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Ca^{2+}-dependent ryanodine binding site: soluble preparation from rabbit cardiac sarcoplasmic reticulum

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The Ca^{2+}-dependent ryanodine binding site of rabbit cardiac sarcoplasmic reticulum is solubilized by treatment with 20 mM CHAPS detergent and 1 M NaCl for 30 min at 0°C. Ca^{2+} added at 5 μM enhances binding, at 0.5 mM increases both the affinity and number of [^{3}H]ryanodine binding sites, while at 10 mM only the number of binding sites is increased. Mg^{2+} up to 1 mM does not significantly affect [^{3}H]ryanodine binding. Radioligand binding is strongly enhanced by all alkali metal chlorides except LiCl. NaCl increases the rate of association of the ligand and the affinity of the binding site but does not influence the dissociation. NaCl and $CaCl_2$ enhance the thermal stability of the [^{3}H]ryanodine-binding protein. Thiol groups are essential for [^{3}H]ryanodine binding. Ruthenium red and Cd^{2+} inhibit binding, while theophylline is stimulatory at low (micromolar) Ca^{2+} concentrations by a mechanism other than phosphodiesterase inhibition. Gel permeation chromatography establishes that the ryanodine binding protein is localized only in the high molecular mass fraction (> 669 kDa). Polyacrylamide gel electrophoresis of the proteins following treatment with SDS and 2-mercaptoethanol indicates that more than 90% are of low molecular mass (34–70 kDa) and that two stain blue with Stains-all as expected of Ca^{2+}-binding proteins.

Introduction

Ryanodine, the insecticidal alkaloid of *Ryania speciosa* [1], is a useful pharmacological probe to study Ca^{2+} release in cardiac sarcoplasmic reticulum [2–8]. A new approach became available with the observation that [^{3}H]ryanodine [9] in the presence of Ca^{2+} binds with high affinity and specificity to heavy sarcoplasmic reticulum preparations of rabbit skeletal and cardiac muscle [10]. We report here the solubilization and properties of the [^{3}H]ryanodine binding site from cardiac sarcoplasmic reticulum.

Methods

Preparation and solubilization of cardiac sarcoplasmic reticulum. Rabbit hearts (frozen immediately after death; Nitabell Rabbitry, Hayward, CA) were homogenized in 10 vol. of pyrophosphate buffer (20 mM sodium pyrophosphate, 20 mM sodium phosphate, 1 mM $MgCl_2$ and 0.5 mM EDTA in 10% sucrose at pH 7.1) [10,11]. Differential centrifugation yielded the heavy sarcoplasmic reticulum fragments (27 000 × *g* pellet) which were resuspended in the pyrophosphate

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; FPLC®, fast protein liquid chromatography (Pharmacia); SDS, sodium dodecyl sulfate; $t_{1/2}$, time for 50% decrease of specific [^{3}H]ryanodine binding.

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buffer (1:4, original heart weight equivalent/volume) and spun again at 27000 × *g* for 45 min. Other studies [10] show that the distribution of vesicle subtypes obtained on discontinuous sucrose gradient centrifugation of the cardiac preparation was similar to that of the skeletal muscle vesicles.

The washed cardiac pellet preparation was resuspended in solubilization buffer (20 mM CHAPS, 1 M NaCl, 0.125 M sucrose and 1 mM histidine, pH 7.1) (modified from Ref. 12) with a Teflon pestle glass homogenizer and incubated with shaking for 30 min at 0°C. Centrifugation at 150000 × *g* for 45 min gave the solubilized preparation, which was stored at 0°C, conditions under which the [^{3}H]ryanodine binding capacity dropped to 50% in about 25 days.

