Vol. 128, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1985 Pages 449-456

THE CALCIUM-RYANODINE RECEPTOR COMPLEX OF SKELETAL AND CARDIAC MUSCLE

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Received March 8, 1985

[^{3}H]Ryanodine binds with high affinity to saturable and Ca^{2+}-dependent sites in heavy sarcoplasmic reticulum (SR) preparations from rabbit skeletal and cardiac muscle. Ruthenium red, known to interfere with Ca^{2+}-induced Ca^{2+} release from SR vesicles, inhibits [^{3}H]ryanodine specific binding in both skeletal and cardiac preparations whereas Mg^{2+}, Ba^{2+}, Cd^{2+} and La^{3+} selectively inhibit the skeletal preparation. The toxicological relevance of the [^{3}H]ryanodine binding site is established by the correlation of binding inhibition with toxicity for seven ryanoids including two botanical insecticides. These findings provide direct evidence for Ca^{2+}-ryanodine receptor complexes that may play a role in excitation-contraction coupling.

Ryanodine (Fig. 1), an insecticidal alkaloid (1), induces progressive contracture in skeletal muscle and loss of contractile tension in cardiac muscle (2,3) by uncoupling the electrical signal of the t-tubule, which itself is unaffected by the alkaloid, from the Ca^{2+}-release mechanism at the SR, i.e., excitation from contraction (4-7). The site of excitation-contraction coupling is proposed to be the triad junctional complex (triad) although the molecular basis remains obscure (8). A Ca^{2+}-induced Ca^{2+} release mechanism is implicated for both skeletal (9-11) and cardiac (12,13) muscle but its physiological relevance to excitation-contraction coupling is controversial (14). Direct examination of the ryanodine binding site became possible only recently with preparation of [^{3}H]ryanodine of high specific activity (15). The present study recognizes and characterizes a Ca^{2+}-ryanodine receptor complex in both skeletal and cardiac muscle.

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Abbreviations: B_{max}, maximal number of binding sites; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; K_d, binding dissociation constant; SR, sarcoplasmic reticulum; t-tubule, transverse tubule.

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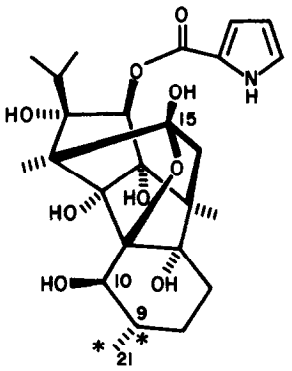

Fig. 1. Ryanodine structure showing positions of tritium labels (9,21), of the epimer(9) and of the N,O-15-dimethyl and 10-keto derivatives.

EXPERIMENTAL PROCEDURES

Ryanoids. Ryanodine and 9,21-didehydroryanodine (>98% purity) were isolated from commercial ryanodine (15). Didehydroryanodine in turn was hydrogenated to prepare 9-epiryanodine or reduced with tritium gas to obtain [^{3}H]ryanodine (60 Ci/mmol) (Fig. 1) (15). Ryanodine was converted to its hydrolysis product ryanodol and rearrangement product anhydroryanodine (procedures reviewed in Ref. 1) and to its dimethyl and 10-keto derivatives (Fig. 1), the latter from treatment with methyl iodide/potassium tertiary-butoxide and oxalyl chloride/dimethyl sulfoxide, respectively. The ryanoids were identified by nuclear magnetic resonance spectroscopy and mass spectrometry.

SR preparations. Heavy SR fragments were prepared from rabbit leg and back white skeletal or cardiac muscle by a modification of the Mitchell et al. (16) procedure. Tissues were ground and then homogenized in ten volumes of pyrophosphate buffer (20 mM Na pyrophosphate, 20 mM Na phosphate, 1 mM $MgCl_2$, and 0.5 mM EDTA in 10% sucrose at pH 7.1). Initial centrifugation at 2,000 g for 10 min was followed by 10,000 g for 15 min and 27,000 g for 45 min. The crude SR pellets obtained at 27,000 g were resuspended as in Ref. 16 and 5 ml aliquots were layered on a discontinuous sucrose gradient (4 ml of 14%, 12 ml of 25%, 5 ml of 28%, 4 ml of 36%, and 3 ml of 45% sucrose w/v in pyrophosphate buffer) and centrifuged in a Beckman rotor SW 25.1 at 40,000 g for 60 min. For each tissue source, two bands in the 36% sucrose layer were collected together, slowly brought to 10% sucrose in pyrophosphate buffer, and pelleted. These heavy SR preparations were resuspended in assay buffer (40 mM Tris/maleate, 10% sucrose at pH 7.1) immediately before analysis.

