Positive Cooperativity of Ryanodine Binding to the Calcium Release Channel of Sarcoplasmic Reticulum from Heart and Skeletal Muscle†

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Received July 20, 1988; Revised Manuscript Received September 27, 1988

ABSTRACT: Ryanodine is a specific ligand for the calcium release channel which mediates calcium release in excitation–contraction coupling in muscle. In this study, ryanodine binding in sarcoplasmic reticulum from heart muscle and skeletal muscle is further compared and correlated with function. The new findings include the following: (1) Two types of binding, high affinity ($K_{D1} \sim 5$–10 nM) and low affinity ($K_{D2} \sim 3$ μM), can now be discerned for the skeletal muscle receptor. K_{D1} is approximately the same as and K_{D2} of similar magnitude to that previously reported for heart. (2) The dissociation rates for the high-affinity binding have been directly measured for both heart and skeletal muscle ($t_{1/2} \sim 30$–40 min). These rates are more rapid than previously reported ($t_{1/2} \sim 14$ h). (3) K_{D1}'s obtained from the ratio of the dissociation and association rate constants agree with the dissociation constant measured by equilibrium binding Scatchard analysis. (4) Ryanodine binding to the low-affinity site can be correlated with a decrease in the dissociation rate constant (k_{-1}) of the high-affinity site, and thereby in the apparent dissociation constant (K_{D1}). The inhibition constant (K_I) for inhibiting the high-affinity off rate obtained from a double-reciprocal plot of the change in off rate vs [ryanodine] is practically the same in heart (0.66 μM) and skeletal muscle (0.64 μM) and in the range of the K_{D2}. The binding of cold ryanodine to the low-affnity site appears to lock the bound [^{3}H]ryanodine onto the high-affinity site rather than to exchange with it. Thus, in this sense, the ryanodine receptor exhibits "positive cooperativity". For both heart and skeletal muscle, the binding of ryanodine to the high-affinity site has previously been correlated with the pharmacologic effect of locking of the channels in the "open" state. We now find that binding to the low-affinity site in skeletal muscle can be correlated with the pharmacologic effect of closing the channel, comparable to that which we have previously reported for heart. The K_m for closing of the channel in heart (1.1 μM) and that in skeletal muscle (3.7 μM) are in the same range as the K_I and K_{D2}. These studies indicate that the low-affinity binding site mediates the closing of the channel and is responsible for the positive cooperativity. The binding characteristics of ryanodine to the receptor (calcium release channel) and their functional correlates (K_m and K_I) are similar in heart and skeletal muscle.

Ryanodine is an alkaloid (Jendon & Fairhurst, 1969) whose toxic action has been pinpointed to the calcium release channel of terminal cisternae of sarcoplasmic reticulum (Fleischer et al., 1985). This finding led to the isolation and characterization of the calcium release channel from both heart (Inui et al., 1987b; Lai et al., 1988a; Hymel et al., 1988b; Fleischer & Inui, 1988) and skeletal muscle sarcoplasmic reticulum (Inui et al., 1987a; Hymel et al., 1988a; Lai et al., 1988b; Imagawa et al., 1987). The channel is morphologically equivalent to the feet structures (Inui et al., 1987a,b), which are involved in the junctional association of the transverse tubules with the terminal cisternae (Franzini-Armstrong & Nunci, 1983). It consists of an oligomer of a single high molecular weight polypeptide (Inui et al., 1987b; Lai et al., 1988b; Imagawa et al., 1987). The purified polypeptide has been reconstituted into phospholipid planar bilayers and found to possess channel-gating activity characteristic of Ca^{2+} release from sarcoplasmic reticulum (Lai et al., 1988a,b; Hymel et al., 1988a,b). Thus, the calcium release channel, which mediates Ca^{2+} release in excitation–contraction coupling in heart and skeletal muscle, has been identified morphologically and in molecular terms (Fleischer & Inui, 1988).

[^{3}H]Ryanodine binds with high affinity (K_D in the nanomolar range) to the calcium release channel (Fleischer et al., 1985; Inui et al., 1987a,b; Lai et al., 1988a,b; Hymel et al., 1988a,b; Fleischer & Inui, 1988; Imagawa et al., 1987; Pessah et al., 1985, 1986). The binding has been characterized as having a very slow dissociation rate ($t_{1/2} > 14$ h) (Pessah et al., 1987). Further, the binding of ryanodine to skeletal terminal cisternae displayed a single type of binding (high affinity) (Pessah, 1985; Fleischer et al., 1985), whereas in heart, two types of binding (high and low affinity) were observed (Pessah et al., 1986, 1987; Inui et al., 1988, 1987b). This study investigates further the binding of ryanodine to sarcoplasmic reticulum fractions of heart and skeletal muscle. We can now detect two types of binding for the channel from skeletal muscle. Furthermore, the low-affinity binding modulates the high-affinity ryanodine binding.

