

How Many Ryanodine Binding Sites Are Involved in Caffeine Induced Calcium Release from Sarcoplasmic Reticulum Terminal Cysternae Vesicles?

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Sarcoplasmic Reticulum, Caffeine, Calcium Release, Ryanodine Binding

The inhibition by ryanodine of caffeine induced calcium release from actively loaded heavy sarcoplasmic vesicles has been studied in order to analyse the relation between the occupancy of the vesicular calcium release channels by ryanodine and channel function. Ryanodine binding was monitored with [³H]ryanodine under ionic conditions favouring the establishment of binding equilibrium. Binding follows 1:1 stoichiometry yielding dissociation constants between 7–12 nM and 12–15 pmol ryanodine/mg vesicular protein as maximum number of ryanodine binding sites. When ryanodine labeling was monitored by measuring the decline of the amplitude of caffeine induced calcium release 50% inhibition occurred at a free ryanodine concentration of 1 nM. At this concentration less than 10% of the available ryanodine binding sites are occupied. Caffeine induced calcium release is completely abolished when 3 pmol ryanodine/mg have reacted. A corresponding divergence between ryanodine binding and its effect on caffeine induced calcium release was observed when the initial rate of ryanodine binding was measured either by labeling the vesicles with [³H]ryanodine or by following the decline with time of caffeine induced calcium release. Caffeine induced calcium release declines four times faster than the fraction of unoccupied ryanodine binding sites, $k = 4.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ versus $1.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. The observed interrelation between the occupation of ryanodine binding sites and its effect on caffeine induced calcium release indicates that the caffeine sensitive calcium channel functions as an assembly of at least 4 ryanodine binding sites whereby the occupation of one site suffices to abolish calcium release. The stoichiometric composition appears to be not fixed but might change according to the size of the fraction of ryanodine receptors exhibiting caffeine sensitivity. The reported data were evaluated according to the algorithm derived by H. Asai and M. F. Morales, J. Biol. Chem. **4**, 830–838 (1965) for the activity of a macromolecule and the extent of an inhibiting reaction.

Introduction

Ample evidence has been provided in the last two decades that muscle's contractile proteins are activated by the release of calcium from specialized structural elements of its sarcoplasmic reticulum membranes. The conditions under which calcium release occurs could be assessed *in vitro* by studying calcium release from passively or actively loaded sarcoplasmic reticulum vesicles, preferentially its heavy fraction enriched in terminal cisternae [1–8]. It has been shown that the rapid efflux of calcium from the preparation depends in a rather complex manner on the composition of the assay media and the physiological or pharmacologi-

cal agents used to induce a rapid net release of calcium such as calcium, ATP or caffeine [2–3]. In a second study line release competent membrane fractions were incorporated into lipid bilayers and single channel activity could be monitored [6, 7]. This analysis revealed that the same agents which effect or modulate net calcium release from calcium loaded vesicles also modify single channel activity. It further turned out, which is also in agreement with observations made with vesicular suspensions, that beside calcium, the channel likewise conducts potassium or sodium ions [8]. Beyond that, single channel recordings seem to reveal that the activity of isolated channels is characterized by at least four subconductance states [7, 9, 10] what, however, has recently been questioned and ascribed to modifications of the channel's protein during isolation and reconstitution [11]. The identification of the channel protein which constitutes only 1–2% of the membrane fraction enriched in

Abbreviations: AcP, acetylphosphate; IP₃, inositol-1,4,5-triphosphate.

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terminal cisternae was very much facilitated by its specific labeling with ryanodine, which is long known as an effective muscle poison [12]. Its reaction with the channel protein as well as its effect on calcium release or channel activity are strongly favoured by the presence of high concentrations of salts of monovalent ions [13]. The high stability of the ryanodine protein complex allowed its purification and its identification with the quadrifollic structures in the terminal cisternae membranes on the one hand and the recent cloning of its c-DNA [10, 14–20]. The large quadrifollic membrane complex is assumed to be composed of a tetrameric complex of the ryanodine binding protein having a molecular weight of 400–500 kDa. The molecular models derived from its amino acid sequence indicate that each monomer is anchored to the membrane by a small assembly of hydrophobic segments from which large hydrophilic extensions protrude into the cytoplasmic space. This peculiar structure together with its observed multiple state ion conductances led to the notion that either each of the four subunits contributes with one conducting pore or that there is only a single large pore formed and controlled by the four protein units [7].

In this study we address the question whether the calcium channel in native heavy, sarcoplasmic reticulum vesicles operates as an oligomeric structure by analyzing ryanodine binding and its effect on caffeine induced calcium release. In contrast to other experimental set ups ryanodine poisoning and its effect on caffeine induced calcium release can be studied under a great variety of well controlled conditions. It is shown that quite different association constants and rate constants are obtained for ryanodine binding at equilibrium and its binding kinetics, respectively, when derived either from the incorporation of radioactively labeled ryanodine or from the inhibition of caffeine induced calcium release. The data support the assumption that the functional releasing unit is at least four times larger than the ryanodine binding unit.

Materials and Methods

Heavy sarcoplasmic reticulum vesicles were prepared from predominantly white rabbit hind leg muscles as described by Hasselbach and Migala

[21] and modified according to Meissner [4]. 100 g muscle were minced for 4×30 s in 400 ml extraction fluid containing 0.1 M KCl, 5 mM potassium phosphate (pH 7.0), 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM sodium azide in a mixer at 4 °C. Myofibrils were removed from the suspension by centrifugation in a Servall GS3 rotor at 6000 rpm for 30 min. The heavy vesicles were separated from the supernatant by subsequent centrifugation in a Spinco type 19 rotor at 10,000 rpm for 30 min.

