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SOLUBILIZATION AND SEPARATION OF Ca^{2+}-ATPase FROM THE Ca^{2+}-RYANODINE RECEPTOR COMPLEX

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Heavy sarcoplasmic reticulum (SR) preparations of rabbit skeletal muscle, which are enriched in Ca^{2+}-release vesicles from the terminal cisternae (TC) and [^{3}H]ryanodine receptor density, exhibit 60% of the Ca^{2+}-ATPase activity, 58% of the *E*P level, and 30% of the steady state Ca^{2+} loading compared to membrane vesicles from the longitudinal SR. The Ca^{2+}-ATPase of TC SR is solubilized and separated from the Ca^{2+}-ryanodine receptor complex in the insoluble fraction on treatment with the detergent $C_{12}E_9$. However, a 50% decrease in receptor density is observed upon removal of the Ca^{2+}-ATPase, suggesting a significant contribution of this protein to maintaining optimal receptor complex density.

Specific probes are required to define the molecular mechanisms of excitation-contraction coupling at the relevant SR Ca^{2+} channels (1) which appear to be localized in the TC of the SR (2-8). Several chemical interventions can trigger Ca^{2+} release from "heavy" SR vesicle preparations, i.e. those enriched in membranes whose origin is the TC. Physiologically-relevant candidates as possible chemical links in excitation-contraction coupling include Ca^{2+} (Ca^{2+}-induced Ca^{2+} release) (2,9), adenine nucleotides (10), and IP_3 (11).

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Abbreviations: B_{max}, maximum number of binding sites; $C_{12}E_9$, polyoxyethylene 9-lauryl ether; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CS, calsequestrin; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; *E*P, phosphorylated intermediates of Ca^{2+}-ATPase; IP_3, 1,4,5-inositol trisphosphate; K_d, equilibrium binding dissociation constant; kDa, kilodalton; Mops, 3-(N-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SR, sarcoplasmic reticulum; TC, terminal cisternae; t-tubule, transverse tubule.

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[^{3}H]Ryanodine (12) is the best probe available to identify SR proteins associated with the Ca^{2+}-release channel(s). It binds to specific sites localized in vesicles originating from the TC of skeletal muscle (13-15) and heavy SR preparations from cardiac muscle (13). Salient features of the ryanodine binding domain include exclusive regulation by Ca^{2+} for both activation and inactivation of the alkaloid binding site, inhibition by Mg^{2+}, modulation by nucleotides, and association with SR vesicles containing Ca^{2+}-induced Ca^{2+} release channels (13-15). We proposed from these findings that ryanodine binds to the Ca^{2+}-activated open state of the channel involved in the release of contractile Ca^{2+} from the SR (15). This results in a change in channel structure possibly preventing its complete closing (16). Solubilization of a functional Ca^{2+}-ryanodine receptor retaining many of the properties of the membrane-associated complex has permitted direct examination of the proteins involved (15). This study evaluates the contribution of the Ca^{2+}-ATPase to the Ca^{2+}-ryanodine receptor complex.

EXPERIMENTAL PROCEDURES

Preparation of SR membranes. Previously-described procedures were used to prepare heavy SR membrane vesicles enriched in CS and morphological (exhibiting the intact junctional feet structures) markers of the TC (15) and SR vesicles enriched in elements of longitudinal origin (17).

Solubilization of SR proteins. Non-junctional SR proteins (primarily the Ca^{2+}-ATPase) were selectively solubilized by treating the TC SR membranes with 1 mM $CaCl_2$, 20% sucrose, and varying amounts of $C_{12}E_9$ in 50 mM Mops at pH 7.0 (18). The $C_{12}E_9$:protein ratio was varied from 0.5:1 to 10:1 keeping the final protein concentration at 5 mg/ml. Junctional proteins were solubilized by treating the $C_{12}E_9$-insoluble proteins in the pellet from the first step with 1M NaCl, 2 mg CHAPS/mg protein and 40 mM Tris/maleate at pH 7.1, with shaking for 30 min at 0°C. In both steps soluble proteins were separated from insoluble materials by centrifugation at 110,000 x g for 60 min.