Materials and Methods

Scatchard Analyses of Ryanodine Binding. Heart microsomes (Chamberlain et al., 1983) and skeletal muscle SR[1]

† This work was supported in part by grants from the National Institutes of Health (DK 14632 and HL 32711) and the Muscular Dystrophy Association of America and by a Biomedical Research Support Grant from the National Institutes of Health administered by Vanderbilt University. Susan McGrew is the recipient of a research fellowship from the American Heart Association, Middle Tennessee Chapter.

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0006-2960/89/0428-1686$01.50/0

containing terminal cisternae vesicles (Saito et al., 1984) were isolated as previously described (Chu et al., 1988). The I4 fraction was selected for binding studies because the B_{max} (maximum number of binding sites) for high-affinity ryanodine binding in this preparation is lower and more comparable to the B_{max} of heart microsomes. The terminal cisternae fraction (R4) which has higher B_{max} gives similar results. The SR microsomes (0.1 mg of protein/mL) were incubated with [^{3}H]ryanodine (1 nM–1.8 μM) in binding buffer (1 M KCl, 10 mM HEPES, 25 μM $CaCl_2$) for 60 min at 37 °C. The samples were then rapidly filtered and washed twice with 2 mL of binding buffer and four times with 2.0 mL of 10% ethanol. The bound radioactivity was measured by liquid scintillation counting as previously described (Fleischer et al., 1985; Inui et al., 1987a). Nonspecific binding was measured at each concentration of [^{3}H]ryanodine by including 100-fold excess ryanodine. The data were analyzed by using the iterative nonlinear least-squares program LIGAND (Munson & Rodbard, 1980), and the dissociation constants (K_{D1} and K_{D2}) and maximum number of binding sites (B_{max1} and B_{max2}) for the high- and low-affinity binding sites were determined.

Measurement of Association Kinetics for [^{3}H]Ryanodine Binding. Heart microsomes or skeletal muscle SR (0.5 mg of protein/mL final concentration) were incubated at 37 °C with [^{3}H]ryanodine (51 nM for heart or 38 nM for skeletal muscle) in binding buffer. The reaction was terminated after 2–240 min, by rapidly filtering duplicate 100-μL (50-μg protein) aliquots. Nonspecific binding, measured in the presence of 100-fold excess cold ryanodine, was found to be constant throughout the time period of the experiment and was measured in subsequent experiments only at the termination of the experiment. Specific binding (total binding minus nonspecific binding) was plotted versus time. The k_{obsd} (observed association rate constant) was calculated from the slope of a first-order plot of these data as described by Weiland (Weiland & Molinoff, 1981); $\ln [(LR)_e/[(LR)_e - (LR)_t]] = k_{obsd}t$ [$(LR)_e$ = amount of radioligand bound at equilibrium; $(LR)_t$ = amount of radioligand bound at time t]. The pseudo-first-order association rate constant (k_1) was calculated from the k_{obsd} by the method of Weiland & Molinoff, 1981); $k_{obsd} = k_1[(L)_T(R)_T/(LR)_e]$ [$(L)_T$ = total concentration of ligand; $(R)_T$ = total concentration of receptor]. The time required for obtaining a plateau for association to the low-affinity [^{3}H]ryanodine binding site in heart and skeletal muscle was measured under comparable conditions in the presence of 1 μM [^{3}H]ryanodine.

Measurement of Dissociation Kinetics. Heart microsomes (Chamberlain et al., 1983) or the I4 fraction of skeletal muscle SR (Chu et al., 1988) (1–2 mg of protein/mL) were incubated in binding buffer with [^{3}H]ryanodine (9 nM for heart; 38 or 231 nM for skeletal muscle) until association was complete (1 h at 37 °C). For the measurement of nonspecific binding, 100-fold excess of cold ryanodine was included in the assay. Aliquots (50 μL) were filtered for measurement of total and nonspecific binding at zero time, and the specific binding at zero time $(LR)_0$ was calculated. Dissociation was initiated by diluting duplicate aliquots of the total and nonspecific binding samples 100-fold (50 μL to 5.0 mL) into binding buffer in the presence or absence of cold ryanodine (concentration 0.15–3 μM). Dissociation was terminated after 15–240 min or longer by rapid filtration of the diluted samples. The nonspecific binding value obtained by diluting the nonspecific sample prior to filtration and washing was approximately 50% of the nonspecific binding obtained when the sample was not diluted and was stable throughout the time course of the experiment. In later experiments, therefore, it was measured only at the termination of the experiment. The percent of specific binding (total minus nonspecific) remaining was calculated and plotted versus time. The dissociation rate constant, k_{-1}, was measured from the slope of the first-order plot of these data as described by Weiland (Weiland & Molinoff, 1981); $\ln [(LR)_t/(LR)_0] = -k_{-1}t$ [t = time after dilution; $(LR)_0$ = maximum amount of ligand bound]. In the absence of cold ryanodine, k_{-1} was calculated from all of the data points. When cold ryanodine was included in the dissociation buffer, the first-order plot was initially curvilinear. The k_{-1} therefore was calculated from the linear portion of the first-order plot which occurred after an initial equilibration period equivalent to the time required for the cold ryanodine to associate to the low- (or high-) affinity ryanodine binding site (60 min for heart or 30 min for skeletal muscle). In skeletal muscle, the k_{-1} measurement was terminated at 180 min (see Results).