Assay of [^{3}H]ryanodine binding. Reaction mixtures for determination of total (specific plus nonspecific) binding consisted of 100 μl membrane preparation (300–600 μg protein in solubilization buffer) and 900 μl assay medium (2–4 nM [^{3}H]ryanodine of spec. act. 60 Ci/mmol, 0.56 mM $CaCl_2$, 0.5 M NaCl and 40 mM Trizma® maleate buffer, pH 7.1). Nonspecific binding was determined at 10 μM ryanodine. After 120 min incubation at 37°C, the binding of [^{3}H]ryanodine was determined by rapid filtration through Whatman GF/C glass-fiber filters pretreated with 5% polyethyleneimine [13] followed by washing the filters with ice cold water (3 × 5 ml) on a Brandell Cell Harvester M-24R (Gaithersburg, MD) and quantitation by liquid scintillation counting. This procedure with single step variations was used throughout to determine optimal conditions for [^{3}H]ryanodine binding as they relate to components and pH of the reaction mixture and the temperature. Unless indicated otherwise the results are the averages of two independent experiments with triplicate samples.

Results

Solubilization and characteristics of [^{3}H]ryanodine binding site. Treatment of the heavy sarcoplasmic reticulum fraction of rabbit cardiac muscle with 20 mM CHAPS and 1 M NaCl for 30 min at 0°C solubilizes 50–60% of the proteins and leaves only 7–25% of the total [^{3}H]ryanodine binding capacity in the insoluble residue. Re-extraction of the latter fraction with CHAPS gives essentially quantitative recovery of the ryanodine binding site. The overall process of membrane isolation and solubilization provides approx. 1.8 mg protein/g rabbit heart. Deletion of either CHAPS or NaCl from the solubilization buffer results in a low yield of solubilized protein with practically no [^{3}H]ryanodine binding. On the other hand, CHAPS at 3–12 mM is inhibitory during the binding assay (Table I) and so dialysis against the solubilization buffer without CHAPS increases the ligand binding. NaCl is also essential for the solubilization process and stabilizes the preparation, e.g., removal of NaCl from the solubilized preparation by dialysis results in protein precipitation and no [^{3}H]ryanodine binding.

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON SPECIFIC [^{3}H]RYANODINE BINDING TO SOLUBILIZED PREPARATION FROM RABBIT CARDIAC SARCOPLASMIC RETICULUM

Determinations were made with 400 μg protein, 4 nM [^{3}H]ryanodine, 0.5 mM added $CaCl_2$, 0.6 M NaCl (0.1 M NaCl for experiments with alkali metal chlorides), 2 mM CHAPS, 12.5 mM sucrose, 0.1 mM histidine and 36 mM Trizma maleate buffer each at pH 7.1, and the indicated additional compounds incubated for 120 min at 37°C. The values given, except where indicated otherwise, are the means ± S.D. for the designated number of experiments with different preparations.

Compound	Binding relative to control [a] (%)	
Alkali metal chlorides added at two concentrations		
	0.5 M	3 M
Li (4) [b]	233 ± 47	0
Na (7)	792 ± 223	1115 ± 817
K (4)	1075 ± 378	863 ± 210
Rb (2)	928 ± 289	800 ± 300
Cs (2)	862 ± 288	531 ± 69
Theophylline and cyclic nucleotides at two $CaCl_2$ concentrations		
	none [c]	0.5 mM
Theophylline (10^{-3} M)	200 ± 8	110 ± 2
+ cAMP (10^{-5} M)	177 ± 0	109 ± 3
+ cGMP (10^{-5} M)	200 ± 8	114 ± 2

[a] Control value of 88 fmol/mg protein.
[b] Number of experiments.
[c] Endogenous micromolar level with no added $CaCl_2$.

[^{3}H]Ryanodine binding to the solubilized preparation is dependent on Ca^{2+} and is maximal at pH 6.5–7.5 and at 37°C (i.e., 37° > 30° > 0°C (practically no binding)). It is saturable (Fig. 1A, giving an apparent B_{max} of 131 ± 48 fmol/mg protein under the routine assay conditions (Fig. 1B, Table II) and undergoes displacement with unlabeled ryanodine (Fig. 1C). More than 90% of the binding is to a high-affinity component (apparent $K_d = 5 \pm 1$ nM) (Table II) but there is also a small and variable portion of low-affinity binding (Fig. 1B, shown as a dotted line). Although not detailed here, the low-affinity site is less stable than the high-affinity component on storage at 0°C.

