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The cardiac sarcoplasmic reticulum calcium-release channel: modulation of ryanodine binding and single-channel activity

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[^{3}H]Ryanodine binding to a preparation of isolated cardiac sarcoplasmic reticulum has been investigated. A method is reported which produces a very high level of specific binding. Scatchard analysis of binding up to 50 nM ryanodine yields data which infer a single class of binding sites with a K_d of 1.4 nM and a B_{max} of 9.7 pmol/mg protein. Micromolar calcium is the principal activating ligand and its effects on binding are modulated by ligands which similarly affect the activity of single calcium-release channels incorporated into artificial planar phospholipid bilayers. The benzimidazole drug, sulmazole, is able to stimulate ryanodine binding in the presence of sub-activating calcium concentrations. Ryanodine binds to the native channel only when it is in its open state and stimulation of maximal ryanodine binding is achieved by ligands which are insufficient to produce full single-channel activation. A model is proposed which relates the modulation of ryanodine binding to the behaviour of single channels.

Introduction

In striated muscle, contraction is initiated by the release of calcium from junctional regions of the sarcoplasmic reticulum (SR) in response to cell excitation. The properties of single calcium-release channels from both skeletal and cardiac muscle have been investigated under voltage-clamp conditions by fusing 'heavy' SR vesicles with artificial planar phospholipid bilayers [1–3]. Ligands, which had previously been demonstrated to modify SR calcium release, either in skinned myocytes or from isolated membrane vesicles, also modulate the gating of the calcium-release channel; open probability is increased by Ca^{2+}, adenine nucleotides and caffeine on the cytosolic side of the channel and is reduced by Mg^{2+} and Ruthenium red.

Ryanodine is a highly toxic neutral plant alkaloid which exerts complex effects on cardiac and skeletal muscle, uncoupling cell excitation from contraction [4]. The development of radiolabelled ryanodine with high specific activity [5] has permitted detailed study of the interaction of this drug with specific binding sites in both cardiac and skeletal muscle. The ryanodine receptor has been identified as a protein with a molecular weight of about 400 000–450 000 [6,7] localised to the triadic structures, where junctional SR and adjacent portions of the t-tubule system of the sarcolemma are joined by foot processes. These proteins have been purified and subsequently reconstituted into artificial planar phospholipid bilayers where they exhibit similar behaviour to the native SR calcium-release channel [8,9]. The calcium-release channel/foot process complex is believed to be formed from four similar ryanodine-receptor protein sub-untis [9]. Ryanodine is, therefore, a marker for the calcium-release channel complex.

The ligands which modulate SR $^{45}Ca^{2+}$ efflux and the gating of the calcium-release channel from skeletal muscle have been shown to have similar effects on [^{3}H]ryanodine binding [10]. It has, therefore, been proposed that ryanodine may only bind to the Ca^{2+}-activated open state of the channel [11]. The results of previous detailed investigations with cardiac SR have reported much lower densities of ryanodine receptors with non-specific binding accounting for up to 30% of total activity [12,13]. Furthermore, these studies have not been able to confirm the effects which would be predicted for certain channel ligands on ryanodine binding. In this study, we report a method which produces an unusually high level of ryanodine binding to a preparation of sheep cardiac SR. This has made it possible to investigate very accurately the factors which affect ryanodine binding. The results of binding studies are discussed in the context of observations of single

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channels. Results from both methods have been correlated to demonstrate that [^{3}H]ryanodine binding can be used as a probe for the activity of the cardiac calcium-release channel.

Experimental Procedures

Materials. [^{3}H]Ryanodine was purchased from New England Nuclear Ltd. Unlabelled ryanodine was purchased from Calbiochem Inc. All other chemicals were of AnalaR or best available grade from BDH Ltd or Sigma Ltd. Phosphatidylethanolamine (bovine heart) was purchased from Avanti Polar Lipids, U.S.A. Aqueous counting scintillant was purchased from Amersham International. Sulmazole was a generous gift from Boehringer Ingelheim.