Binding assay. Binding determinations were performed at 37°C for 80 min with shaking. One ml incubation mixtures were quickly diluted with 5 ml of ice-cold assay buffer, filtered under vacuum through Whatman GF/B glass fiber filters using an Amicon VFM1 filtration manifold, rapidly rinsed with 5 ml ice-cold buffer, air dried and radioassayed by liquid scintillation counting. Specific binding is defined as the difference between total binding (with [^{3}H]ryanodine alone) and nonspecific binding (with [^{3}H]ryanodine fortified with 10 μM unlabeled ryanodine). Values for B_{max} and K_d were determined by least squares linear regression analysis with skeletal preparations and a computerized nonlinear iterative procedure with cardiac preparations. Each inhibitor was tested at 4 concentrations giving between 10 and 90 percent inhibition with 4 replicates each in 2 separate experiments. Values for the median inhibitory concentrations (IC_{50}) were determined directly from Hill plots.

RESULTS AND DISCUSSION

The heavy SR fragments of rabbit skeletal and cardiac muscle contain highly specific binding sites for [^{3}H]ryanodine. This receptor is localized in the 10,000 to 27,000 g heavy SR fraction and does not appear in lighter fragments. The preparation procedure uses pyrophosphate to dissociate myofibrillar contaminants from triads (16) and is reported to yield highly purified, morphologically-intact triads which retain structures identifiable as junctional feet (17). Similar distributions of vesicle subtypes are obtained on discontinuous sucrose gradient centrifugation of the skeletal and cardiac preparations. Two brown, turbid bands appear with each organ preparation in the 36% sucrose gradient layer. Vesicles from the two bands contain most of the total sites for specific [^{3}H]ryanodine binding recovered from the gradients, i.e., 76% and 85% for skeletal and cardiac heavy SR, respectively.

Our preparations of rabbit skeletal and cardiac muscle bind [^{3}H]ryanodine in a saturable manner. Binding equilibrium is reached within one hour at 37°C under optimal Ca^{2+} concentration. Scatchard plots reveal that radioligand binding to skeletal preparations is to a single class of high affinity receptors (K_d of 21 nM). Cardiac preparations, however, yield nonlinear Scatchard plots indicating the presence of multiple receptor sites having greatly different affinities for the ligand (K_d of 36 and 339 nM). Skeletal membrane preparations are a much richer source than cardiac membranes of [^{3}H]ryanodine receptors (Fig. 2).

Specific [^{3}H]ryanodine binding has an absolute dependence on the presence of Ca^{2+} with threshold and optimal concentrations of 0.6 and 70 μM, respectively, for the skeletal preparation, and 6 and 100 to 2000 μM, respectively, for the cardiac preparation (Fig. 3). The dissociation of [^{3}H]ryanodine from skeletal preparations is very slow, with a k_{-1} of 0.044 per hr at optimal Ca^{2+} concentration ($t_{1/2}$ = 15.6 hr). Scatchard analysis indicates that Ca^{2+} is an uncompetitive modulator of radioligand binding in skeletal muscle preparations. For example, the B_{max} and K_d for radioligand