Contaminating contractile proteins were extracted by treating the pellet with 10 volumes of 0.6 M KCl, 0.3 M sucrose, 3.0 mM ATP, 3 mM $MgCl_2$, 0.1 mM benzethonium-Cl and 0.1 mM benzamidinium-HCl, 0.1 mM dithiothreitol for 20 min. Subsequently, the vesicles were sedimented by centrifugation in a Spinco rotor Ti 60 at 55,000 rpm for 50 min and resuspended in 0.1 M KCl (30–50 mg/ml).

Calcium uptake and release experiments were performed as described by Su and Hasselbach [3]. The standard media contained 0.1 M potassium gluconate, 0.1 M sucrose, 50 mM imidazole-sulfate or Tris-Mops (pH 7.0), 2–3 mM ATP, 8 mM phosphoenolpyruvate, 0.04 mg/ml phosphoenolpyruvate kinase, 2–3 mM magnesium gluconate, 20 μ M $^{45}CaCl_2$. Calcium uptake was started by adding 0.2 mg/ml vesicles to 15 ml of the assay solution. Aliquots were taken at appropriate times and filtered through Schleicher & Schuell BA 85 nitrocellulose filters 0.45 μ m. Caffeine induced calcium release was initiated by the addition of 10 mM caffeine. Release was interrupted at different times by filtration. Time resolved calcium release was measured with a rapid filtration instrument from Bio-Logic, Grenoble, France. Vesicles were loaded with AcP as energy yielding substrate in solution containing 3 mM AcP, 3 mM Mg-gluconate, 0.2 M sucrose, 0.1 M K-gluconate at 30 °C. After cooling to 20 °C aliquots of 0.3 mg were layered on mixed ester filters Me 26, 0.65 μ m and subsequently perfused for 25–1000 ms. ^{45}Ca was measured by liquid scintillation counting of the filters and or of the filtrate.

Ryanodine binding to the vesicles was performed at room temperature, 20–22 °C and in one experiment at 37 °C, in solutions containing 0.6 M KCl, 0.3 M sucrose, and 5–10 mg/ml vesicular protein. Medium pH was adjusted to 6.3 and 7.0

with 0.1 M imidazole sulfate buffer, respectively. Medium pCa was measured with a calcium sensitive electrode and adjusted to 4.0. [^3H] ryanodine bound to the vesicles was determined by separating the vesicles in aliquots of 0.3 mg from the medium using Schleicher & Schuell nitrocellulose filters BA 85, 0.45 μm . Nonspecific binding was measured in media containing 2 mM EGTA. The radioactivity on the filter or, and in the filtrate was registered by liquid scintillation counting. To monitor the dependence of the amplitude of caffeine induced calcium release on the reaction time at given ryanodine concentrations, or after equilibrium binding was achieved after an incubation period of at least 5 h at different ryanodine concentrations, the binding reaction was interrupted by diluting the high salt media (0.6 M) to 0.03 M.

Subsequently the vesicles were loaded with 90–100 nmol/mg ^{45}Ca for 10–15 min in the above described uptake medium. Caffeine induced calcium release was initiated by the addition of 10 mM caffeine and terminated as described above. Ryanodine receptor protein was isolated and purified by combining the procedures applied by Lai *et al.* [15] and Inui *et al.* [16]. Ryanodine binding to the purified protein (0.02–0.05 mg/ml) was performed in 0.6 M KCl, 0.2 M sucrose, pH 7.0, pCa 4 at 20 °C. Binding was terminated by dilution and coprecipitation with vesicular protein (0.2 mg/ml) using 15% polyethyleneglycol (6000) as precipitating agent [10]. After formation of coarse flocculi in the cold the protein was separated by filtration as described above.

Ryanodine and [^3H]ryanodine were purchased from Cal. Biochem. and from Buchler & Co., Frankfurt, respectively. ATP was obtained from Pharma-Waldhof, Mannheim (F.R.G.). Phosphoenolpyruvate and pyruvate kinase were purchased from Boehringer, Mannheim (F.R.G.). All other reagents were analytical grade and bought from E. Merck, Darmstadt; Sigma Chemical Company, Deisenhofen (F.R.G.) and Serva, Heidelberg (F.R.G.). $^{45}\text{CaCl}_2$ was supplied by Buchler & Co., Frankfurt (F.R.G.).

Results

Calcium release from heavy sarcoplasmic reticulum vesicles actively loaded with 90–100 nmol calcium per mg was initiated by the addition of

10 mM caffeine and terminated manually by filtration after 15 s. (Fig. 1). The vesicles were actively loaded in media containing 2–3 mM ATP which was regenerated from 8 mM phosphoenolpyruvate in the presence of 0.1 M K-gluconate and 2–3 mM magnesium gluconate. ATP does not only effect calcium uptake by the calcium pump but also essentially modulates the properties of the calcium release channel. Thus, caffeine only acts as releasing agent in the presence of ATP or of its closely related analogues [22, 23]. Depending on the concentration of ATP, calcium release can be resolved into several phases by rapid filtration procedures (Fig. 2). The quantity of calcium that can be released from actively loaded vesicles by 10 mM caffeine – 30–50 nmol/mg – is set free in approximately one second. Hence, provided that the amount of calcium found in the filtrate 15 sec after caffeine addition is neither affected by active reuptake nor by slow calcium losses, it correctly measures the vesicular fraction which responds to the releasing agent. Fig. 1 demonstrates that reuptake proceeds slowly and thus does not significantly reduce the release amplitude. The half time of reup-