Membrane preparation. Sheep hearts were obtained from a local abattoir and transported directly to the laboratory in an ice-cold cardioplegic solution composed of: 102 mM NaCl, 29 mM sodium lactate, 20 mM KCl, 16 mM $MgCl_2$, 2 mM $CaCl_2$. SR vesicles were prepared from left ventricular tissue as previously described [14]. A mixed microsomal preparation was isolated from nuclear material, mitochondria and contractile proteins by differential centrifugation. These membranes were then fractionated by further centrifugation on a discontinuous sucrose density gradient. Heavy SR vesicles were collected from the 30/40% interface, suspended in a solution of 400 mM sucrose, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes)-tris(hydroxymethyl)methylamine (Tris) (pH 7.2) snap frozen in liquid nitrogen and stored at −70 °C.

[^{3}H]Ryanodine binding assay. Vesicles were diluted to 60–100 μg protein/ml and incubated at 37 °C in a buffered medium containing 1 M KCl, 5 μM phenylmethylsulphonyl fluoride (PMSF), 25 mM piperazine-*N,N'*-bis(2-ethanesulphonic acid) (Pipes)-KOH (pH 7.4). Unless otherwise specified, incubation time was 90 min with 5 nM [^{3}H]ryanodine; other additions to the medium are described under individual experiments. Calcium concentrations below 100 μM were buffered with 1 mM ethyleneglycolbis(aminoethyl ether)tetraacetic acid (EGTA)and free calcium was determined using the method of Bers [15]. At the completion of incubation, the medium was diluted with 5 ml of ice-cold buffer and filtered through Whatman GF-B filters, presoaked in buffer. Filters were washed with 3 × 5 ml aliquots of buffer and counted in 10 ml aqueous counting scintillant the following day. Non-specific binding was determined from incubations to which 2.5 μM unlabelled ryanodine had been added. All incubations were performed in triplicate and the number of experiments is detailed with individual results.

Single-channel recordings. Experiments were performed at 22 °C. Planar phospholipid bilayers, composed of phosphatidylethanolamine, dispersed in decane at a concentration of 30 mg/ml, were painted across a 200 μm diameter hole in the styrene co-polymer septum between two experimental chambers containing 50 mM choline chloride, 5 mM $CaCl_2$, 10 mM Hepes-Tris (pH 7.2). Vesicles were then added to the designated *cis* chamber and the solution fortified with choline chloride to produce a 7:1 gradient across the membrane. Vesicle fusion was detected by the appearance of a chloride selective conductance. Calcium channels were observed by perfusing the *cis* chamber with 250 mM Hepes-Tris (pH 7.4) and the *trans* chamber with 250 mM glutamic acid, 10 mM Hepes, adjusted to pH 7.4 with $Ca(OH)_2$, to give a solution with approx. 67 mM free calcium. SR membrane vesicles incorporate into planar phospholipid bilayers with a fixed orientation so that the *cis* chamber is equivalent to the cytosolic environment of the channel [16,17]. The composition of this chamber can then be modified, as described in Results, to observe the effect of various ligands on channel function. Channel opening results in a flow of ions, which was amplified [18] and recorded on to FM tape. Single channel recordings shown in the figures were obtained with the *cis* chamber voltage clamped at 0 mV relative to the grounded *trans* chamber. Single channel data were displayed on a Hewlett Packard 7475A plotter following digitisation at 2 kHz using a PDP 11/73 based computer system (Indec, Sunnyvale CA, U.S.A.).

Enzyme and protein assay. Na^+/K^+-ATPase and Ca^{2+}-ATPase assays were performed using the methods described by Mahony and Jones [19]. Protein assays were performed using the modification of the Lowry method described by Markwell et al. [20].

Results and Discussion

The time course of ryanodine binding to heavy SR vesicles is shown in Fig. 1. The plot represents the mean of three experiments in which 5 nM [^{3}H]ryanodine was incubated with SR vesicles in media with different channel ligands: 100 μM calcium alone, 10 μM calcium + 3 mM ATP and 100 μM calcium + 1 mM magnesium. Differences in rates between each experiment were not significant. The equilibrium point for these experiments was taken as the level of binding after 3 h incubation. Under the experimental conditions used, ryanodine binds to available sites with the kinetics of a first-order reaction. By allowing the heavy SR to incubate for at least 90 min, equilibrium will have been reached.