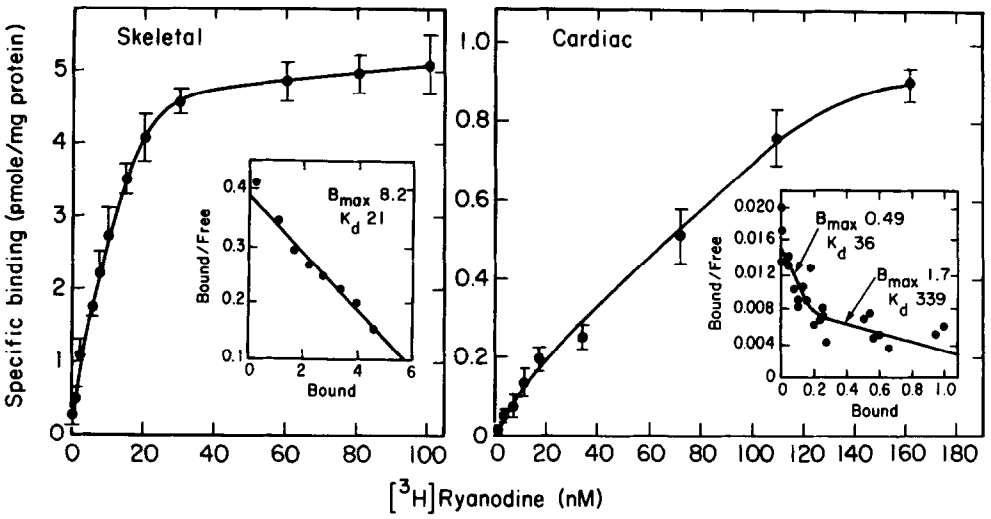

Fig. 2. Specific binding of [^{3}H]ryanodine to rabbit skeletal and cardiac muscle preparations shown as saturation isotherms and Scatchard plots. The conditions used were optimal for specific binding of 5 nM [^{3}H]ryanodine by heavy SR preparations, i.e., 100 µg protein/ml (22) and 60 µM added $CaCl_2$ for skeletal muscle and 500 µg protein/ml (22) and 300 µM added $CaCl_2$ for cardiac muscle. Standard deviations (n = 3) for specific binding are indicated by error bars. Nonspecific binding with skeletal and cardiac preparations averaged 15 and 30%, respectively, of total binding at 5 nM [^{3}H]ryanodine. Nonspecific binding was linear with radioligand level and varied within 10% of the mean. For Scatchard insets, B_{max} and bound ligand are given as pmole per mg protein and K_d as nM.

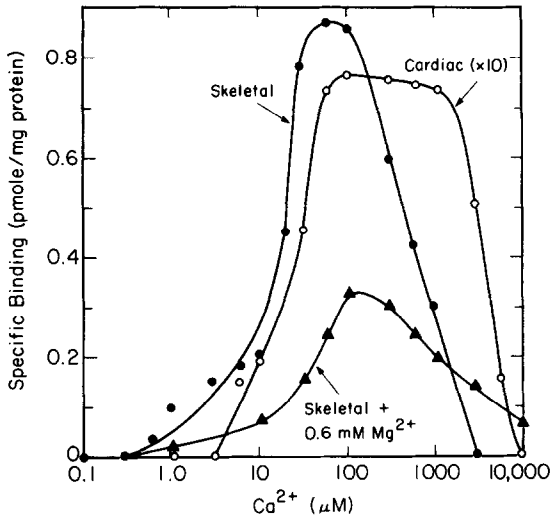

Fig. 3. Calcium modulation of and effect of magnesium on [^{3}H]ryanodine specific binding to rabbit skeletal and cardiac muscle preparations. The assay conditions were as in Fig. 2 with 5 nM [^{3}H]ryanodine. In varying the Ca^{2+} concentration, levels over 40 µM were obtained by adding $CaCl_2$ and of 0.1 to 40 µM by using 40 µM $CaCl_2$ titrated as appropriate with EGTA based on an apparent binding constant of 3.9×10^6 M^{-1} at pH 7.1 (23). Mg^{2+} at 1 mM does not appreciably alter the curve for the cardiac preparation. Nonspecific binding was independent of Ca^{2+} concentration.

binding to skeletal muscle membranes are altered from 8.2 pmole per mg protein and 21 nM at optimal Ca^{2+} levels (Fig. 2) to 4.1 and 90, respectively, at the suboptimal Ca^{2+} concentration of 6 μM. These results suggest an obligatory sequence to ligand binding, i.e., formation of a Ca^{2+}-receptor complex is prerequisite to ryanodine binding. We therefore propose that this system should be referred to as the Ca^{2+}-ryanodine receptor complex.

The dependence of [^{3}H]ryanodine binding on Ca^{2+} concentration is consistent with the observation on skeletal muscle that in the absence of an applied stimulus there is no progressive contracture regardless of the amount of ryanodine present (2,18). Although localized concentrations of the physiologically relevant cations in muscle are difficult to accurately determine, it is generally agreed that the Ca^{2+} concentration of the sarcoplasm is reduced by the Ca^{2+}/Mg^{2+} ATPase of the SR to 0.1 μM or lower during relaxation and increased in response to the action potential at the t-tubule to 1 μM or higher. Interestingly, the same range of Ca^{2+} concentration regulates the action of intact muscle and the [^{3}H]ryanodine binding site *in vitro*.