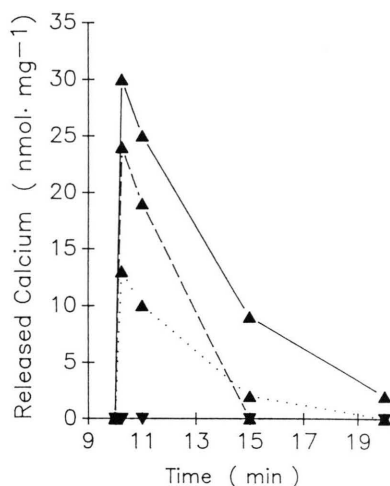


Fig. 1. Time course and decline of caffeine induced calcium release from actively loaded vesicles following ryanodine treatment. 10 mg/ml heavy vesicles were treated with 1 μM ryanodine in 0.6 M KCl, pH 6.3 for 0 (▲—▲), 5 (▤—▤), 10 (▥—▥) and 30 (▼) min. Subsequently the vesicles were loaded for 15 min with 90 nmol ^{45}Ca /mg in solutions containing 2 mM ATP and 3 mM Mg-gluconate as described in Materials and Methods. Calcium release was initiated by the addition of 10 mM caffeine and terminated by filtration at times indicated on the abscissa.

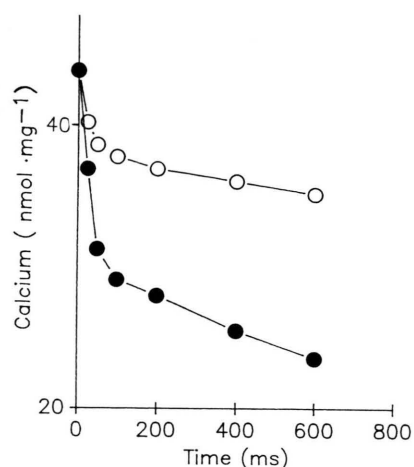


Fig. 2. Initial phases of caffeine induced calcium release from actively loaded vesicles. Calcium loading was performed with 3 mM AcP in solutions containing 3 mM Mg-gluconate, 0.1 M K-gluconate 50 mM Tris Mops at 30 °C for 10 min. After cooling to 20 °C aliquots of the vesicular suspension (0.3 mg/ml) were layered on Schleicher and Schuell BA 0.6 μ m filters and rapidly perfused for the times given on the abscissa with solutions containing 5 mM ATP, 5 mM EGTA, 0.1 M K-gluconate (○) or 5 mM ATP, 5 mM EGTA, 10 mM caffeine and 0.1 M K-gluconate (●). Ordinate: calcium remaining in the vesicles.

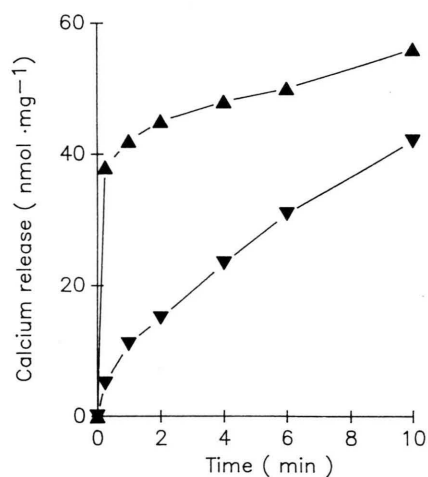


Fig. 3. Time courses of caffeine induced calcium release and calcium leakage. Calcium loading was performed in uptake media containing 2 mM ATP and 3 mM Mg-gluconate as given in Materials and Methods. Release was initiated by adding four volumes of 0.1 M K-gluconate, 0.1 M sucrose, 50 mM imidazole-sulfate with 1 mM EDTA (▼), or with 1 mM EDTA + 10 mM caffeine (▲). Release initiated with EDTA alone represents calcium leakage. Rapid caffeine induced calcium release is followed by a slow calcium loss.

take (2 min) is furthermore not affected when the release amplitudes are reduced by prior treating the vesicles with ryanodine (Fig. 1, see below). The release amplitude is also not falsified by losses of calcium occurring through unspecific pores through which it permeates in a time scale of several minutes as shown in Fig. 3. A quantitative correlation between the release amplitude and the action of caffeine further requires as discussed by Meyer *et al.* [24] that the time constants for the binding of the releasing agent as well as for its efficacy are short compared to the measuring time. This can safely be assumed since the adopted release interval is ten times longer than the observed reaction times (Fig. 2). Two experimental approaches were pursued to evaluate the relation between the occupancy by ryanodine of the calcium release protein and the releasing action of caffeine. i) Measurements of the dependence of both reactions on the concentration of ryanodine should verify if ryanodine exhibits identical or different affinities for binding and effectiveness, respectively. ii) The stoichiometric relation between the release protein's receptors for ryanodine and caffeine, respectively, might be established by the evaluation of the dependence of calcium release on the occupancy of the ryanodine binding sites. The first approach requires measurements of ryanodine binding after attainment of binding equilibrium with radioactively labeled ryanodine and in parallel the same preparation labeled under identical conditions with non-labeled ryanodine was used to probe caffeine induced calcium release. This protocol avoids interferences between the measurements of ^{45}Ca activity in the uptake and release assays and the ^3H -activity of bound ryanodine. Fig. 4 shows that at pH 7.0 and 20 °C approximately 5 h are required at ryanodine concentrations 40 nM to approach binding equilibrium in media containing 0.6 M KCl and 0.1 mM CaCl_2 . The binding isotherm is not further raised by prolonging the reaction time. It rather starts to decline and significantly lower values were observed after 23 h. At higher concentrations of ryanodine (40 nM), binding equilibrium is more rapidly approached. Maximum specific binding of 10 pmol/mg is reached at 100 nM free ryanodine which is comparable to results obtained with preparations of purified terminal cisternae [25–27]. Computer approximation of the concentration binding rela-