Fig. 2 compares specific [^{3}H]ryanodine binding and the activity of marker enzymes between the mixed microsomal preparation and heavy SR membranes. Although Ca^{2+}-ATPase activity (a marker for the SR) is concentrated approximately 2-fold in the heavy SR

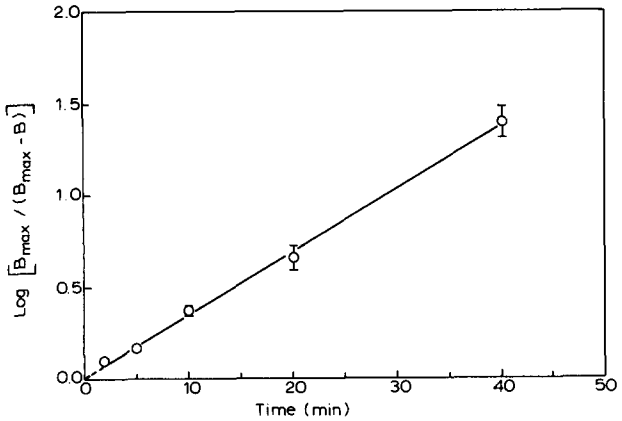

Fig. 1. Time course of binding (B) of 5 nM [^{3}H]ryanodine to heavy SR. Data represents the means and standard errors from three experiments performed with different ligands in the incubation media. B_{max} values are taken as the level of binding after 180 min incubation. The time constant of the first order reaction is calculated to be 0.08 s^{-1}.

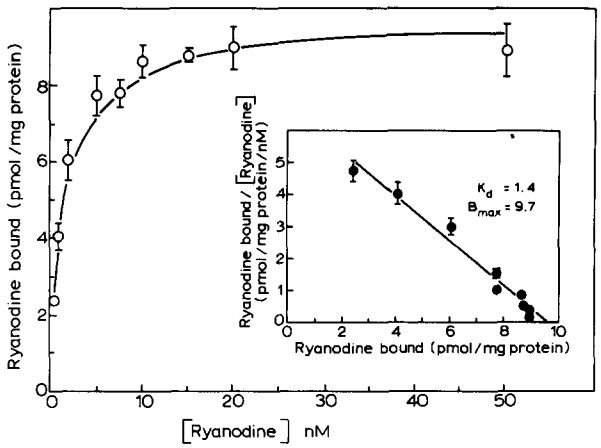

Fig. 3. Specific binding of ryanodine to heavy SR. The data are the means and standard errors from three different preparations. Scatchard analysis of the data (inset) infers a K_d of 1.4 nM and a B_{max} of 9.7 pmol/mg protein.

fraction, Na^+/K^+-ATPase activity (a marker for the sarcolemma) is similar to the mixed microsomal preparation. Despite this apparently poor purification of membranes, the method produces a heavy SR fraction with a high level of specific ryanodine binding.

Cross-contamination between the sarcolemma and junctional SR has been reported previously from other cardiac preparations [21–23]. In skeletal muscle, Moczydlowski and Latorre [24] investigated saxitoxin and ouabain binding sites and showed that French press treatment was more effective than mild homogenisation in reducing cross-contamination. Use of the French press decreased the yield of intact triads and the number of binding sites.

In the experiments reported here, [^{3}H]ryanodine binding was performed in media containing 1 M KCl. Michalak et al. [13] have previously investigated the effect of ionic strength on ryanodine binding. In skeletal muscle SR, binding increased considerably over the range tested up to 500 mM NaCl; in cardiac muscle SR there was very little effect. In the present study, ryanodine binding in media containing 150–500 mM KCl produced inconsistent results and levels of binding of approximately 0.6 pmol/mg protein (data not shown), which are similar to some previous reports of binding to cardiac SR [12,13]. It has been suggested that high ionic strength may buffer surface membrane charges which might otherwise shield receptor sites, or alternatively it may interact directly with either ryanodine or its receptor to increase their affinity [13]. However, working with skeletal muscle, Campbell et al. [25] demonstrated that heavy SR vesicles were decorated with membrane projections which were removed by washing the vesicles in 0.6 M KCl. It is possible that, in preparation methods which produce intact triads, ryanodine binding sites may be mainly concealed and exposure to media with high ionic strength is required to reveal them. Previous reports of significant differences in [^{3}H]ryanodine binding site density between skeletal and cardiac muscle [12,13] may, in part, reflect the closeness of the association of the t-tubule and SR derived membrane in the triads and hence the relative ease with which binding sites can be exposed.