ATPase rate determinations. Ca^{2+}-independent ATPase (Mg^{2+}-ATPase) and total (Ca^{2+} + Mg^{2+}) ATPase activities were measured at 37°C and pH 7.0 using a coupled-enzyme method described previously (19). For measuring Mg^{2+}-ATPase rates, the assay medium (1 ml) consisted of 10 µg SR protein, 110 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 2 mM MgATP, 2 mM phosphoenolpyruvate, 5 IU pyruvate kinase and 10 IU lactate dehydrogenase in 50 mM Mops at pH 7.0. For measuring total ATPase activity, the assay medium just described contained in addition 1.1 mM $CaCl_2$, to give about 90 µM free Ca^{2+}. In some experiments the Ca^{2+} ionophore A23187 (calimycin) was also included at 1 µg/ml.

$^{45}Ca^{2+}$-loading and release measurements. SR vesicles (0.2 mg protein) were incubated at 37°C in an assay medium (0.96 ml) consisting of 0.3 µCi $^{45}Ca^{2+}$, 0.1 mM $CaCl_2$, 50 mM KCl, 0.6 mM $MgCl_2$, and 0.1 mM EGTA in 50 mM Mops at pH 7.0. Active Ca^{2+} loading was initiated by addition of 40 µl of 50 mM

MgATP. Ca^{2+}-loading measurements were conducted in the above medium where Ca^{2+} was adjusted based on a computer program with published EGTA binding constants (20) to give final free Ca^{2+} levels ranging from 0.7 to 80 μM. Sample aliquots of 900 μl were rapidly filtered through Millipore HAWP cellulose acetate filters at times ranging from 30 sec to 5 min and the filtrates were assayed for $^{45}Ca^{2+}$ by liquid scintillation counting (21).

[^{3}H]Ryanodine binding assays. Equilibrium binding to membranes and $C_{12}E_9$-insoluble proteins was determined as previously described (13) except that the assay medium (1 ml) consisted of 50 μg protein, 5 nM [^{3}H]ryanodine (60 Ci/mmol), 300 μM $CaCl_2$, 115 mM KCl, 15 mM NaCl, 1.6 mM $MgCl_2$, 1 mM ATP, 100 μM cAMP, and 10% sucrose in 40 mM Tris/maleate at pH 7.1. Appropriate additions of unlabeled ryanodine gave final concentrations as specified. Binding to soluble receptors was assayed by filtration through polyethylenimine-treated glass fiber filters (15) with the exception that the incubation medium was composed of 50 μg protein, 5 nM [^{3}H]ryanodine (with unlabeled ryanodine added as required), 300 μM $CaCl_2$, 0.1% CHAPS, 0.5M NaCl, 1.6 mM $MgCl_2$, 1 mM ATP and 100 μM cAMP in 40 mM Tris/maleate at pH 7.1. Values of B_{max} and K_d were determined by least squares linear regression analysis of Scatchard plots.

SDS-PAGE. Proteins (1 mg/ml) (denatured at 95°C for 5 min in 1 mM EDTA, 1% SDS, 5% 2-mercaptoethanol, and 0.2 mM bromophenol blue in 10 mM Tris-HCl at pH 8.0) were electrophoresed on 3-10% linear-gradient polyacrylamide slab gels (Isolab, Akron, OH) in 2 mM EDTA, 0.2% SDS and 20 mM Na acetate in 40 mM Tris at pH 7.4. Samples (30 μg protein) were electrophoresed at 300 volts for 10 min followed by 150 volts for 2.5 hr (22). Gels were stained with Coomassie blue.

RESULTS AND DISCUSSION

Preparations of heavy SR vesicles enriched in TC membranes differ from longitudinal SR vesicles in their lower ATPase activity and steady state Ca^{2+} loading capacity (Table I) and their higher [^{3}H]ryanodine binding site density (15). The TC SR preparations exhibit 60% of the Ca^{2+}-ATPase activity on a protein basis (Table I) and 58% of the *E*P level (4.2 *vs* 7.2 nmol mg protein^{-1}) compared to the longitudinal SR preparations. The lower Ca^{2+}-ATPase level in TC SR is consistent with findings on highly purified TC membranes isolated by a different protocol and is due only in small part to the presence of junctional face regions of the TC membrane which lack the Ca^{2+}-ATPase protein (23). The principal function of Ca^{2+}-ATPase in the longitudinal cisternae of the SR is to decrease the myoplasmic free Ca^{2+} concentration. In the presence of the Ca^{2+} ionophore A23187, Ca^{2+}-ATPase activity is markedly increased in longitudinal SR vesicles (to 9.77 ± 0.06 μmol P_i mg protein^{-1} min^{-1}) as well as in TC SR vesicles (to 4.82 ± 0.96 μmol P_i mg protein^{-1} min^{-1}), suggesting that the TC SR membranes are not freely permeable to Ca^{2+}. However, TC SR vesicles have approximately a 3-fold