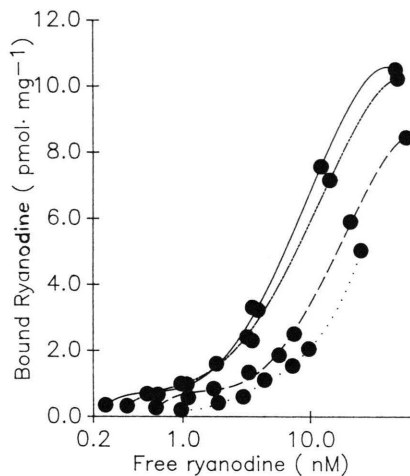


Fig. 4. Effect of reaction time on ryanodine binding at increasing free ryanodine concentrations. 5 mg/ml vesicles were reacted with increasing concentrations of [^3H]ryanodine for 30 min (●—●), 1 h (●—●), 5 h (●—●) and 16 h (●—●) in 0.6 M KCl, pH 7.0, pCa 4.0. Bound ryanodine was determined as described in Materials and Methods. Binding data were analyzed assuming simple ligand binding and applying Enzfitter, Elsevier-Biosoft for approximation.

tionship assuming ligand binding to one site yielded a dissociation constant of 7–10 nM (Table). Significantly higher values were obtained when instead of KCl, NaCl was used to promote ryanodine binding (not shown). Markedly lower values appear to prevail for the isolated and purified receptor, 4 nM [15] and 2.5 nM (Table), respectively.

To monitor the binding of ryanodine by measuring its effect on caffeine induced calcium release, the heavy vesicular fractions were reacted with cold ryanodine under the same conditions as described above for measuring the binding of radioactively labeled ryanodine. As shown in Fig. 1 and previously reported, the amplitude of caffeine induced calcium release is progressively reduced by treating the vesicles with ryanodine in the incubation medium [21]. In Fig. 5 the release amplitude is plotted *versus* the prevailing free concentration of ryanodine. There is considerable scattering of the data points which is due to uncertainties in the determination of both variables especially at low ryanodine concentrations and small release increments. Yet, the free ryanodine concentration of

Table. Ryanodine binding and efficacy parameters. The collected data were obtained by simultaneously measuring caffeine induced calcium release from actively loaded heavy sarcoplasmic vesicles preceded by ryanodine treatment and its registration using [^3H]ryanodine as described in Materials and Methods. The free calcium concentration of 0.8 nM (c_d) at which caffeine induced release is reduced by 50% corresponds to the binding of 0.7 pmol ryanodine/mg protein as shown in Fig. 6. The number of sites involved in caffeine induced calcium release (n), given in the text, were obtained from the graph in Fig. 6 and the tabulated data. Eqn. (1) was used to derive (n) from the decline of caffeine induced calcium release and the fractional occupation O/T of the ryanodine binding sites.

$$n = 0.3 / \log (K_d + c_d) / K_d \quad (2)$$

which results from Eqn. (1) under the assumption that all ryanodine binding sites were equally reactive was applied to derive (n) from the apparent dissociation constants emerging from ryanodine binding (K_d) and caffeine induced calcium release measurements (c_d), respectively. Data are given as means \pm SEM.

Activity	Preparation	App. dissociation constant nM	Max. capacity pmol · mg ⁻¹	Binding rate constant M ⁻¹ · s ⁻¹
Ryanodine binding	Vesicles Ryanodine receptor	10.2 \pm 1.3 (K_d)	13.0 \pm 0.7	(1.3 \pm 0.16)10 ⁴
		2.6 \pm 1.0 (K_d)	133 \pm 5.2	—
Caffeine induced calcium release	Vesicles	0.8 \pm 0.2 (c_d)	2.5 \pm 3.0	(4.3 \pm 0.4)10 ⁴

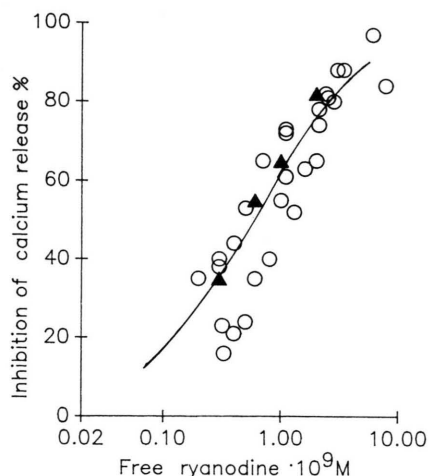


Fig. 5. Dependence on free ryanodine of inhibition of caffeine induced calcium release. 10 mg/ml heavy vesicles were reacted with [^3H]ryanodine in 0.6 M KCl, pH 7.0, pCa 4.0 for 5 h. Free ryanodine remaining in the solution was determined as described in Materials and Methods. Aliquots were taken and added to ATP-containing uptake media. After a loading period of 15 min, calcium release was initiated by adding 10 mM caffeine. Release was terminated by filtration 15 s subsequent to caffeine addition. Release amplitudes (%) (○) are given on the ordinate. Mean values from previously reported experiments [21] (▲) are included.