The K_I for inhibition of the high-affinity ryanodine dissociation rate with excess cold ryanodine (positive cooperativity) was obtained from the x intercept of a double-reciprocal plot of the change in the dissociation rate constant (Δk_{-1}) as a function of ryanodine concentration.

Ca^{2+} Loading Assay. Ca^{2+} loading was measured with a Hewlett-Packard UV/visible spectrophotometer Model 8450A or Model 8451A diode array spectrophotometer by using antipyrylazo III as the metallochromic indicator and measuring the difference in absorbance between 710 and 790 nm at 37 °C (Fleischer et al., 1985; Scarpa, 1979). Terminal cisternae of skeletal muscle SR (R4) (Saito et al., 1984) (1 mg of protein/mL) were preincubated in binding buffer in various concentrations of ryanodine (0–3 μM) for 60 min at 37 °C. Aliquots (50 μg) were then added into the Ca^{2+} loading assay medium (Fleischer et al., 1985; Inui et al., 1988). The Ca^{2+} loading assay medium in 1 mL contained 100 mM potassium phosphate buffer (pH 7.0), 1 mM $MgCl_2$, 5 mM NaN_3, and 0.2 mM antipyrylazo III. Na_2ATP (1 mM) was then added, and the reaction was initiated by addition of 50 μM $CaCl_2$. The apparent K_m value for stimulation of Ca^{2+} loading by high concentrations of ryanodine was obtained by a double-reciprocal plot of the Ca^{2+} loading rate versus ryanodine concentration.

Results

Scatchard analyses of ryanodine binding to skeletal muscle SR have previously been reported to have a single high-affinity binding (K_D = 10–50 nM)[2] whereas in heart, both high- (K_{D1} = 5–10 nM) and low-affinity (K_{D2} approximately micromolar) binding were reported (Fleischer et al., 1985; Inui et al., 1987b; Pessah et al., 1985). Ryanodine binding studies to heart microsomes and skeletal muscle SR are presented in Figure 1. We now report that the K_D for the high-affinity skeletal muscle site is approximately the same as the high-affinity K_D for heart when performed under identical conditions (K_D 5–10 nM). Furthermore, the presence of additional low-affinity binding in skeletal muscle in the micromolar range is indicated by the saturable specific binding with a K_D in the micromolar range ($K_D \sim 3$ μM) (see Figure 1 insets).

[1] Abbreviations: SDS–PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; SR, sarcoplasmic reticulum; HMW polypeptide, high molecular weight polypeptide.

[2] The high-affinity binding constant (K_{D1}) for ryanodine binding is very dependent on the conditions used. Binding studies carried out under identical conditions (37 °C for 1 h) indicate that the high-affinity binding of ryanodine to the receptor is about the same ($K_{D1} \sim 6$ nM) for both heart and skeletal muscle (McGrew et al., 1987).