TABLE I

ATPase and Ca^{2+}-Loading Activities of Longitudinal and TC Membranes and ATPase Activities of $C_{12}E_9$ Detergent-Treated TC Membranes

Type of activity	Longitudinal membranes	TC		
		Membranes	$C_{12}E_9$-treated	
			Soluble	Insoluble
ATPase (μmol P_i mg protein^{-1} min^{-1})				
Total	3.50 ± 0.21	2.24 ± 0.36	4.85 ± 0.63	1.08 ± 0.54
Mg^{2+}-ATPase	0.46 ± 0.35	0.39 ± 0.07	0.28 ± 0.02	0.67 ± 0.03
Ca^{2+}-ATPase	3.04	1.85	4.57	0.41
Steady state Ca^{2+}-loading (nmol Ca^{2+} mg protein^{-1})	120	40		

TC membranes were extracted with $C_{12}E_9$ (3 mg detergent/mg protein) as described under Materials and Methods. Ca^{2+}-ATPase was determined as the difference between Mg^{2+}-ATPase (with Mg^{2+} alone) and the total ATPase (with Ca^{2+} and Mg^{2+}). SD values from at least 3 experiments. Steady state Ca^{2+} loading values were obtained in a medium containing 1 μM free Ca^{2+}.

higher density of [^{3}H]ryanodine binding sites (15) and exhibit 33% of the steady-state Ca^{2+}-loading level of the longitudinal SR when assayed under the same conditions (Table I), suggesting that the TC SR vesicles are more permeable to Ca^{2+}.

TC SR vesicles loaded in a medium buffered at a high level of free Ca^{2+} (80 μM) display large oscillations in Ca^{2+} uptake levels (not shown) while at lower concentrations of Ca^{2+} (0.7 or 3.4 μM, Fig. 1) a stable level of filling is attained. Ca^{2+} release from loaded vesicles is triggered by increasing the Ca^{2+} concentration to 7 μM (Fig. 1A). The rapid release of Ca^{2+} is followed by re-uptake to the original steady state loading level. However, when 100 μM ryanodine is added simultaneously with the trigger Ca^{2+}, Ca^{2+} re-uptake is reduced by ~70% (Fig. 1A). TC SR vesicles preincubated for 30 min with 3 μM ryanodine prior to Ca^{2+} loading exhibit similar initial uptake rates to those of control vesicles but then quickly lose their ability to retain Ca^{2+} (Fig. 1B). These results indicate that Ca^{2+} channels of the TC SR are influenced by the free Ca^{2+} concentration in the medium and that

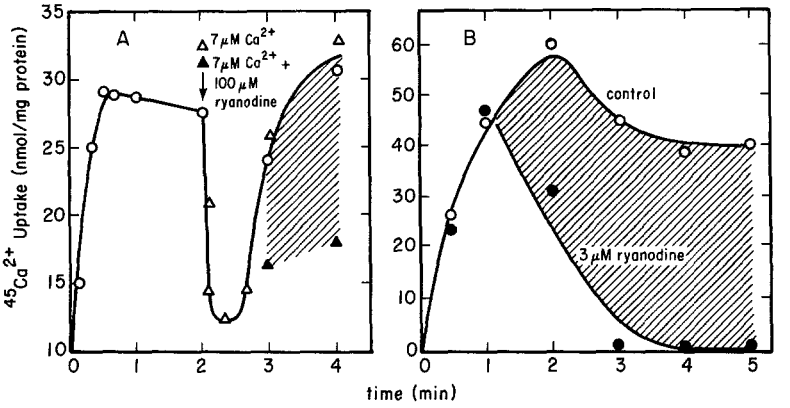

Fig. 1. Effect of Ca^{2+} and ryanodine on $^{45}Ca^{2+}$-loading of TC SR vesicles. A gives the level of ATP-dependent Ca^{2+} uptake for 4 min at 0.7 μM free Ca^{2+} (o) or for 2 min after increasing the free Ca^{2+} to 7 μM (arrow) in the presence (▲) or absence (Δ) of 100 μM ryanodine. B gives the level of ATP-dependent Ca^{2+} uptake at 3.4 μM free Ca^{2+} for 5 min following 30 min preincubation of vesicles at 37°C in the presence of 100 μM cAMP with (•) or without (o) 3 μM ryanodine prior to initiating Ca^{2+} uptake with MgATP. Shaded areas designate ryanodine inhibition of uptake.

ryanodine interferes with the inactivation (or complete closing) of the Ca^{2+}-induced open state of these channels. These effects are induced almost immediately at high alkaloid concentrations (Fig. 1A) but are also observed at low concentrations when the alkaloid is preincubated under conditions which optimize receptor binding (Fig. 1B) (16). The action of ryanodine on Ca^{2+} loading is not a direct effect on the Ca^{2+} pump protein since Ca^{2+}-ATPase activity is not inhibited even at 100 μM ryanodine.