Skeletal and cardiac muscle preparations differ in the effect of selected cations on [^{3}H]ryanodine binding. [^{3}H]Ryanodine does not bind to either receptor in the presence of Mg^{2+} alone at any concentration. However, Mg^{2+} at 0.6 mM, the estimated ionized intracellular concentration (19,20), markedly inhibits [^{3}H]ryanodine binding to skeletal muscle preparations at physiological Ca^{2+} concentrations while having little effect on cardiac muscle preparations (Fig. 2, Table I). Importantly, there is still appreciable [^{3}H]ryanodine binding to both receptor preparations at physiologically-relevant Ca^{2+} and Mg^{2+} concentrations. The lower sensitivity of the cardiac receptor extends to other cations examined (Table I) indicating fundamental differences at the Ca^{2+}-ryanodine binding sites of the two organs.

Neither nifedipine nor verapamil, representing two classes of voltage-dependent Ca^{2+} channel antagonists, inhibits [^{3}H]ryanodine binding. Although

TABLE I

Pharmacological Profile of [^{3}H]Ryanodine Binding to Skeletal and Cardiac Muscle Preparations

Compound	IC_{50} Skeletal	IC_{50} Cardiac
Cations	μM	
Ruthenium red	0.41 (0.23-0.72)	0.29 (0.16-0.53)
Cadmium chloride	1.5 (0.54-4.4)	23 (15-34)
Lanthanum chloride	17 (6.6-44)	>1000
Barium chloride	122 (48-308)	>1000
Magnesium chloride	308 (185-511)	>1000
Ruthenium chloride	>500	>500
Ryanoids	nM	
9,21-Didehydroryanodine	23 (14-36)	7.0 (2.2-21)
Ryanodine	27 (19-36)	22 (15-34)
10-Ketoryanodine	73 (60-89)	60 (22-167)
9-Epiryanodine	76 (64-89)	61 (30-126)
N,O-15-Dimethylryanodine	384 (326-451)	240 (210-270)
Anhydroryanodine	>10,000	>10,000
Ryanodol	>10,000	>10,000

Receptor binding assays were performed as in Fig. 2 with [^{3}H]ryanodine at 5 nM. The 95% confidence limits are given in parentheses as determined by computer-assisted linear regression analysis. Hill numbers with their standard deviations for the active compounds in the skeletal preparations were 0.88 ± 0.11 for the cations and 0.97 ± 0.05 for the ryanoids. Compounds without effect on the skeletal or cardiac muscle preparations were: procaine and two Ca^{2+} channel antagonists (nifedipine and verapamil) at 100 μM; nicotine and caffeine at 1 mM.

the preparations exhibit appreciable [^{3}H]nitrendipine binding, ryanodine does not inhibit this binding, i.e., the Ca^{2+}-ryanodine receptor is pharmacologically distinct.

Ruthenium red is a potent inhibitor of [^{3}H]ryanodine binding in both skeletal and cardiac muscle preparations whereas $RuCl_3$ is not active (Table I). This polycationic dye inhibits the Ca^{2+}-induced Ca^{2+} release mechanism of both cardiac heavy SR vesicles (21) and skeletal junctional SR vesicles (9,10). [^{3}H]Ryanodine is therefore a powerful new probe for the Ca^{2+}-induced Ca^{2+} release mechanism.

The high specificity of the ryanodine receptors is evident from displacement experiments with several ryanoids. Similar results are obtained with skeletal and cardiac muscle preparations (Table I). Didehydroryanodine

is similar to ryanodine itself in effectiveness for displacing [^{3}H]ryanodine, while ryanodine's 10-keto derivative and 9-epimer are about 3-fold less potent. The dimethyl derivative is 10- to 14-fold less potent while anhydroryanodine and ryanodol do not show displacement at 10 μM. These results closely parallel the toxicity of the ryanoids to mice. Median lethal dose (LD_{50}) values as mg/kg for mice treated intraperitoneally are: 0.10 for didehydroryanodine and ryanodine; 0.47, 0.55 and 17 for epiryanodine, ketoryanodine and dimethylryanodine, respectively; >20 for anhydroryanodine and ryanodol. The ryanodine binding site is therefore of both pharmacological and toxicological relevance.

ACKNOWLEDGEMENT

The authors thank Dr. Josef Seifert of this laboratory for reviewing the manuscript.

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