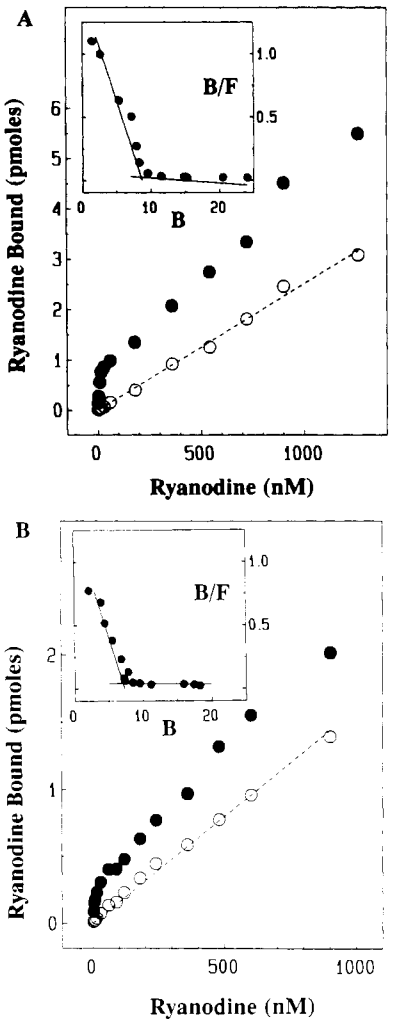

FIGURE 1: Binding of ryanodine to SR from muscle: (A) cardiac microsomes; (B) skeletal muscle SR. Ryanodine binding was measured at a range of [^{3}H]ryanodine concentrations (3 nM–900 nM for skeletal muscle and 1.5 nM–1.26 μM for heart) in an assay containing binding buffer (1 M KCl, 25 μM $CaCl_2$, and 10 mM HEPES, pH 7.4) and 0.1 (heart) or 0.034 (skeletal muscle) mg of SR. Nonspecific binding was measured at each [^{3}H]ryanodine dose by the inclusion of 100-fold excess cold ryanodine. After 60-min incubation at 37 °C, the samples were filtered and the bound ryanodine was measured by liquid scintillation counting. The main panel gives the total (●) and nonspecific (○) binding as a function of [^{3}H]ryanodine dose. In the inset, a Scatchard plot of the data (inset) is presented. The bound (pmol/mg of protein)/free (nM) is expressed as a function of the bound (pmol/mg of protein). The dissociation constant K_{D1} (high-affinity site), K_{D2} (low-affinity site), maximum number of binding sites B_{max1} (for high affinity), B_{max2} (for low affinity) and number of receptor subtypes for the ryanodine were calculated by using iterative, nonlinear, least-squares computer analysis [LIGAND (Munson & Rodbard, 1980)]. For this set of experiments, the K_{D1} was 5.7 and 5.5 nM for heart and skeletal muscle, respectively, and the B_{max1} was 8.7 and 7.3 pmol/mg of protein. The K_{D2} was 1.9 and 3.0 μM and the B_{max2} was 13.3 and 63.8 pmol/mg of protein for heart and skeletal muscle, respectively.

The association kinetics for the high-affinity binding site was measured for heart microsomes and skeletal muscle SR using $\geq 5K_D$ (50 nM). Ryanodine reaches a plateau binding value within 60 min. The binding is exponential and therefore pseudo first order for the receptor from both types of muscle (Figure 2). The association rate constant (k_1) calculated from the slope (k_{obsd}) of the semilog plot is similar for heart and skeletal muscle (Table I). The association of [^{3}H]ryanodine to the low-affinity ryanodine binding site at 1 μM ryanodine reaches a plateau between 30 and 60 min for heart and skeletal muscle (Figure 3).

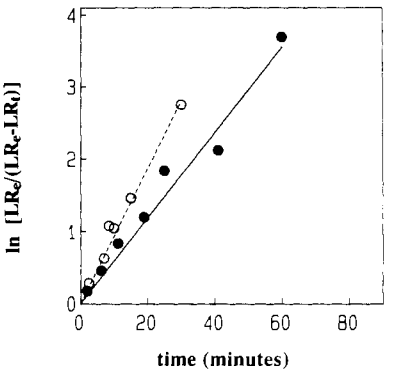

FIGURE 2: First-order plot of the association of ryanodine to the high-affinity binding site for heart and skeletal muscle sarcoplasmic reticulum. Ryanodine (51 nM for heart or 38 nM for skeletal muscle) was added to heart microsomes (●) or skeletal muscle SR (○) in binding buffer and incubated at 37 °C. After varying periods of time, aliquots were removed and assayed for binding (see Materials and Methods). The maximum amount of ryanodine bound at the plateau was 4.9 pmol/mg of protein for heart and 9.6 pmol/mg of protein for skeletal muscle. The data were plotted according to the pseudo-first-order equation ln $[(LR)_e/[(LR)_e - (LR)_t]] = k_{obsd}t$ (see Materials and Methods). The correlation coefficients are 0.98 (heart, ●) and 0.99 (skeletal muscle, ○). k_{obsd} (equal to the slope) is 0.0586 min^{-1} for heart and 0.0897 min^{-1} for skeletal muscle. The pseudo-first-order rate constant for association, calculated from k_{obsd} (see Materials and Methods), is 0.00111 and 0.00236 min^{-1} nM^{-1} for heart and skeletal muscle, respectively.

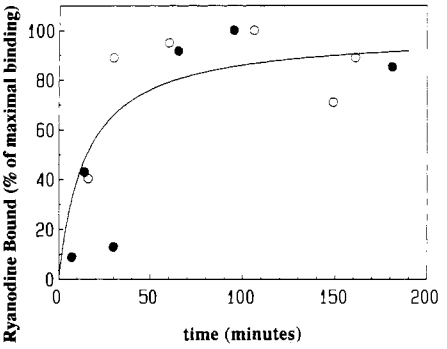

FIGURE 3: Association rate of low-affinity binding site. Heart (●) or skeletal muscle (○) SR was incubated with 1 μM [^{3}H]ryanodine in binding buffer at 37 °C. At the indicated times duplicate 100-μg protein (heart) or 50-μg protein (skeletal muscle) aliquots were rapidly filtered and washed. Nonspecific binding was measured by the inclusion of 100-fold excess of cold ryanodine at selected time points and was constant (~75% of the total binding) throughout the period of the experiment. Displayed is the specific binding (percent of maximal binding) as a function of time. The plateau value for binding [^{3}H]ryanodine was 10.2 pmol/mg of protein (heart) and 23.4 pmol/mg of protein (skeletal muscle).