Treatment of TC SR membranes with $C_{12}E_9$ results in selective solubilization of Ca^{2+}-ATPase activity (Fig. 2A) and concomitant loss from the insoluble fraction of the 110 kDa protein band of Ca^{2+}-ATPase (Fig. 3). Protein solubilization increases with $C_{12}E_9$ concentration in the extraction medium to an optimum at 3 mg $C_{12}E_9$/mg protein, but reaches a maximum of 60% of the total proteins even at 10 mg $C_{12}E_9$/mg protein (Fig. 2A). The majority (~90%) of the [^{3}H]ryanodine binding sites remain associated with the unextractable proteins in the pellet, although at very low detergent to protein ratios (i.e., 0.5:1) ~10% of the total specific binding sites are consistently observed in the supernatant (Fig. 2B). These lower levels of $C_{12}E_9$ also solubilize an appreciable amount of CS with the Ca^{2+}-ATPase (not

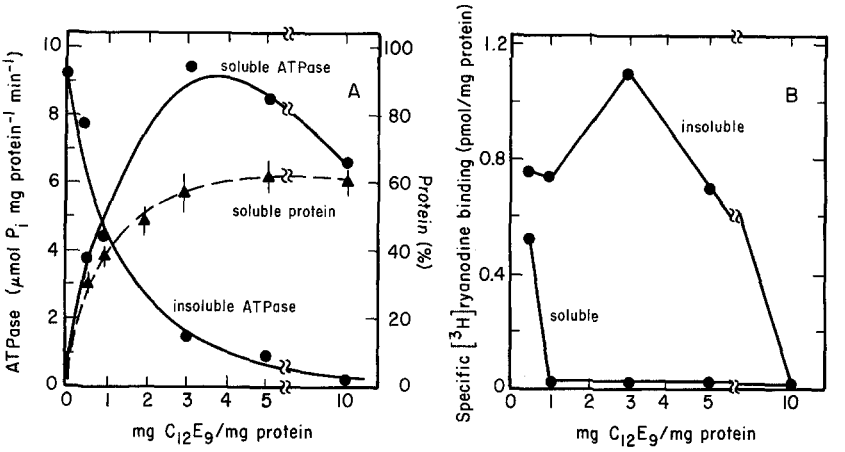

Fig. 2. Effect of extraction of TC SR membranes with increasing amounts of $C_{12}E_9$ on solubilization of proteins, ATPase activity and binding of [^{3}H]ryanodine. A gives protein solubilization and soluble and insoluble ATPase activities and B gives binding of 5 nM [^{3}H]-ryanodine in the soluble and insoluble fractions.

shown), supporting earlier findings which implicate CS in maintaining a functional receptor complex (15).

Mg^{2+}-ATPase activity is a minor component of the total ATPase of the TC SR preparations, but it distributes with and is enriched 1.7-fold in the $C_{12}E_9$ protein residue (Table I); these insoluble proteins also contain most of the [^{3}H]ryanodine binding sites (Fig. 2B). This Mg^{2+}-ATPase appears to be

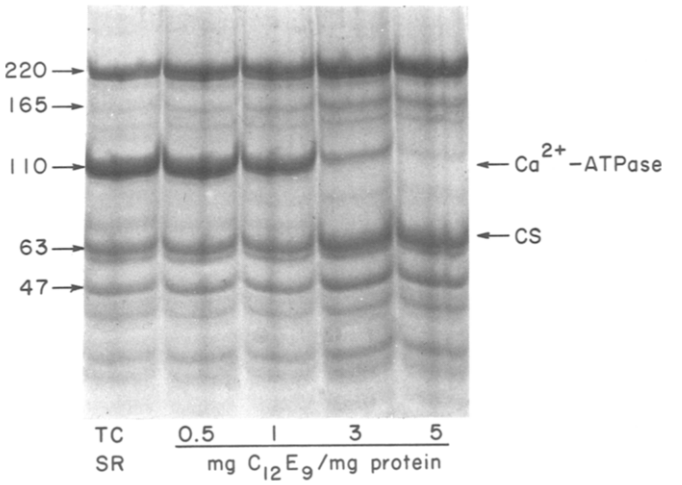

Fig. 3. SDS-PAGE of TC SR membranes and insoluble protein residues from extraction with increasing amounts of $C_{12}E_9$. Each lane was loaded with 30 μg protein. Molecular weights are given as kDa.