Association of [^{3}H]ryanodine with its binding site (Fig. 2). The association proceeds according to pseudo-first-order kinetics, reaching equilibrium

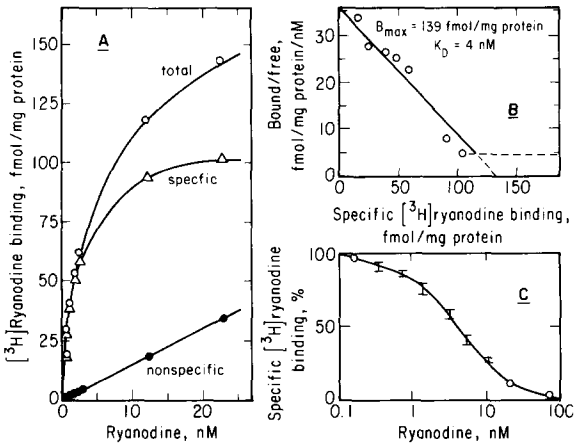

Fig. 1. Binding of [^{3}H]ryanodine to solubilized heavy sarcoplasmic reticulum of rabbit cardiac muscle. A. Typical curves for total binding and nonspecific binding (with additional 10 μM unlabeled ryanodine) assayed in the presence of 0.5 mM added $CaCl_2$ and 0.6 M NaCl after 120 min incubation at 37°C by filtration through polyethyleneimine-treated glass fiber filters. Saturating concentrations of between 24 and 100 nM ryanodine are not illustrated. Specific binding is defined as the difference between total and nonspecific binding. Values are averages of triplicate samples. B. Scatchard plot for experiment shown in A. Apparent K_d and B_{max} values for the high-affinity site are calculated by linear regression ($r = 0.98$). There is also a small and variable portion of low-affinity binding designated by the dotted line. C. Displacement of 2 nM [^{3}H]ryanodine by unlabeled ryanodine under the conditions described above. [^{3}H]Ryanodine binding with 2 nM radioligand was 36 fmol/mg protein.

TABLE II

EFFECT OF NaCl AND $CaCl_2$ ON SPECIFIC [^{3}H]-RYANODINE BINDING TO SOLUBILIZED PREPARATION FROM RABBIT CARDIAC SARCOPLASMIC RETICULUM

Determinations were made as in Fig. 1. The values given are the means ± S.D. of the indicated number of experiments. Apparent K_d and B_{max} values and Hill numbers are calculated from Scatchard plots by linear regression with correlation coefficients of 0.91 for 0.1 M NaCl and 0.96–0.99 in other cases.

Salt	K_d (nM)	B_{max} (fmol/mg)
NaCl (M)		
0.1	21 ± 5 (4) [a]	
0.2	7 ± 2 (2)	131 ± 48 (15) [b]
0.6	5 ± 1 (6)	
1.1	1.2 (1)	
3.1	1.1 ± 0.4 (4)	
$CaCl_2$ added (mM)		
none [c]	8 ± 1 (3)	43 ± 4 (2)
0.5	5 ± 1 (6)	151 ± 35 (5)
10	5 ± 1 (3)	252 ± 40 (3)

[a] Number of experiments.

[b] Mean ± S.D. of findings at indicated NaCl concentration. The high variability is between different preparations rather than within an experiment.

[c] Endogenous micromolar level with no added $CaCl_2$.