Table I: Comparison of Kinetic Binding Data in Heart and Skeletal Muscle[a]

source	association rate constant k_1 (min^{-1} nM^{-1})	dissociation rate constant k_{-1} (min^{-1})	dissociation constant K_{D1} (nM)
heart	0.00111 (1)	0.0140 ± 0.003 (3)	12.7
skeletal muscle	0.00236 (1)	0.0247 ± 0.004 (4)	10.5

[a] The association (k_1) and dissociation (k_{-1}) rate constants for the high-affinity ryanodine binding site were calculated from association and dissociation data similar to those presented in Figures 2 and 4 and as described under Materials and Methods. The mean value and standard deviation are given for k_{-1}. The number of replicates is given in parentheses. The dissociation constant for the high-affinity binding (k_{D1}) was calculated from the ratio of the off and on rate constants ($K_{D1} = k_{-1}/k_1$).

We next measured the dissociation rate of [^{3}H]ryanodine from the high-affinity ryanodine binding site. The high-affinity binding was obtained by preincubation of [^{3}H]ryanodine with

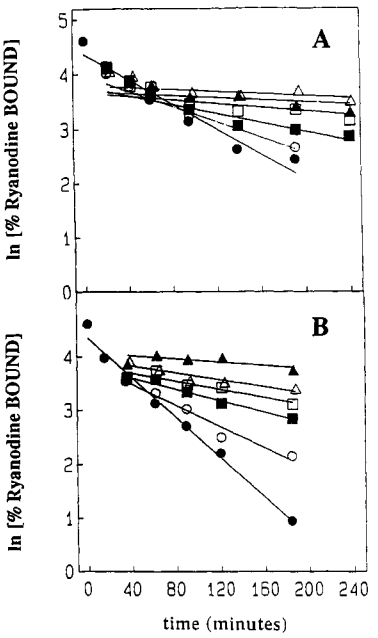

FIGURE 4: Influence of low-affinity ryanodine binding on the dissociation rate of high-affinity [^{3}H]ryanodine binding for heart (Figure 4A) and skeletal muscle SR (Figure 4B). Heart (2 mg of protein/mL final concentration) or skeletal muscle SR fractions (1 mg of protein/mL final concentration) were incubated at 37 °C with [^{3}H]-ryanodine (9 nM for heart or 231 nM for skeletal muscle) in binding buffer (1 M KCl, 10 mM HEPES, and 25 μM $CaCl_2$) until association with the high-affinity binding site was complete (1 h). Similar results were obtained when 38 nM ryanodine was used for association for skeletal muscle. Aliquots were diluted 100-fold (50 μL to 5 mL total volume) in binding buffer containing various concentrations of ryanodine, between 0 and 3 μM. The dissociation rate was measured by rapid filtration after different time intervals, and the percentage of specifically bound [^{3}H]ryanodine (total minus nonspecific) that remained was measured. The first-order plot of the percent [^{3}H]-ryanodine binding remaining versus time is displayed for each concentration of ryanodine in the dilution medium: (●) zero ryanodine; (○) 0.4 μM; (■) 0.8 μM; (□) 1.4 μM; (▲) 2 μM; (△) 3 μM ryanodine. For heart, the off rate was linear for 60–520 min (correlation coefficient 0.92 or greater with one exception, see Table II). The initial nonlinear portion reflects the time of association to the low-affinity binding site, which is approximately 60 min. In skeletal muscle, the off rate was linear from 30 to 180 min (correlation coefficient 0.93 or greater with one exception, see Table II). The off rate constant, k_{-1}, has been calculated from the slope of the first-order plot via the formula ln $[(LR)_t/(LR)_0] = -k_{-1}t$ [$(LR)_t$ = amount of ligand bound at time t; $(LR)_0$ = maximal amount bound] and is given in Table II as a function of ryanodine dosage.

the sample for 1 h. The dissociation rate, measured by 100-fold dilution in the absence of cold ryanodine, is exponential and more rapid than previously reported for both heart (Figure 4A) and skeletal muscle (Figure 4B). The half-time for dissociation ($t_{1/2}$) is on the order of 30–40 min under these conditions. The dissociation was virtually complete in 180 min, and the first-order plots were linear for both tissues, indicating a single population of high-affinity ryanodine binding sites which do not exhibit cooperativity. The dissociation rate constant, k_{-1} was similar for heart and skeletal muscle. The dissociation constant for ryanodine binding for the high-affinity binding site (K_{D1}) calculated from the kinetic rate constants ($K_D = k_{-1}/k_1$) is similar for heart (12.7 nM) and for skeletal muscle (10.4 nM) (Table I).