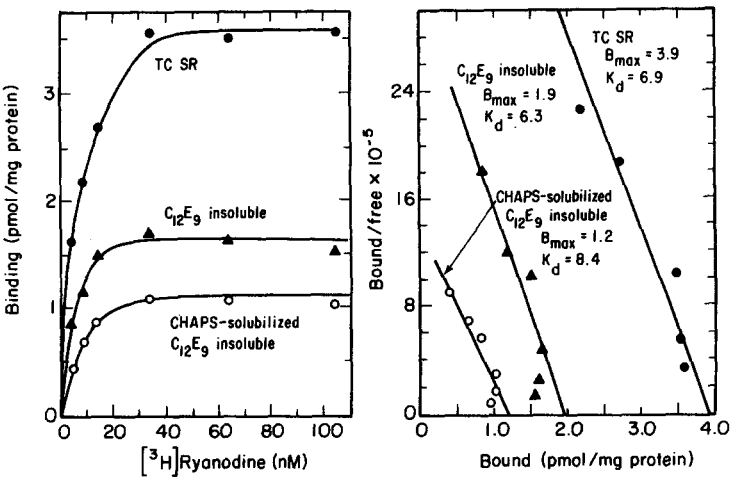

Fig. 4. Influence of extraction of TC SR with $C_{12}E_9$ (3 mg detergent/mg protein) on specific binding of [^{3}H]ryanodine shown as binding isotherms and Scatchard plots giving B_{max} (pmol/mg protein) and K_d (nM) values. The $C_{12}E_9$-insoluble fraction was assayed directly or after solubilization with CHAPS/NaCl.

of SR origin since it is insensitive to conconavalin A at a level (5 µg/mg protein) known to activate the t-tubule Mg^{2+}-ATPase (24) and electron microscopy reveals that the TC SR preparations used are free of myosin contamination. These findings can be rationalized on the basis of a relationship between the Ca^{2+}-ATPase released to the supernatant by $C_{12}E_9$ and both the [^{3}H]ryanodine binding domain (Fig. 2B) and the Mg^{2+}-ATPase (Table I) remaining as insoluble proteins in the pellet. Low $C_{12}E_9$ to protein ratios release [^{3}H]ryanodine binding sites into the soluble fraction whereas the high levels destroy binding in both the soluble and insoluble fractions, indicating that perhaps optimization of the Ca^{2+}-ryanodine receptor complex reflects the aggregational state of the protein components of the TC SR.

Removal of Ca^{2+}-ATPase on $C_{12}E_9$ solubilization of TC SR membranes results in ~ 50% reduction in receptor density in the insoluble fraction (3.9 vs 1.9 pmol mg protein^{-1}) with no significant change in affinity (6.9 vs 6.3 nM) (Fig. 4). Subsequent solubilization of these proteins with CHAPS and high ionic strength results in an additional 37% reduction in original (TC SR) receptor density (Fig. 4); this reduction is consistent with our previous findings on direct CHAPS/NaCl solubilization of TC SR membranes (15). The [^{3}H]ryanodine binding site on CHAPS solubilization of the $C_{12}E_9$-

insoluble pellet is a high molecular weight oligomer of >1000 kDa based on Superose gel high pressure liquid chromatography. SDS-PAGE of $C_{12}E_9$-insoluble proteins reveals major bands at 220, 165, 63 (CS), and 47 kDa (Fig. 3).

This study indicates that Ca^{2+}-ATPase (in its pump form) contributes significantly to optimization of the [^{3}H]ryanodine receptor site density, and hence the density of functional channels, without directly comprising the binding domain. Large changes in the tryptophan fluorescence and $E\tilde{P}$ level of the Ca^{2+}-ATPase of the TC just prior to Ca^{2+} release suggest that it undergoes a conformational change which may be critical to the Ca^{2+}-release process (25). The Ca^{2+}-ATPase protein clearly plays a functional role in the Ca^{2+}-ryanodine receptor complex and possibly also in Ca^{2+} release at the TC SR.

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