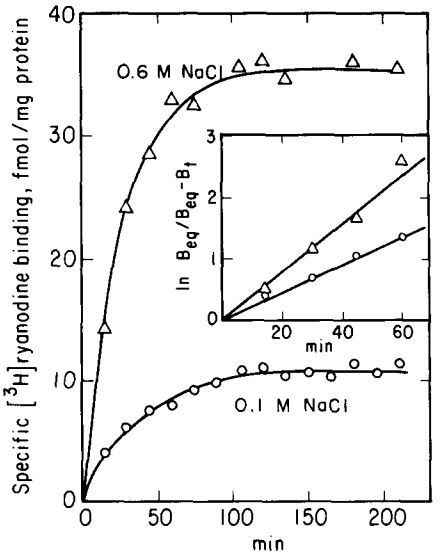

Fig. 2. Association of [^{3}H]ryanodine with its binding site in solubilized rabbit preparation. Determinations were made with 2 nM [^{3}H]ryanodine, 0.5 mM added $CaCl_2$ and 0.1 or 0.6 M NaCl. Lines of the inset plot are based on linear regression ($r = 0.99$) and their slopes are the rates of association uncorrected for dissociation.

in 100–120 min. The rate of [^{3}H]ryanodine binding is increased about 1.7-times by 0.6 M NaCl.

Dissociation of [^{3}H]ryanodine from its binding site. The dissociation appears to proceed in two phases, one ($\approx 30\%$ of the overall binding) with $t_{1/2} < 20$ min and the other approx. 178 min ($k_{-1} = 3.9 \cdot 10^{-3}$ min^{-1}). The course of dissociation is not affected by 0.6 M NaCl.

Thermal stability of [^{3}H]ryanodine-binding protein complex (Fig. 3). [^{3}H]Ryanodine binding undergoes little or no change within 60 min under the standard assay conditions (NaCl + $CaCl_2$) with fresh membrane at 40°C but a progressive inactivation occurs on deleting either $CaCl_2$ or NaCl ($t_{1/2}$ for the major phase = 88 and 43 min, respectively). Membrane preparations stored 17–28 days at 0°C are more sensitive than fresh preparations to thermal inactivation at 40°C ($t_{1/2}$ for 'aged' preparation with NaCl and $CaCl_2$ is only 18 min), and in both cases the stability order is dependent on the ionic composition of the medium, i.e., NaCl + $CaCl_2$ > NaCl > $CaCl_2$ for 28-day aged membranes. The thermal inactivation curve follows apparent first-order kinetics and is monophasic with NaCl + $CaCl_2$, while possibly two phases appear on deleting either of these salts. Additional studies (not illustrated) with the 28-day aged membranes showed that more than 90% of the [^{3}H]ryanodine binding capacity is lost after 10 min at 45°C.

Effect of various compounds on [^{3}H]ryanodine binding. [^{3}H]Ryanodine binding is optimal at 0.5–10 mM added $CaCl_2$, independent of the NaCl level (Fig. 4). Mg^{2+} is not an activator but at high concentrations (> 10 mM) it is an inhibitor (Fig. 4).

Four monovalent cations (Na^+, K^+, Rb^+ and Cs^+) enhance [^{3}H]ryanodine binding by 500–1100% when added at 0.5–3 M (Table I). LiCl is less stimulatory at 0.5 M and totally abolishes binding at 3 M (Table I). Theophylline enhances [^{3}H]ryanodine binding in preparations without added Ca^{2+} (Table I). ATP (1 μM) and cAMP and cGMP (10 μM) have no significant effect on the binding under the present assay conditions, even in combination with theophylline. Other inhibitors gave the following percentages of binding relative to the control: CHAPS, 47 and 0% at 1 and 5 mM, respectively; *p*-hydroxymercuribenzoate, less than