$0.8 \pm 0.2 \text{ nM}$ at which calcium release is reduced by 50% can be determined with considerable confidence (Table). It is significantly smaller than the ryanodine concentration at which half saturation of its binding sites is reached. The slope of the graph is somewhat steeper than to be expected for the occupation of one independent binding site. The results of binding experiments performed in parallel with radioactive ryanodine could also be used to directly correlate caffeine induced calcium release and ryanodine binding. In Fig. 6 results of experiments are collected in which the vesicles were labeled with ryanodine at pH 7.0 and at pH 6.3. The subsequent loading of the vesicles with calcium and its release were performed at pH 7.0. The initial calcium load did neither depend on the degree of labeling nor on the pH of the labeling medium. It is evident that the labeling conditions with respect to medium pH do not affect the relation between the degree of labeling and the reduction of the release amplitude. Thus, changing of medium pH does not interfere with the specificity of ryanodine binding in the sense that it might lead

to the occupation of different sets of binding sites although medium pH greatly affects the rate of labeling (see below). The midpoint of calcium release inhibition is reached when only 0.5 pmol ryanodine/mg protein are bound and tends to completion at an occupancy of 3 pmol/mg. Thus the occupation of only a small fraction of the ryanodine binding sites suffices to abolish caffeine induced calcium release. The large divergences between the dissociation constants as well as the number of reactive sites obtained from the evaluation of ryanodine binding and its effect on calcium release, respectively, led to suspect similar divergences between the time course of ryanodine binding and the time course of the decline of caffeine induced calcium release. We have therefore evaluated its dependence on the concentration of added ryanodine, for both functions under identical conditions. Reaction rates could most conveniently be measured at ryanodine concentrations be-

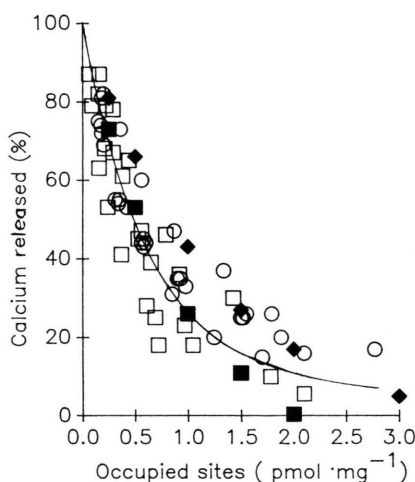


Fig. 6. Relation between caffeine induced calcium release and the occupancy of the vesicles' ryanodine binding sites. Ryanodine binding and the determination were performed as described in Materials and Methods 10 mg/ml vesicles were incubated with ryanodine in 0.6 M KCl, pCa 4 at pH 6.3 and pH 7.0 [19]. Release amplitudes (%) are given on the ordinate for preparations reacted with ryanodine at pH 7.0 (○) and pH 6.3 (□). The relation between calcium release and the occupation number (pmol/mg) was fitted by Eqn. (1). The experimental data are quite well approximated by exponents between $n = 8$ (◆) and $n = 12$ (■) under the assumption that all ryanodine binding sites were equally reactive. Assuming that only half of the sites participate in calcium release the data could be fitted with $n = 4$ or 6. The latter approximation yielded results which were identical with those for $n = 12$ assuming homogeneous sites.

tween 0.1 and 1 μM at 20–22 °C. Labeling with active and non-active ryanodine was started by the addition of the agent to 5 mg/ml heavy vesicles suspended in 0.6 M KCl, pH 7.0 or in some experiments at pH 6.3. The applied concentrations are only little reduced during initial binding which we measured between 5 and 100 s. Binding is considerably accelerated when temperature is raised to 37 °C making rate measurements more difficult. The reaction was terminated by diluting the sample with 0.2 M sucrose at least 10-fold. The complete stop of the reaction after dilution is illustrated in Fig. 7 also showing the acceleration of the reaction at elevated temperature. The minimal reaction time is 2 sec. Fig. 8 shows that the binding of labeled ryanodine follows first order kinetics between 0.2 and 1 μM . The same rate parameters prevail between 3 nM and 10 μM whereby the measurements in the higher concentration range become increasingly difficult to follow by our manual procedure (not shown). A straight line is obtained when the observed rates are plotted *versus* the respective concentrations yielding a rate constant $k_1 = 1.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for the binding reaction (Fig. 9). The line intersects the ordinate very near

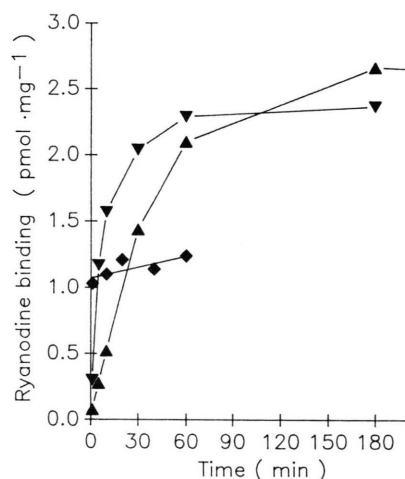


Fig. 7. Time course of ryanodine binding at a low ryanodine concentration. 5 mg/ml vesicles were reacted with 15 nM [^3H]ryanodine in 0.6 M KCl, pH 7.0 pCa 4.0 for the time indicated on the abscissa at 37 °C (▼) and 20 °C (▲). The reaction was interrupted after 1 min by a 10-fold dilution with 0.2 M sucrose. Subsequently the vesicular suspension was filtered to determine bound [^3H]ryanodine at the times given on the abscissa. The line depicted by (◆) shows the complete stop of the reaction after dilution.

