Interaction of Ryanodine with the Calcium Releasing System of Sarcoplasmic Reticulum Vesicles

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Sarcoplasmic Reticulum, Ryanodine, Caffeine, Calcium Release

Heavy sarcoplasmic reticulum vesicles were reacted with ryanodine in 0.6 M KCl 0.3 M sucrose at pH 6.3 and pH 7.0 at 20 °C.

The inhibition of caffeine induced calcium release from actively loaded vesicles by ryanodine was applied to monitor time course and attainment of equilibrium of the interaction of ryanodine with its receptors in the vesicular membranes.

At ryanodine concentrations rising from 0.1–100 μM, the logarithms of the release amplitudes linearly decline with time. The dependence of the inactivation reaction on the concentration of ryanodine did not saturate in the applicable concentration range. The reaction halflife times are concentration dependent. At pH 7.0, the half times decline from 100 to 10 s when the ryanodine concentration is raised from 0.1 to 1 μM. At pH 6.3 a corresponding decline occurs between 3 μM and 100 μM. The marked dependence of the inactivation reaction on medium pH requires reaction times of one and five hours at pH 7.0 and 6.3, respectively for the attainment of reaction equilibrium at low ryanodine concentrations. The dependence of the amplitude of calcium release on the concentration of added ryanodine has been evaluated as proposed by Gutfreund (Enzymes: Physical Principles, p. 71, Wiley-Interscience, London 1972) for the preparation's affinity for ryanodine and its number of binding sites. At pH 7.0, preparations appear to contain only 0.7 pmol sites per mg protein having an affinity for ryanodine of 0.33 nM^{-1}. The titration curves for caffeine induced calcium release, initial calcium uptake and final calcium level are identical, indicating that the three functions are controlled by the same receptor. Calcium induced calcium release, however, is only partially and differently affected by the occupancy of the high affinity ryanodine binding sites.

The kinetic and equilibrium data for the effects of ryanodine were combined and analyzed on account of a two step reaction sequence. The corresponding dissociation and rate constants were evaluated and combined with reported data of [^{3}H]ryanodine binding (Pessah *et al.*, J. Biol. Chem. **261,** 8643–8648 (1986)).

Introduction

Ryanodine is one of the most active agents interfering with muscle function (*cf.* [1]). Concentrations as low as 10 nM were found to reduce tension development in heart muscle [2, 3] and micromolare concentrations are required to cause irreversible contractures in skeletal muscle [1]. It was quite early suspected that the target of ryanodine might reside in the muscles' cytoplasmic calcium transport system located in the sarcoplasmic reticulum [4]. Yet in order to affect isolated sarcoplasmic calcium transport in the presence of physiological concentrations of magnesium and ATP, drug concentrations between 0.1–1 mM had to be applied [5–7].

In a recent study [8] we made use of the finding of Pessah *et al.* [9] who showed that ryanodine binding is considerably enhanced in media of high ionic strength in the presence of calcium ions. We found that by a pretreatment of the isolated membranes in 0.6 M KCl for 30 min at room temperature, the drug at concentrations of 100 nM in magnesium and ATP containing media produces half maximal effects on calcium uptake, calcium concentrating ability and caffeine induced calcium release. In pursuing this study we found that a prolonged treatment of the membranes at high ionic strength results in a further enhancement of the drug's effectiveness.

In this report we show that the apparent affinity of the drug derived from dose response curves for caffeine release, calcium uptake and calcium concentrating ability coincide with the affinity found in binding studies using the radioactively labeled compound. We further report that in contrast, to the

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caffeine induced calcium release, the release induced by calcium itself is only partially reduced by the drug.