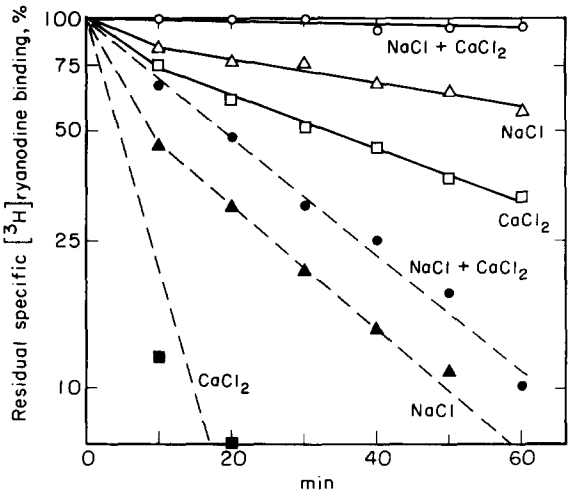

Fig. 3. Thermal stability of solubilized rabbit preparation. The solubilized preparation was incubated with either 0.5 mM added $CaCl_2$ or 0.6 M NaCl, or with both 0.5 mM added $CaCl_2$ and 0.6 M NaCl for various times at 40°C, then [^{3}H]ryanodine was added (2 nM) and binding was determined after 120 min at 37°C. Results are shown as solid lines for fresh preparation (stored 1–2 days at 0°C) and dotted lines for 'aged' preparation (stored for 28 days at 0°C). Values for membranes stored 17–18 days (not shown) lie between those for the fresh and aged preparations.

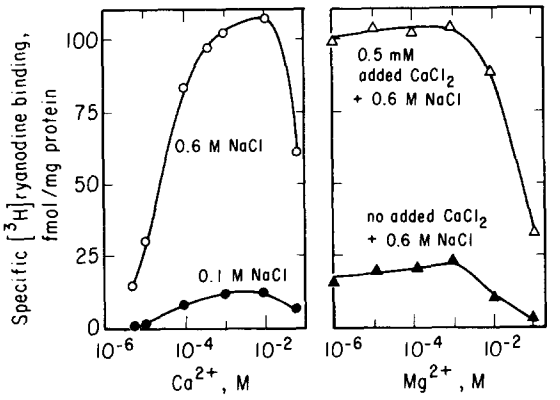

Fig. 4. Effect of varying concentrations of added $CaCl_2$ and $MgSO_4$ on [^{3}H]ryanodine binding with solubilized rabbit preparation. Determinations were made with 2 nM [^{3}H]ryanodine at the indicated concentrations of added $CaCl_2$, $MgSO_4$ and NaCl with incubation for 120 min at 37°C. The background binding due to endogenous Ca^{2+} (μmolar levels) is deducted (approx. 2 and approx. 10 fmol/mg protein with 0.1 and 0.6 M NaCl, respectively). Values are averages of 2–5 independent experiments with triplicate samples.

5% at 10 μM; ruthenium red, 88 and 52% at 1 and 5 μM, respectively; $CdCl_2$, less than 5% at 10 μM.

Radioligand binding is enhanced by 2-mercaptoethanol (120 ± 2% binding at each of 0.1 and 1 mM).

Scatchard analysis of effects of NaCl and $CaCl_2$ on ryanodine binding site. NaCl increases the affinity of the binding site for ryanodine, i.e., the apparent K_d decreases from 21 to 1 nM for 0.1–3.1 M NaCl, with no significant change in the number of binding sites (Table II). $CaCl_2$ acts in a different way, increasing the binding capacity (from 43 fmol/mg protein for preparations without added $CaCl_2$ to 252 fmol/mg protein at 10 mM $CaCl_2$) while only moderately changing the affinity for the ligand in preparations with $CaCl_2$ compared to preparations without $CaCl_2$ addition (Table II). The Hill numbers are 1.00 ± 0.01 at each NaCl or $CaCl_2$ concentration examined (Table II).