When dissociation of [^{3}H]ryanodine is studied by including cold ryanodine (10 times or greater the concentration of K_{D1}) in the dissociation buffer, there is a marked concentration-dependent slowing of the dissociation rate for both heart and skeletal muscle (Figure 4). The dissociation of ryanodine slows within the first half-hour subsequent to dilution in cold ryanodine and is first order for the next 2.5 h (Figure 4). These observations complement the direct binding studies referred to above which show that binding to the low-affinity site is not complete for a half-hour. The k_{-1}'s are inversely proportional to the concentration of ryanodine in the dilution medium (Table II). The low-affinity binding leads to a decrease in the dissociation rate of the high-affinity binding. We conclude that the binding at the low-affinity site induces positive cooperativity at the high-affinity binding site.

Table II: Influence of Ryanodine on the Dissociation Rate Constant of High-Affinity [^{3}H]Ryanodine Binding in Heart and Skeletal Muscle[a]

source of SR	ryanodine concn in diluent (μM)	k_{-1} (min^{-1})	corr coeff[b]	Δk_{-1}
heart	0	0.0113	0.97	
	0.40	0.00698	0.98	0.0044
	0.80	0.00411	0.95	0.0072
	1.4	0.00169	0.92	0.0096
	2.0	0.00098	0.82	0.0104
	3.0	0.00096	0.94	0.0104
skeletal muscle	0	0.0186	0.99	
	0.40	0.0098	0.98	0.0087
	0.80	0.0050	0.96	0.0136
	1.40	0.0038	0.93	0.0147
	2.0	0.0016	0.83	0.0170
	3.0	0.0033	0.93	0.0153

[a] The dissociation rate constant (k_{-1}) is calculated from the data in Figure 4 via the formula ln $[(LR)_t/(LR)_0] = k_{-1}t$. $(LR)_t$ = the amount of ligand bound at time t, and $(LR)_0$ is the maximal amount bound (immediately prior to dilution). The k_{-1} in the absence of ryanodine in the diluent was measured from 0 to 180 min, at which time dissociation is virtually complete. The off rate constant in the presence of increasing concentrations of ryanodine in the dissociation buffer, k_{-1}, is calculated after association to the low-affinity binding site is complete (30–60 min subsequent to dilution into dissociation buffer containing ryanodine). In skeletal muscle the k_{-1} was determined between 30 and 180 min because the off rate of the first-order plot is curvilinear after 180 min (markedly slowing) whereas in heart the k_{-1} is linear from 60 min to termination of the experiment (520 min). The K_I for the inhibition of the dissociation of ryanodine from the high-affinity binding site is calculated from a double-reciprocal plot of Δk_{-1} vs ryanodine concentration. The Δk_{-1} is the difference between k_{-1} in the absence vs presence of cold ryanodine. For this plot, the K_I is 0.50 μM for heart and 0.49 μM for skeletal muscle. [b] Correlation coefficient.

Double-reciprocal plots of the change in dissociation rate constants (Δk_{-1}) for high-affinity ryanodine binding as a function of ryanodine concentration are linear for heart and skeletal muscle. The inhibition constant (K_I) for ryanodine to inhibit the dissociation rate of the high-affinity binding site (calculated from the x intercept) is similar for heart (0.66 ± 0.28 μM) and skeletal muscle (0.64 ± 0.15 μM) (Table III).

We have previously correlated binding at the high-affinity ryanodine binding site (nanomolar range) for both heart and skeletal muscle terminal cisternae with the pharmacological effect of locking the channel in the open state. This means that ruthenium red added subsequent to ryanodine cannot close the channels and Ca^{2+} loading is not enhanced by ruthenium red (Fleischer et al., 1985). The low-affinity ryanodine binding in heart terminal cisternae was correlated with a closing of the channels, thereby enhancing Ca^{2+} loading (Inui et al., 1988). We now correlate the low-affinity ryanodine binding in skeletal muscle SR also with the pharmacologic effect of closing the channels. Preincubation with higher concentrations of ryanodine leads to enhanced calcium loading of skeletal muscle microsomes (Figure 5A). The Ca^{2+} loading is enhanced as a function of ryanodine concentration (Figure 5B). The double-reciprocal plots of enhanced loading as a function of ryanodine concentration give a K_m of about 3.7 μM (Table III).

Table III: Comparison of Binding Data for the Ryanodine Receptor in Heart and Skeletal Muscle[a]

source	high-affinity binding site: Scatchard analysis, K_{D1} (nM)	high-affinity binding site: kinetic analysis, K_{D1}[b] (nM)	high-affinity binding site: B_{max1} (pmol/mg of protein)	low-affinity binding site: K_{D2} (μM)	low-affinity binding site: B_{max2} (pmol/mg of protein)	K_m for stimulation of calcium loading (μM)	K_I for inhibition of high-affinity [^{3}H]ryanodine off rate (μM)
heart	5.7 ± 1.5 (2)	12.7	7.8 ± 1.3 (2)	1.2 ± 2.7[c] (2)	11.1 ± 17.7 (2)	1.11 ± 0.63 (3)[d]	0.66 ± 0.28 (3)
skeletal muscle	4.6 ± 1.3 (2)	10.5	6.8 ± 0.9 (2)	2.7 ± 1.4[c] (2)	102.3 ± 43.5 (2)	3.67 ± 0.68 (2)	0.64 ± 0.15 (3)

[a] Values are the mean ± the standard error. The number of replicates is given in parentheses. [b] $K_{D1} = k_{-1}/k_1$. See Table I. [c] This value is the standard error in the line. [d] Data from Inui et al. (1988).