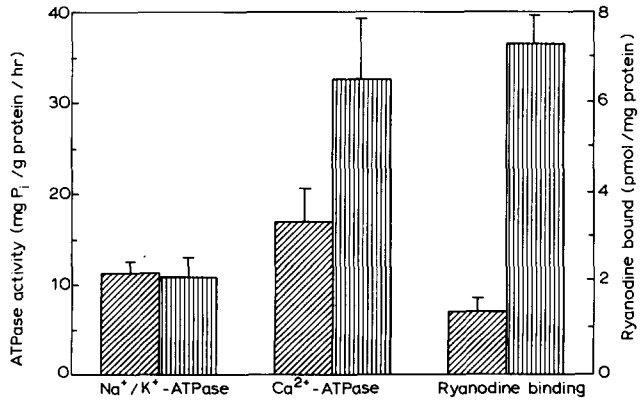

Fig. 2. Comparison of ryanodine binding and biochemical analysis between the mixed microsomal preparation ((hatched bar) and the heavy SR fraction (vertical lines). Data are the means and standard errors from four different preparations.

Fig. 3 shows ryanodine binding to heavy SR as a function of ryanodine concentration. Scatchard analysis of these data is shown as the inset. From this graph, a single ryanodine binding site is inferred with maximal binding of 9.7 pmol/mg protein and a K_d of 1.4 nM. Each data point is the mean of results from three different preparations. [^{3}H]Ryanodine alone was used to obtain the data points up to concentrations of 7.5 nM; at higher concentrations, [^{3}H]ryanodine was admixed with unlabelled ryanodine. The proportions of each were varied so as to ensure that error was not introduced as a result of differences in purity between the [^{3}H]ryanodine and the unlabelled ryanodine.

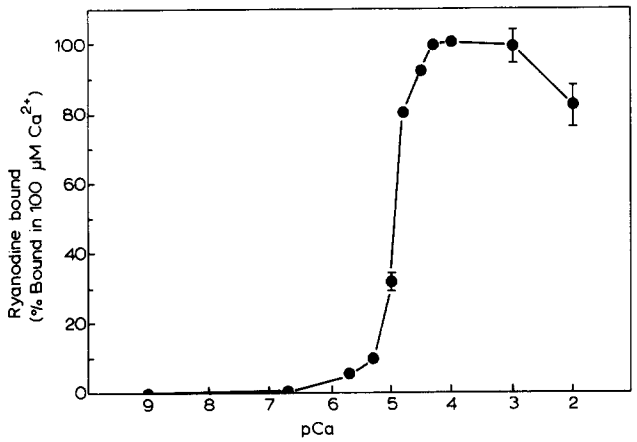

Fig. 4. Activation of ryanodine binding by calcium. Means and standard errors for three sets of experiments are shown.

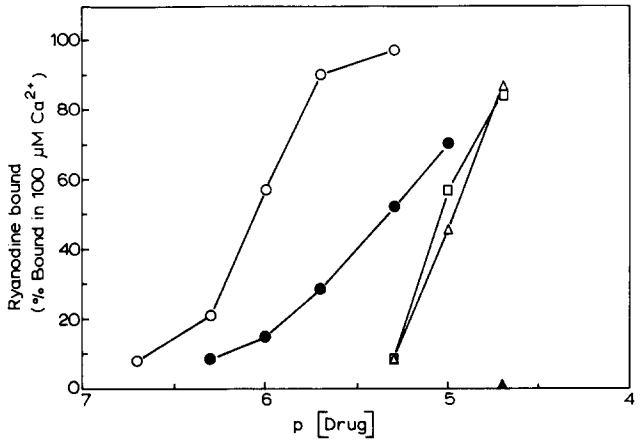

Fig. 5. Modulation of ryanodine binding by caffeine (solid symbols) and sulmazole (open symbols). The means of results from two experiments are shown. Experiments were performed in 2 μM (●, ○), 10 nM (□) or 1 nM (▲, △) free calcium.

Some previous reports have suggested the presence of two ryanodine binding sites with K_d values in the nanomolar range [12,13]. The presence of a single binding site with a K_d of 6.8 nM was previously reported by Alderson and Feher [26]. On the basis of ion flux experiments, which showed a stimulation of calcium uptake into vesicles treated with high concentrations of ryanodine, they proposed a low-affinity binding site for ryanodine with a K_d of approx. 17 μM. Occupancy of this binding site would result in closure of the calcium-release channel thereby abolishing the leak of calcium from the heavy SR vesicles. The error in binding studies increases as the ryanodine concentration is raised due to a combination of rising non-specific binding and possible errors introduced by differences in purity between [^{3}H]ryanodine and unlabelled ryanodine. Despite low non-specific binding (approx. 3% at 10 μM ryanodine) and low standard errors between individual samples within single data points, the identification of a possible binding site with micromolar affinity for ryanodine was beyond the sensitivity of the assay.