Gel permeation chromatography. Specifically bound [^{3}H]ryanodine is eluted in the void volume of a Sephadex G-25 column, as expected for association with a protein fraction (Fig. 5). Chromatography on Sephadex G-200 yields a mixture of proteins of low molecular mass (major components of apparent molecular weight 45–70 kDa) and one of high molecular mass (> 440 kDa, eluted at V_0), only the latter binding [^{3}H]ryanodine (99 fmol/mg protein). FPLC on Superose 6^{TM} (Pharmacia) more effectively resolves the low molecular mass region (Fig. 5 inset). Although not illustrated, a profile similar to that shown for Sephadex G-200 is also obtained with Sepharose

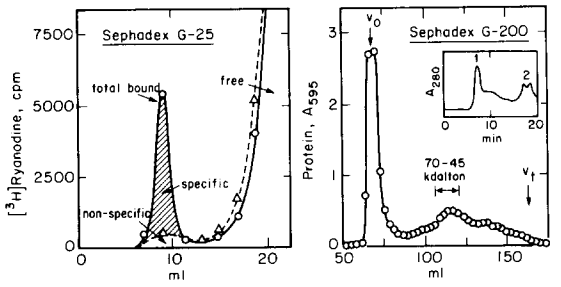

Fig. 5. Gel permeation chromatography of solubilized rabbit preparation. Sephadex G-25: the reaction mixture of solubilized preparation (400 μg protein), 2 nM [^{3}H]ryanodine (alone or with 10 μM unlabeled ryanodine for nonspecific binding), 0.5 mM added $CaCl_2$ and 0.6 M NaCl was incubated for 120 min at 37°C, then applied to the Sephadex G-25 column (V_0 = 9 ml, V_t = 19 ml; Pharmacia, Piscataway, NJ) and eluted with the same buffer (containing no ryanodine or 10 μM unlabeled ryanodine for nonspecific binding) using in the incubation. Elution of free and bound [^{3}H]ryanodine was monitored by liquid scintillation counting (2 ml of a fraction collected in 10 ml of Aquasol, New England Nuclear, Boston, MA). The elution profile for total bound [^{3}H]ryanodine was identical to that for total protein determined by the Coomassie blue method [14]. Sephadex G-200: 1 ml of solubilized preparation (approx. 2 mg protein) was added to a Sephadex G-200 column (V_c = 65 ml, V_t = 172 ml; Pharmacia) and eluted with 20 mM CHAPS, 1 M NaCl, 0.125 M sucrose and 1 mM histidine buffer, pH 7.1. Protein was monitored by the Coomassie blue method and molecular masses were determined with protein standards (Pharmacia). The V_0 and 45–70 kDa fractions were further examined for [^{3}H]ryanodine binding. The inset is the separation of 0.2 ml of solubilized preparation by FPLC (Superose 6^{TM}, Pharmacia) monitored at 280 nm (peak 1 corresponds to V_0 and peak 2 to the 45–70 kDa protein fractions as separated by Sephadex G-200 chromatography).