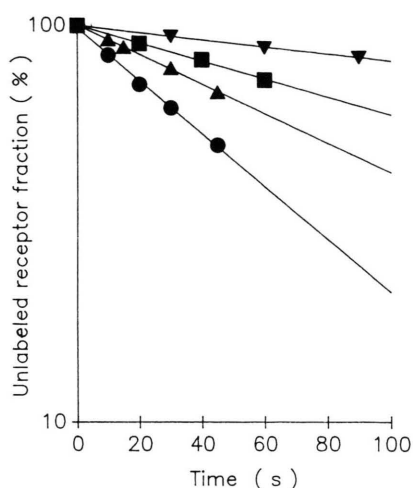


Fig. 8. Initial rate of ryanodine binding at different ryanodine concentrations. 5 mg/ml vesicles were incubated in reaction media containing 0.6 M KCl, pH 7.0, pCa 4.0 and 0.2 (▼), 0.4 (■), 0.6 (▲), 1.0 (●) μM ryanodine for the times given on the abscissa and for 5 h to reach binding equilibrium. The reaction was interrupted by dilution and filtration. The logarithm of the unreacted fraction of receptors $(R_{y_{00}} - R_{y_t})/R_{y_{00}}$ was plotted on the ordinate.

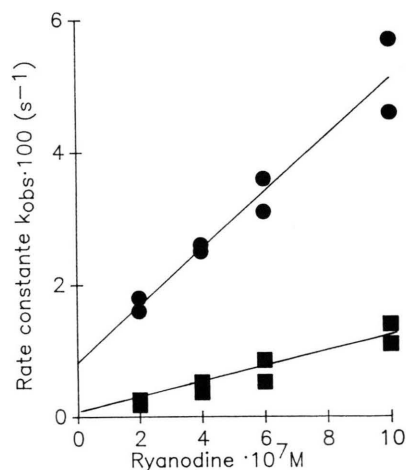


Fig. 9. Dependence on free ryanodine of the rate constants for ryanodine binding and the decline of caffeine induced calcium release. The rate constants for ryanodine binding were obtained from the half times τ of the binding experiments depicted in Fig. 8; (■) $k_{\text{obs}} = 0.69/\tau$. The constants for the decline of caffeine induced calcium release were derived from the experiments shown in Fig. 11 (●).

to the origin indicating a small value for the dissociation rate constant. Its magnitude is also difficult to obtain by the alternative procedure of directly measuring the decay of the ryanodine protein complex. Due to the high affinity of ryanodine to its receptor, it is hardly possible to achieve dissociation of the complex by dilution sufficiently large to be reliably measurable. It proves also not practical to follow dissociation of the radioactively labeled complex by adding thigh concentrations of cold ryanodine. McGrew *et al.* [27] have shown that this procedure lowers the dissociation rates presumably due to the occupation of low affinity binding sites. We have therefore induced dissociation by withdrawing from the assay calcium which is needed to stabilize the complex. Fig. 10 shows the decay of the complex logarithmically plotted *versus* time. The straight line yields $5 \times 10^{-5} \text{ s}^{-1}$ as dissociation rate constant. Together with k_1 a dissociation constant K of 4 nM is obtained which agrees reasonably with the results of the equilibrium measurements. Yet, this agreement does not prove that ryanodine binding in fact occurs in a single reaction step. The formation of a labile primary complex is suggested by the low value of k_1 and the fact that the observed rates do not exhibit

any sign of saturation up to a concentration of $10 \mu\text{M}$.

The decline of the release amplitude of the caffeine induced calcium release with reaction time is shown in Fig. 11. Its comparison with Fig. 8 reveals that the release amplitude declines much faster with time than the fraction of non-occupied ryanodine receptors. The rate constant emerging from the decline of calcium release is approximately 4 times higher than that monitored for ryanodine binding (Fig. 9). Thus we are confronted with the question whether several ryanodine binding sites constitute the caffeine sensitive ryanodine receptor complex or if the caffeine sensitive sites more rapidly react with ryanodine than the non-sensitive fraction. The latter alternative appears to be excluded by the observation that the same k_1 on-rate was obtained for the whole concentration range from 3 nM to $10 \mu\text{M}$. Likewise the dissociation reaction proceeds with an uniform rate.

Discussion

The results of the reported experiments demonstrate that occupation of the calcium release channel by ryanodine is not assigned directly to its reac-

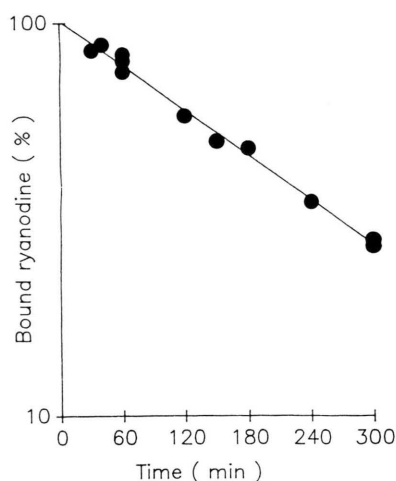


Fig. 10. Decay with time of the ryanodine complex. 2 mg/ml vesicles were reacted with 10 nM [^3H]ryanodine for 1 h in 0.6 M KCl, pH 7.0 pCa 4.0 at 20°C . Dissociation of the complex was initiated by complexing medium calcium with 2 mM EGTA. The undissociated fraction of the complex was determined after dilution and removal of unbound ryanodine by filtration. Bound ryanodine is plotted logarithmically on the ordinate.