In Fig. 4, the dependence of ryanodine binding on calcium is demonstrated. 1 μM calcium is the threshold for the activation of binding, and maximal binding is achieved across a range from 30 μM to 1 mM calcium. A small, but significant decrease in binding was seen when the vesicles were incubated with 10 mM calcium. Table I shows the effects of magnesium, ATP and Ruthenium red on ryanodine binding. Added to 30 μM

TABLE I

Modulation of ryanodine binding by ATP, Mg^{2+} and Ruthenium red

All data are means and standard errors of three experiments.

Incubation medium	[^{3}H]Ryanodine binding (pmol/mg protein)		
	Control	+3 mM ATP	
100 μM Ca^{2+}	7.4 ±0.2	7.6 ±0.4	
5 μM Ca^{2+}	0.61±0.01	7.0 ±0.2	
1 nM Ca^{2+}	0	0	
	Control	+1 mM Mg^{2+}	% of control
30 μM Ca^{2+}	7.2 ±0.4	7.3 ±0.6	101
15 μM Ca^{2+}	5.9 ±0.6	4.7 ±0.2	80
10 μM Ca^{2+}	2.1 ±0.1	1.1 ±0.1	53
2 μM Ca^{2+}	0.37±0.02	0.14±0.03	39
	Binding		% of control
100 μM Ca^{2+}	7.6 ±0.2		100
100 μM Ca^{2+} +1 μM Ruthenium red [a]	5.9 ±0.1		78
100 μM Ca^{2+} +1 μM Ruthenium red [b]	2.8 ±0.07		37
100 μM Ca^{2+} +10 μM Ruthenium red [a]	1.3 ±0.06		17

[a] In these experiments heavy SR vesicles were added to incubation media already containing Ruthenium red and [^{3}H]ryanodine.

[b] In these experiments heavy SR vesicles were preincubated for 15 min at 37 °C with Ruthenium red prior to the addition of [^{3}H]ryanodine.

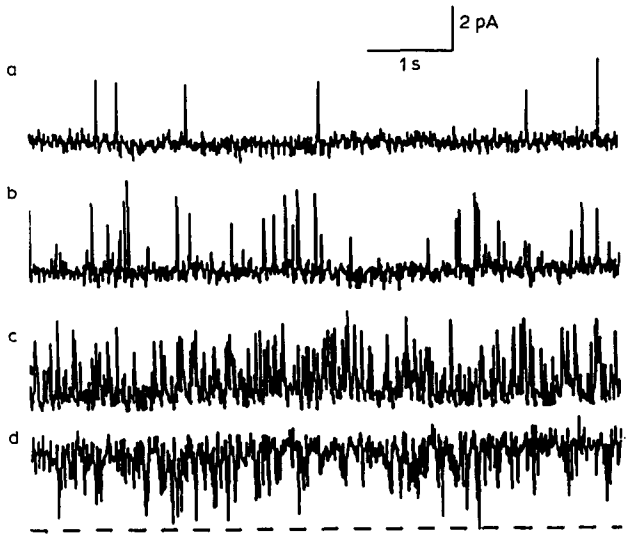

Fig. 6. Effect of cytosolic calcium and ATP on a single channel. Current due to channel opening is shown by upward deflections of the trace from the closed state. The *cis* calcium was raised from (a) 1 μM to (b) 10 μM to (c) 100 μM. In (d) 1 mM ATP was added to 100 μM calcium in the *cis* chamber; the dashed line indicates the current level of the closed channel state.