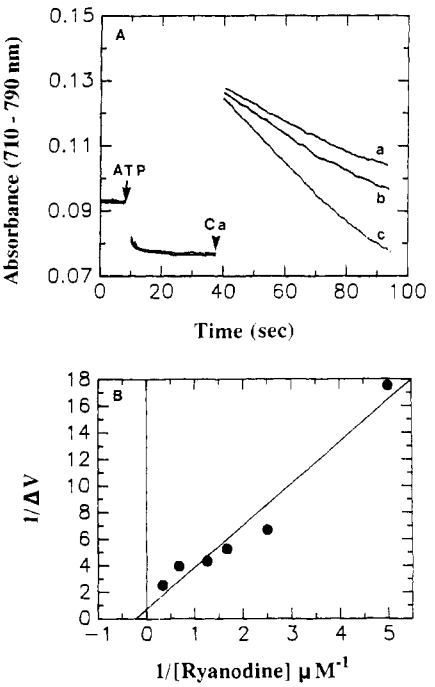

FIGURE 5: High concentrations of ryanodine close the calcium release channels in terminal cisternae of skeletal muscle SR. (A) Ca^{2+} loading is enhanced by preincubation with ryanodine. Terminal cisternae of skeletal muscle SR (1 mg of protein/mL) were preincubated for 60 min at 37 °C with various concentrations (0–3.0 μM) ryanodine. An aliquot (50 μg of protein) of the sample was added to 1 mL (final volume) of Ca^{2+} loading assay medium. Ca^{2+} loading in the presence of 100 mM potassium phosphate buffer was measured at 37 °C by using antipyrylazo III. Na_2ATP was added (arrow) to give a final concentration of 1 mM. Ca^{2+} loading was initiated by adding $CaCl_2$ (arrowhead) to a final concentration of 50 μM. (a) Control; (b) 0.4 μM ryanodine (c) 2 μM ryanodine. (B) Double-reciprocal plot of ΔV vs ryanodine concentration (Fleischer et al., 1985; Inui et al., 1988). ΔV is the loading rate measured after preincubation with ryanodine minus that of the control without ryanodine. The Ca^{2+} loading rate (V) was determined as in (A). The apparent stimulation constant (K_m) for ryanodine was 4.35 μM. The correlation coefficient was 0.98.

Discussion

Ryanodine is a specific ligand for the Ca^{2+} release channel of sarcoplasmic reticulum (Fleischer et al., 1985; Inui et al., 1987a,b; Lai et al., 1988a,b; Hymel et al., 1988a,b; Fleischer & Inui, 1988). The binding has previously been localized to the terminal cisternae (Fleischer et al., 1985; Inui et al., 1988). In this study, the nature of ryanodine binding in heart and skeletal muscle has been further characterized and compared. The new findings include the following: (1) High-affinity binding is similar in heart and skeletal muscle, and an additional low-affinity binding site in the micromolar range is indicated from the binding data in heart and skeletal muscle. (2) The low-affinity binding can be correlated with closing of the channels. (3) The dissociation rates of the high-affinity binding have been directly measured for both heart and skeletal muscle and can be much more rapid than previously observed. (4) The ryanodine receptor exhibits "positive cooperativity". That is, binding at the low-affinity site decreases the dissociation rate of the ligand from the high affinity site. Previous reports of the slow dissociation of ryanodine can be explained in terms of the positive cooperativity. The binding of cold ryanodine to the low-affinity site locks [^{3}H]ryanodine onto the high-affinity site rather than exchanging with it.

The low-affinity binding can now be detected for the skeletal muscle receptor. The K_{D2} is of similar magnitude in heart and skeletal muscle. K_{D2} is about 100-fold greater than K_{D1}. This binding measurement is therefore subject to great uncertainty because it is difficult to discriminate low-affinity binding from displaceable nonspecific binding (Molinoff et al., 1981). Furthermore, at high concentration of ryanodine the binding to the filter becomes very high, obscuring the specific binding. Previous studies with skeletal muscle (Fleischer et al., 1985; Pessah et al., 1985; Michalak et al., 1988) missed the low-affinity binding because of these inherent problems, and our K_{D2} values contain substantial experimental error (Table III).