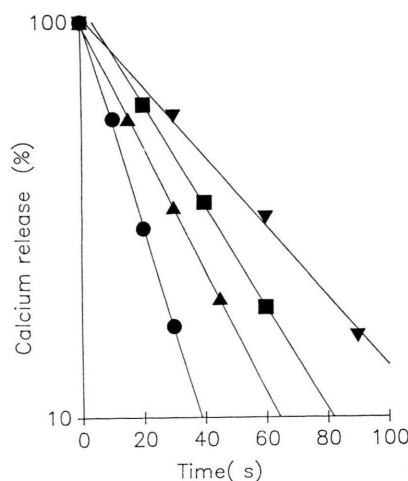


Fig. 11. Dependence on reaction time of caffeine induced calcium release at different ryanodine concentrations. 10 mg/ml vesicles were reacted in 0.6 M KCl, pH 7.0, pCa 4 with $0.2 \mu\text{M}$ (∇), $0.4 \mu\text{M}$ (\blacksquare), $0.6 \mu\text{M}$ (\blacktriangle) and $1 \mu\text{M}$ (\bullet) ryanodine for the times given on the abscissa. The reaction was arrested by dilution. Caffeine induced calcium release was subsequently measured as described in Materials and Methods and the release amplitudes were logarithmically plotted on the ordinate.

tion with caffeine measured as calcium release from actively loaded heavy vesicles. This has been shown under quite different experimental conditions with respect to the extent of ryanodine binding. We found that ryanodine binding monitored with radioactive ryanodine furnished 4–12-fold higher values for the dissociation constant of the ryanodine receptor complex than when registered by following the concentration dependence of the decline of caffeine induced calcium release. The same divergence was found when the initial rate of ryanodine binding was studied either by labeling the channel with radioactive ryanodine or by following the decline of its sensitivity for caffeine. The most striking finding underlining the multimodal relation between the inhibition of caffeine induced calcium release and the occupancy of the ryanodine binding sites resulted from the experiments where both functions were simultaneously measured on the same preparation. The mean maximal number of ryanodine binding sites 10–15 pmol/mg of our preparation well agrees with values found by others under similar conditions [14, 16, 25, 27]. The occupation of only a fraction of 3 pmol/mg suffices to completely block caffeine induced calcium release. 50 percent blockage is already achieved when only 0.8 pmol ryanodine/mg have reacted with the channel protein. Before drawing conclusions concerning the functional implications of these findings we have to ascertain that they are not affected by methodical or experimental artifacts. Since widely varying values for the dissociation constant of the ryanodine complex of the channel protein measured with radioactive ryanodine have been reported [13, 16, 10, 25–27], we had to repeat these measurements under the conditions which we had adopted to study the effect of ryanodine on caffeine induced calcium release [21]. Labeling was therefore performed in media containing 5–10 mg protein/ml, 0.2 M sucrose, 0.6 M KCl, pH 7.0, pCa 4 at 20 °C. The relatively high protein concentration was required, in order to minimize changes in the composition of uptake and release media when aliquots of the labelling assay were added to establish a protein concentration of 0.2 mg/ml and KCl concentrations not exceeding 0.03 mM. The labeling period could be extended up to 20 h under these conditions. The establishment of binding equilibrium was thus assured. The quite large differences be-

tween the values of previously reported binding constants are presumably caused at least in part by not reaching binding equilibrium. It proves also necessary to carefully adjust the pH of the labeling medium since the binding reaction exhibits a marked pH dependence [21]. It becomes much faster with rising pH whereby the extent of labeling is not affected. We also limited our analysis of the action of ryanodine to concentrations below 1 μ M to be sure that only the high affinity binding sites were involved in the reaction. We considered the contribution of unspecific binding by subtracting the amount of ryanodine which was bound at each concentration in the presence of 2 mM EGTA. It is a small correction as long as the concentration does not exceed 10 μ M. By defining unspecific binding as the binding in the absence of calcium and not by adding high concentration of cold ryanodine we circumvent the complication arising from the increase of unspecific binding and its interference with the establishment of binding equilibrium [27]. To measure the time course of ryanodine binding one must be able to rapidly and completely interrupt the reaction. This could be done most efficiently by diluting 10-fold the high ionic strength labeling medium. At ionic strength values below 0.1 the channel proteins reactivity towards ryanodine is completely blocked (comp. Fig. 7 and [21]). It was thus possible to study calcium release after the vesicles were labeled in high salt media for different time intervals. For the evaluation of binding rate measurements it was necessary to determine binding equilibrium for each concentration. This was done after an incubation period of at least 5 h. The applied monitoring of the activity of the calcium release channel by measuring the amplitude of calcium release requires the correlation of this event with accepted reaction parameters. The amplitude of the release of various effectors from different organelles has repeatedly been used to quantitate the effect of the releasing agents. For IP_3 -induced calcium release it has been shown that the quantity of calcium released after 15 s represents the fraction of open channels if the interaction of the agent with the channel and its response occur on a much shorter time scale than the registration of the release [24]. This requirement is fulfilled for the interaction of caffeine with the channel as shown by our time resolved rapid filtration experiments, in agreement

with the recent results of Wyskovsky *et al.* [28], see also [23], caffeine induces a three phasic release. The two fast components are completed after approximately 1–4 s. They are followed by a loss of a small fraction of calcium which proceeds in a time scale of minutes. The distortion of the release amplitude by calcium reuptake can also be disregarded because reuptake by the vesicular fraction having no or non-activated channels is slow due to the inhibiting action of the high calcium concentrations inside the actively loaded vesicles. Our analysis, furthermore, does not depend on the actual mechanism of ryanodine action. There is a general agreement that ryanodine arrests the calcium release channel in an open state configuration when monitored in the absence of ATP [29]. Yet when ryanodine treated vesicles were actively loaded in ATP-containing media the initial retardation of calcium uptake is followed by a phase of accelerated uptake and terminates at a reduced calcium level [21]. Subsequently caffeine induced calcium release can no longer be elicited. In this study we discussed the possibility of an ATP induced channel closing [21]. This mechanism is difficult to rule out. Yet, caffeine induced calcium release would equally be affected by both permanent channel opening or closing. By comparing the dependence on caffeine of the calcium release amplitude with other manifestations of channel activity, additional arguments can be obtained which support our basic assumption that the amplitude of caffeine induced calcium release represents a quantitative measure of channel function. The recent studies of Rousseau *et al.* [30] who measured the dependence on caffeine of channel open probability in planar bilayers furnished the same concentration dependence as previously reported for the calcium release amplitude [3]. In both studies the threshold concentration of caffeine was found to amount to 0.5 mM and half saturation was achieved with 10 mM. In the same concentration range caffeine accelerates the rate of calcium release from passively loaded vesicles in the presence of 5 mM ATP [23] and stimulates the calcium dependent ATPase of heavy vesicles being previously suppressed by high internal calcium (not shown). The opening of the channels by caffeine result in a decline of the intraluminal calcium concentration which lifts trans-inhibition.