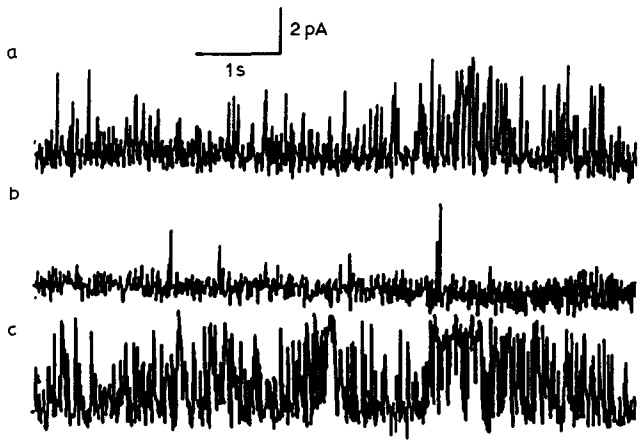

Fig. 7. Effect of cytosolic magnesium on a single channel. Current due to channel opening is shown by upward deflections from the closed state. The composition of the *cis* chamber has been altered: (a) 10 μM calcium, (b) 10 μM calcium + 1 mM magnesium, (c) 100 μM calcium + 1 mM magnesium.

calcium, 1 mM magnesium had no effect on binding, but in incubation media with lower calcium concentrations, it had an increasingly potent inhibitory effect. In media containing very low (10 nM) calcium, 3 mM ATP produced no stimulation of binding; furthermore, no additional binding sites were recruited when 3 mM ATP was added to 100 μM calcium in the medium. However, at a sub-maximal activating (5 μM) calcium concentration, 3 mM ATP increased ryanodine binding to maximal levels. Ruthenium red was effective in reducing binding. At a concentration of 1 μM, inhibition was greater when vesicles were allowed to pre-incubate with ruthenium red prior to the addition of the ryanodine.

The ability of caffeine to release calcium from heavy SR vesicles [27] and also to increase the open probability of the single calcium-release channel [28,29] has been described. The benzimidazole drug, sulmazole [30], has also been demonstrated to increase single-channel open probability [31]. In Fig. 5, the effects of these drugs on [^{3}H]ryanodine binding at different calcium concentrations is shown. Both drugs increased binding at sub-maximal activating calcium concentrations. In 10 nM calcium, the potency of caffeine was dramatically reduced, 20 mM caffeine produced only 1.4% of maximal binding. The potency of sulmazole in very low calcium concentrations was also reduced; however, the drug still produced considerable stimulation of binding and its potency was similar at both 1 nM and 10 nM calcium. Neither drug (data not shown) was able to recruit additional ryanodine binding sites when added to media containing 100 μM calcium.

Activation of the sheep cardiac calcium-release channel by elevation of the *cis* calcium concentration is shown in Fig. 6. As previously reported [32], calcium, as sole ligand, was unable to activate the channel fully. 1 mM ATP added to the *cis* chamber produced a marked increase in channel open probability even when added to media with optimal calcium concentrations. 10 nM *cis* calcium is too low to stimulate any channel openings and, under these conditions, the addition of ATP was without effect (data not shown).

The addition of millimolar magnesium to the *cis* chamber reduces channel open probability. As shown in

Fig. 8. Single-channel recordings in 60 pM *cis* calcium in the presence of 5 μM ryanodine. Upper trace, the channel shows no opening events over a 10-min period. Lower trace, 4 mM sulmazole was added to the *cis* chamber immediately prior to the recording. Frequent channel opening events are seen followed by a ryanodine blocking event after which the channel remained open with a reduced conductance.

Fig. 7, this effect is dependent on the level of *cis* calcium, being most marked at low calcium concentrations. The effect of ryanodine on the calcium-release channel in bilayers is to modify the normal rapid gating pattern, holding the channel in a fixed open state with a reduced conductance relative to the native open state [33]. Once 'blocked' by ryanodine, channels are insensitive to control by other ligands.

In Fig. 8, current fluctuations from a single channel in the presence of very low (60 pM) *cis* calcium is shown. The channel remained closed and 5 μM ryanodine added to the *cis* chamber had no effect. The addition of 4 mM sulmazole to the *cis* chamber activated the channel, despite the very low, sub-activating, calcium concentration and shortly afterwards the channel was blocked by ryanodine. As in this experiment, within the resolution of our apparatus (approx. 1 ms), ryanodine always appeared to block the channel from the open state.