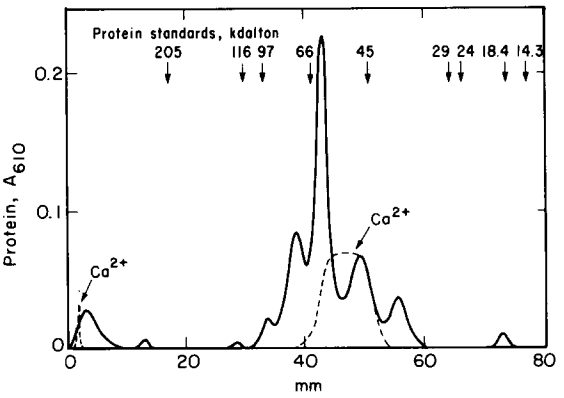

Fig. 6. SDS-polyacrylamide gel electrophoresis of the solubilized rabbit preparation. The solubilized preparation (500–900 μg protein in 100 μl) was combined with 300 μl of SDS-glycerol-bromophenol mixture and heated at 100°C for 10 min [15], then 8 μl of 2-mercaptoethanol were added and the mixture was maintained for 12 h at 25°C. These denatured samples (10–20 μg protein) were applied to 3–10% linear gradient polyacrylamide gels (Isolab, Akron, OH) prerun in 0.1% SDS/pH 8.2 glycine buffer prior to electrophoresis in the glycine buffer. The gels were stained with Coomassie blue and/or with 'Stains-all' dye (Sigma, St. Louis, MO) [16]. The Coomassie blue-stained gels were scanned at 610 nm with the LKB Ultrascan XL (carried out by courtesy of LKB Instruments, Inc., Rockville, MD). Molecular mass profile standards were treated identically and in parallel with the solubilized preparation. The dotted lines show the positions of fractions stained blue with the Stains-all dye.

6B-CL where the high molecular mass peak was more than 669 kDa, i.e., more than the V_e for thyroglobulin.

SDS-polyacrylamide gel electrophoresis. Four components of apparent molecular masses 34, 44, 61 and 70 kDa comprise approx. 90% of the Coomassie blue-stained proteins (Fig. 6). Their ratio varies in different preparations from that shown in Fig. 6 to a more equal distribution among the components. Stains-all dye gives a diffuse band in the 44–61 kDa region and a sharp band for a high molecular mass component (> 205 kDa).

Discussion

The [^{3}H]ryanodine binding site of cardiac heavy sarcoplasmic reticulum is almost quantitatively solubilized on treatment with 20 mM CHAPS and 1 M NaCl, as with the skeletal muscle preparation [12]. This CHAPS concentration is sufficient for solubilization of the protein complex, with minimal inhibition of [^{3}H]ryanodine binding by residual CHAPS carried forward into the assay. NaCl is important for both membrane solubilization and stabilization of the solubilized protein.

The properties of the solubilized cardiac preparation described here are very similar to those of the membrane-bound binding site reported earlier [10] relative to dependency on Ca^{2+}, interaction with Mg^{2+} and inhibition by Cd^{2+} and ruthenium red. As with the membrane preparation, there also appear to be two populations of binding sites based on Scatchard analysis and the biphasic course of radioligand dissociation. The proportion of high- and low-affinity sites varies with the preparation procedure, apparently because the low-affinity site is less stable. As an alternative, this study used whole hearts so there could be some contribution by coronary arteries and veins to the [^{3}H]ryanodine binding profile.

The ryanodine binding site is influenced in different ways by mono- and divalent cations and theophylline. Low Ca^{2+} concentrations increase both the affinity and the number of binding sites, whereas high Ca^{2+} levels only increase the number of binding sites. The 0.5 mM Ca^{2+} routinely added was a compromise between levels that are required for high binding and those of physiological relevance. NaCl enhances [^{3}H]ryanodine binding by 10-fold by increasing receptor affinity for the ligand, enhancing association with no effect on dissociation, and improving protein stability. Other alkali metal chlorides appear to modulate [^{3}H]-ryanodine binding in the same way as NaCl, probably by influencing the tertiary or quaternary structure of the radioligand-binding site complex. Increased [^{3}H]ryanodine binding by theophylline at low Ca^{2+} concentrations is not related to phosphodiesterase inhibition, since there is no change in binding with cyclic nucleotides and theophylline.

Solubilized ryanodine-binding protein complex becomes more susceptible to thermal inactivation during prolonged storage at 0°C. This process is not prevented by 0.1 mM phenylmethanesulfonyl fluoride, indicating that the impaired thermal stability is not initiated by serine-hydrolases. It might be related to changes in functionally essential thiol groups of the binding site.

Gel permeation chromatography separates high and low molecular weight fractions of the solubilized preparations; ryanodine is bound only to the high molecular weight fractions. SDS-polyacrylamide gel electrophoresis after SDS and 2-mercaptoethanol treatment provides further protein fractionation; a Ca^{2+}-binding protein appears in each of the high and low molecular weight fractions. The relationship between these components and those reported earlier [16] remains to be established relative to their contribution to the Ca^{2+} release mechanism in regulation of muscle contraction.

Acknowledgements

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