ*. This is seen as a lower density of pumping activity in the isolated SR. The pump may also become inactivated upon isolation (8). Conflicting results can also arise out of differences in (a) kinetics, regulation and density of the

Table 5 : Fatty acid profile of caprine cardiac SR Peaks obtained in the chromatograms

Standard Components	Chain Length	Sample Components
Octanoate	C8:0	Octanoate
Decanoate	C10:0	Decanoate
Laurate	C12:0	Laurate
Myristate	C14:0	Myristate
Palmitate	C16:0	Palmitate
Palmitoleate	C16:1	Stearate
Stearate	C18:0	Oleate
Oleate	C18:1	Linoleate
Linoleate	C18:2	Linolenate
Linolenate	C18:3	Arachidate
Arachidate	C20:0	
Behenate	C22:0	
Erucate	C22:1	
Lignocerate	C24:0	

Ca^{2+} pump, (b) surface area of the membrane per volume of tissue, (c) volume of organelle per volume of tissue, (d) amount and characteristics of SR-associated binding proteins and (e) kinetics, regulation and density of calcium channels (18).

Approximately twenty bands, of clear visibility and others of

fairly high intensity, were seen when isolated caprine cardiac SR samples were run on SDS polyacrylamide gel (figure1). When compared to earlier research work, a similar pattern was obtained in rabbit ventricle SR samples (3), though the intensity and hence concentration of individual proteins was different. The components of the canine cardiac SR have been identified and characterised. Prominent among these are a 105kD Ca^{2+} -ATPase, phospholamban of approximately 27-29kD, a 55kD calsequestrin, two glycoproteins of 130kD and 53kD and a high molecular weight ryanodine receptor of >400kD (19). Species differences in protein components are very prominent in SR samples. Rabbit skeletal, canine and human cardiac SR samples, when run simultaneously (19), showed varied differences in banding pattern. Human cardiac

Figure 4. Scan of High Performance Thin Layer Chromatographic Plate of SR Samples after derivatisation with ninhydrin

Lanes 1, 2 and 3 (starting from the baseline): standards; Lane 1: Standard Mix (Phosphatidylethanolamine [PE]); Lane 2 : Phosphatidylserine [PS]; Lane 3 : Not visible; **Lanes 4 to 9:** SR samples [only PE and PS are visible]

Figure 5: Standard methyl esters of fourteen fatty acids

SR samples show many more bands in the region between 97.4kD and 45kD than its canine cardiac counterpart. In contrast rabbit skeletal muscle triads showed a sparse distribution of bands in the region from the front to 45kD and no visible bands between 97.4kD and 45kD when stained with Coomassie Brilliant Blue.

In a preliminary study on the ryanodine receptors, data from three SR samples were evaluated. The Scatchard plot A of the averages of the data obtained, showed distinctly different maximal binding activities (B_{max}) and dissociation constants (K_d) as seen in Table 3. Other studies showed a similar pattern, though values differ due to a difference in the populations of SR used in their study. Many researchers have made a comparative study on the skeletal and cardiac SR to assess the difference/similarity between the two (20). Cardiac SR is approximately 7 times more sensitive to ryanodine and display two binding sites, high affinity and low affinity binding sites as against its skeletal counterpart that shows only one binding site. This is attributed to the slight variation in the function of the SR in both

muscle systems (20).

The major type of lipids encountered in our isolated SR membranes was phospholipids (Table 4). The phospholipid to protein ratio in the SR is approximately 1.7. Cholesterol and Triglycerides were also present in low concentrations. After subjecting our SR samples to high performance thin layer chromatography, we noted a predominance of phosphatidylethanolamine, phosphatidylinositol and phosphatidylcholine over sphingomyelin and phosphatidyl-serine in the phospholipid bilayer. The phospholipid composition of caprine cardiac SR is similar to that obtained by other researchers for the canine system (7).

Since the SR is actively involved in excitation-contraction coupling, the maintenance of calcium ion concentration within and external to the SR is very important. Hence, the SR phospholipid bilayer is required to alter its function suitably. This is most likely accomplished by a predominance of phosphatidylethanolamine, an amphoteric

Figure 6 : methyl esters of fatty acid components of SR sample

Figure 7. EM OF NORMAL (i.e. UNTREATED) CAPRINE CARDIAC SR SAMPLES (n=6)

phospholipid in the membrane. Phosphatidylethanolamine has an amphoteric headgroup that might be able to assume either a negative or a positive charge as per the demand of the cytosolic environment. The high occurrence of acidic phospholipid (phosphatidylinositol) may form calcium binding sites (21) and facilitate the SR in its function of sequestering the positive calcium ions.

Phospholipids and cholesterol form the major membrane lipids of the caprine cardiac SR. Phospholipids predominate almost eight times over cholesterol. Phospholipids influence conformational changes in phospholamban, a key regulator of the SR Ca^{2+} -ATPase (22). The phospholipids contained in the SR are known to stabilize the pentameric form of phospholamban (23), a conformation that does not inhibit the SR Ca^{2+} -ATPase thus facilitating Ca^{2+} uptake into the SR lumen. Phospholipid headgroups stabilise phospholamban and other membrane proteins through interaction with their cytoplasmic domains; thus conferring on phospholipids an ability to modulate the function of biological membranes (24).

Membrane fluidity is dictated by the fatty acyl chains of the phospholipids: their chain length, conformation of the hydrocarbon chain tails, degree of bond saturation in the tail and type of substituent in the head groups. The fluidity, in turn, influences the transport rate of component transmembrane proteins (25). In view of this, a pilot GLC study was performed to assess the fatty acid composition of the three SR samples. The SR was found to contain octanoate, decanoate, laurate, myristate, palmitate, stearate, cis-oleate, linolenate, linoleate and arachidate. Linoleate, stearate, palmitate and cis-9-oleate were found to be present in higher concentrations (Table 5 and figure 6). These fatty acids allow for enzymes (viz. Ca^{2+} -ATPase) to switch between activated

and inactivated conformation states as per the requirement of the membrane.

Cholesterol was estimated by spectrophotometric analysis. This too is a key regulator of membrane fluidity. It acts as a moderator molecule in membranes producing intermediate states of fluidity. High cholesterol:phospholipid ratios abolish phase transitions altogether (26). The ratio (1:8) in our isolated SR samples was low enough to maintain the fluidity of the membrane and allow for phase transitions within the bilayer.

The SR from caprine heart has been successfully isolated and biochemically characterized on the basis of both its protein and lipid contents. The caprine SR has not yet been characterized in depth. This study provides an insight into the SR membrane of yet another mammalian system and a deeper understanding of this SR in comparison to other highly researched animal systems viz. canine and male Sprague Dawley rats.

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