Pessah et al. (1987) measured the dissociation rate of ryanodine from the high-affinity receptor by adding excess cold ryanodine and reported a much slower off rate (k_{-1}) than that obtained here. Their kinetic K_{D1} was 20-fold lower than the K_{D1} that they obtained by Scatchard analysis. We believe this discrepancy is at least in part due to the positive cooperativity exerted by the low-affinity binding on the high-affinity binding as described in our study. Our conditions for measuring the dissociation rate differ in that dilution was carried out in the absence of ryanodine in the dilution medium. The K_{D1}, calculated from the ratio of the off to the on rate (K_{D1} by kinetic analysis) obtained in our study, is similar to that obtained directly by Scatchard analysis; i.e., K_{D1} = 12.7 nM by kinetic analyses vs 5.7 ± 1.5 nM by Scatchard analysis for heart, and K_{D1} = 10.4 nM by kinetic analysis vs 4.6 ± 1.3 nM by Scatchard analysis for skeletal muscle (Table I).

Ryanodine binding to the low-affinity site decreases the dissociation rate constant of the high-affinity site (k_{-1}) (Table II) and thereby the apparent dissociation constant (K_{D1}). The inhibition constants (K_I's) for this positive cooperativity in heart (0.66 μM) and skeletal muscle (0.64 μM) are in a similar concentration range (Figure 4 and Table III) and likewise in the range of the K_m's for closing the calcium release channel in heart (1.1 μM) and skeletal muscle (3.7 μM) (Table III). These values are also in the same concentration range as the dissociation constant for the low-affinity ryanodine binding site (K_{D2}), measured directly from our binding data by Scatchard analysis. Thus, the positive cooperativity appears to be mediated by the same low-affinity ryanodine binding which can be correlated with the closing of the calcium release channel in heart and skeletal muscle. Although binding in the micromolar range is difficult to detect and resolve by Scatchard analysis, we believe that the low-affinity binding is genuine

since it can be correlated with the pharmacologic effect (closing the Ca^{2+} release channel) which occurs in this higher dose range.

In both heart and skeletal muscle, the K_{D2}'s are about 2 orders of magnitude greater than the K_{D1}'s. We have correlated K_{D2} with the closing of the channel in heart (Inui et al., 1988) and skeletal muscle (this study), whereas K_{D1}'s have been correlated with locking the channels in the open state (Fleischer et al., 1985; Inui et al., 1988). The K_m's for closing the channels and the inhibition constants (K_I) for positive cooperativity are likewise in the same concentration range as K_{D2} (Table III). As already discussed above, the binding parameters (K_{D2} and B_{max2}) for the low-affinity site have a large error associated with the measurements. Nonetheless, the maximum number of bindng equivalents for the weaker binding (B_{max2}) is severalfold greater than B_{max1}. Taking note that the foot structure/calcium release channel approximates 4-fold symmetry [see especially Saito et al. (1988)], one interpretation consistent with our data is that the initial high-affinity binding represents only one of four equivalents. Ryanodine binding to the first equivalent would then result in negative cooperativity with respect to the remaining three equivalents, giving rise to weaker binding and a lower K_{D2}. If this explanation is valid, then K_{D2} would be an average of several K_{D2}'s which cannot be resolved experimentally because of the large error associated with the binding measurement for the weaker binding. By contrast, the K_I and K_m values are more accurate than the K_{D2}'s. The significantly lower value of K_I for positive cooperativity of K_{D1} as compared with K_m might then be explained in terms of occupancy of but one of the equivalents of K_{D2} whereas the K_m for closing of the channels might require binding of all three equivalents of the weaker binding.

The higher K_{D1} reported by us previously for skeletal muscle (Fleischer et al., 1985; Inui et al., 1987b) is referable to different conditions of binding than used in this study: (1) the temperature used here is higher, 37 vs 25 °C, and (2) the time for binding is longer (1 h vs 30 min). Thus, when the conditions used in this study are similar to those of other investigators (Lai et al., 1988b; Imagawa et al., 1987; Michalak et al., 1988), a value for K_{D1} in the 5–10 nM range is obtained with our preparations.

Positive cooperativity in which binding at one site influences the subsequent binding at equivalent sites can sometimes be detected on Scatchard plots by a slope that is initially positive and changes to negative with increased drug dose. We do not observe this in our studies. The positive cooperativity that we find is mediated at a different binding site which does not become occupied until much greater drug dosages are used (>0.15 μM). At this higher drug concentration, the high-affinity ryanodine binding site is practically saturated, and therefore no further effect on the amount of ligand bound is observed.

Positive cooperativity in ligand binding is a rare phenomenon, albeit an important characteristic of ryanodine binding to the calcium release channel of both heart and skeletal muscle sarcoplasmic reticulum. From the perspective of the studies reported here, the ryanodine binding characteristics to the calcium release channels from heart and skeletal muscle and their functional correlates are quite similar.

Registry No. Ca, 7440-70-2; ryanodine, 15662-33-6.

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