As ryanodine binding and bilayer experiments were performed under different conditions, the results may not necessarily be directly comparable. Nevertheless, there are obvious parallels between the two sets of results. All agents which either increase or decrease channel open probability have similar effects on ryanodine binding. Thus calcium, either as sole ligand or together with caffeine or ATP, stimulates, while magnesium and Ruthenium red inhibit binding. The reduction of [^{3}H]ryanodine binding from maximal levels in the presence of 10 mM calcium is in accord with previous observations [26] and is consistent with reports that very high calcium concentrations reduce the open probability of the cardiac calcium-release channel [32]. The activation of channel openings and ryanodine binding by sulmazole at very low calcium concentrations is of interest (a detailed account of the actions of sulamzole on single calcium-release channels will be published elsewhere [37]). The effect of sulmazole on ryanodine binding at sub-micromolar calcium concentrations appears to be calcium independent as drug potency is similar in 1 nM and 10 nM calcium. The observation that no additional binding sites are recruited when 10 mM sulmazole is added to binding media containing 100 μM calcium provides additional evidence that [^{3}H]ryanodine binding activated by sulmazole in low calcium media involves the same pool of receptor sites that are available for calcium activated binding. It is therefore possible to extend the observation on ryanodine binding dependency to include open-channel states other than those produced by calcium activation.

1 μM Ruthenium red reduced ryanodine binding more effectively when pre-incubated with vesicles before the addition of [^{3}H]ryanodine. This difference suggests that once ryanodine has bound to the calcium-release channel, ruthenium red in unable to displace it, this is consistent with previous reports that pre-incubating heavy SR vesicles with ryanodine inhibits the stimulation of calcium loading produced by Ruthenium red [34].

The major difference between ryanodine binding and single-channel behaviour, is that maximal ryanodine binding is achieved with ligands that do not appear sufficient to activate the single channel maximally. As evidence suggests that ryanodine must bind to the channel open state, maximal binding may well be achieved under conditions which permit some minimum degree of channel opening for all individual channels. This would obviously not require full activation of all channels. Ryanodine binding studies would be insensitive to additional channel activation above this level.

This model explains why Michalak et al. [13] showed minimal effects of ATP on [^{3}H]ryanodine binding in cardiac muscle when added to incubation media containing 50 μM calcium, as this calcium concentration is sufficient to produce maximal binding on its own. Similarly, the report that magnesium did not reduce [^{3}H]ryanodine binding in the presence of 50 μM calcium [13], is explained because, at this calcium concentration, magnesium may not be able to reduce channel open probability below the threshold which permits maximal binding. In skeletal SR, Pessah et al. [12] found that magnesium inhibited [^{3}H]ryanodine binding and that this effect was due to direct competition with calcium for the binding site. Our results which show that the inhibitory effect of magnesium is most marked at low calcium concentrations on both [^{3}H]ryanodine binding and single-channel activity are in accord with this mechanism. The relatively low sensitivity of [^{3}H]ryanodine binding to modulation by magnesium and ATP in cardiac, as opposed to skeletal, SR is, however, consistent with previous comparisons of the effects of these ligands on calcium efflux and single-channel activity [3]. Activation of the cardiac channel was dependent to a greater extent on cytosolic calcium concentrations than the skeletal channel which showed a more marked response to ATP and magnesium.

The kinetics of single calcium-release channels have been investigated in both skeletal and cardiac muscle SR [32,35,36]. Given the resolution possible with planar bilayer experiments, at least two open states are proposed for the calcium-activated channel. The effect of increasing the cytosolic calcium concentration is to increase the frequency of opening events without altering the distribution of their durations. In the presence of calcium concentrations which produce only a relatively low open probability, all channels exhibit some opening events and it would be predicted, therefore, that equilibrium ryanodine binding would be similar although the time taken to reach equilibrium would be dependent on the calcium concentration. As has been shown previously [26] and is also demonstrated in Fig. 1, in which

different combinations of ligands result in similar rates of binding, this does not occur. The evidence suggests that, although ryanodine appears to bind only to the open channel, it cannot bind to all open states. Open states which can and cannot bind ryanodine are, however, not distinguished by the analysis of single-channel data.

In conclusion, ryanodine binding studies can provide a very sensitive guide to the degree of activation of the calcium-release channel. The results presented here extend and qualify previous observations of [^{3}H]ryanodine binding to cardiac SR. A model has been proposed to relate the results of binding studies to present and previous reports of single-channel behaviour. The model adequately explains differences in the binding results reported here with those previously reported for skeletal muscle preparations. The use of [^{3}H]ryanodine binding as a probe for channel activation makes it a powerful tool for the investigation of ligands such as novel drugs, which may interact with the calcium-release channel.

Acknowledgement

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