The most striking disparity between ryanodine

binding and the suppression of caffeine induced calcium release is seen when the total number of ryanodine binding sites, 12 pmol/mg, is compared with the number of sites the occupation of which abolishes caffeine induced calcium release, 3 pmol/mg. The possibility that the latter assembly of sites has a higher affinity than the remaining majority is unlikely. In titration experiments with ryanodine under identical conditions no second set of binding sites could be detected in our preparation. Following the considerations of Liu *et al.* [7] it is suggestive to assume that the calcium releasing unit is composed of several ryanodine binding units and that as shown here, the occupation of one unit results in the abolition of the release function. The steep decline of the caffeine response which reaches already 50% when only 1 pmol ryanodine has reacted with one mg protein is in line with the proposed structure of the calcium releasing unit. The composition of the complex was estimated from the relation

$$a = (1 - O/T)^n \quad (1)$$

a = activity, O = number of occupied sites, T = total number of sites, n = number of sites involved in activity

given by Asai and Morales [31], describing the relation between the activity of a macromolecule and the extent of an inhibiting reaction. The evaluation by this relation of the results shown in Fig. 6 depends on the occupation ratio O/T . Only if all ryanodine binding sites were equal in their reactivity the release unit would consist of approx. 8–12 ryanodine binding units. Smaller figures result if the ryanodine binding sites were not homogeneous, *i.e.* if only a fraction of them can form caffeine sensitive calcium channels and which preferentially react with ryanodine. However, the results of our kinetic studies do not support the latter possibility. The experimental findings depicted in Fig. 6 can well be approximated by exponents of either 8–12 or 4–6 if we alternatively assume that all or only half of the ryanodine binding units, which is less likely, were involved in caffeine induced calcium release. Support for a multimeric structure of the calcium release unit is furthermore obtained when the differences of the apparent binding constants are considered which ryanodine exhibits in binding experiments on the one hand and calcium release experiments on the other hand. This concept is

reenforced by the differences of the corresponding binding rate constants. After Pessah's finding [13] that ryanodine binding is accelerated by high salt concentrations many studies were performed to establish the channel's affinity for ryanodine. Yet, in most studies binding equilibrium was not achieved mainly because incubation was too short under the applied ionic conditions. The smallest values for dissociation constants of the ryanodine receptor complex in vesicular membranes range between 4 nM and 10 nM and well agree with the values obtained in this study [15, 27]. Our finding that the apparent affinities of ryanodine depend on the tested functions and differ by a factor of 10 is also in line with the notion that the caffeine sensitive calcium release unit is composed of several (up to 12) ryanodine binding units. The smallest numbers concerning subunit composition of the calcium release channel were furnished by experiments in which we measured the rate of ryanodine binding on the one hand and the time course of the decay of caffeine induced calcium release on the other hand. Ryanodine concentrations between 0.1 and 1 μM proved to be most suitable for studying the time course of both reactions. The composition of the reaction media with respect to the concentrations of protein and ryanodine was chosen in such a way that the concentrations of ryanodine did not significantly change in the course of the reaction on the one hand and that the composition of the test medium was not significantly disturbed by the addition of aliquots of labeling medium having a high salt content. In addition to the initial progress of ryanodine binding, binding equilibrium had to be assessed for the evaluation of the respective time constants. The fact that the apparent rate constants which were obtained from the results of Fig. 9 differ by a factor of 3.5 clearly indicates that the caffeine sensitive calcium release unit react approximately 4 times faster than the ryanodine receptor. This suggests that the caffeine sensitive unit presents a four times larger target for ryanodine than the ryanodine receptor itself.

A similar ratio emerges when the apparent affinities of ryanodine for the calcium release unit and for its isolated and purified receptor are compared (Table). Our analysis clearly demonstrates that the caffeine sensitive calcium release channel is composed of multiple ryanodine receptor units. Yet, the stoichiometry of the channel could not unambiguously be established. An aggregation of 4 ryanodine receptors appears to be the smallest possible structure as it emerges from initial rate measurements of ryanodine action and when the high affinity observed for ryanodine binding to the isolated receptor is considered. On the other hand, the largest structure appears to be a dodecameric assembly of ryanodine receptors. It is supported by the observed relation between equilibrium ryanodine binding and the concomitant abolition of caffeine induced calcium release.

The relatively small rate constants for both reactions which enabled us to follow them by manual procedures indicates that the formation of the ryanodine receptor complex is not formed in a single reaction step. The fact that no saturation of the reaction rates could be detected up to a ryanodine concentration of 10 μM suggests the initial formation of a transition complex with an affinity not higher than 10^4 M^{-1} which in a subsequent step leads to the formation of the quite stable ryanodine complex and to the abolition of calcium release. If these considerations were correct binding equilibrium constants derived from measurements of formation and dissociation rates must be considered with care. In fact deviation were observed, but due to the scattering values of the reported binding constants they were considered to be not significant.

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