Materials and Methods

Heavy sarcoplasmic reticulum vesicles were prepared from predominantly white rabbit hind leg muscles as described by Hasselbach and Migala [8] by treating 100 g minced muscle 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 in a mixer at 4 °C. Myofibriles were removed from the suspension by centrifugation in a Sorval 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, 3.0 mM $MgCl_2$, 0.1 mM benzethonium-Cl and 0.1 mM benzamidine-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 [10]. The standard medium contained 50 mM potassium gluconate, 50 mM KCl, 100 mM sucrose, 50 mM imidazole sulfate (pH 7.0), 2 mM ATP, 8 mM phosphoenolpyruvate, 0.04 mg/ml phosphoenolpyruvate kinase, 3.0 mM magnesium gluconate, 20 μM $^{45}CaCl_2$ (~ 20,000 cpm/ml). 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 a glass nitrocellulose filter combination (Schleicher & Schuell, GF 9 glass filter and BA 85 nitrocellulose filter, 0.45 μm). Ryanodine treatment of the vesicles was performed at room temperature, 20–22 °C, in solutions containing 0.6 M KCl, 0.3 M sucrose, and 10 mg/ml vesicular protein. Medium pH was adjusted to pH 6.3 and to pH 7.0 with 0.1 M imidazole sulfate buffers, respectively. The dependence of the amplitude of caffeine, and of calcium induced calcium release, the residual calcium level and the initial calcium uptake on the reaction time of ryanodine with the vesicular membranes was determined by incubating the vesicular protein with different concentration of ryanodine for various times. The reaction was interrupted by adding aliquots of the incubation medium to the uptake solution yielding 0.2 mg protein/ml. Initial calcium uptake was monitored by terminating calcium uptake after 1 min. The residual calcium level was determined after uptake has proceeded for 10–15 min. Caffeine induced calcium release was initiated by the addition of 10 mM caffeine at 10 min and stopped at 10 min 15 s. Calcium induced calcium release was accomplished by the addition of 0.5 mM EGTA + 0.45 mM $CaCl_2$ + 0.1 M imidazole sulfate, pH 7.0. The amount of calcium released after 15 s was calculated from the released $^{45}Ca^{2+}$ assuming that the specific activity of the stored calcium was not reduced by calcium uptake during the release period [10]. ^{45}Ca was determined by liquid scintillation counting of the filtrates [2, 7].

Ryanodine was generously given to us by Dr. G. Isenberg, Köln (FRG), Dr. L. R. Jones, Indiana (USA) and Dr. J. Y. Su, Seattle (USA). ATP was obtained from Pharma-Waldhof, Mannheim (FRG); phosphoenol pyruvate and pyruvatekinase were purchased from Boehringer Mannheim (FRG). All other reagents were of analytical grade and bought from E. Merck, Darmstadt (FRG); Sigma Chemical Company, Deisenhofen (FRG), and SERVA, Heidelberg (FRG). $^{45}CaCl_2$ was supplied by Buchler and Co., Frankfurt (FRG).

Results

Under all conditions applied in this study more than 90% of the calcium present in the medium were taken up during a loading period of 10–15 min by the vesicular fraction enriched in release competent structures. On addition of a final concentration of 10 mM caffeine 30–40% of the stored calcium are released under control conditions in 15 s in accordance with [10]. Calcium uptake and release are not significantly affected when the vesicles prior to calcium loading were incubated for 3 to 5 h in 0.6 M KCl + 0.3 M sucrose at pH 6.3 or pH 7.0, respectively. Calcium uptake and release of thus treated vesicles were taken as control values. The change of calcium uptake and release affected by different concentrations of the drug in the incubation media and the time of incubation were related to the release and uptake of the respective controls. Fig. 1 demonstrates the time dependent decline of the amplitude of calcium release induced by caffeine from vesicles pretreated with 1 μM ryanodine in 0.6 M KCl – 0.3 M sucrose containing 1 mM calcium chloride at pH 6.3

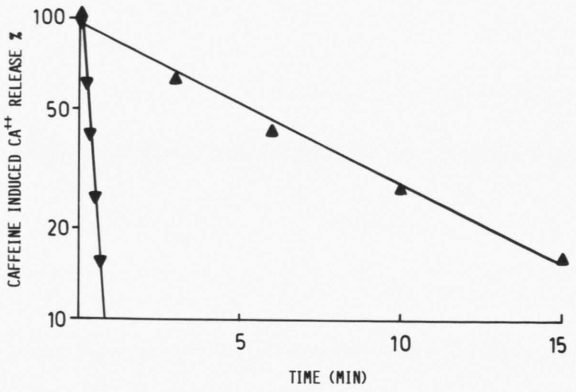

Fig. 1. Effect of pH on the time dependent decline of the amplitude of caffeine induced calcium release from sarcoplasmic reticulum vesicles treated with ryanodine. 10 mg/ml of heavy sarcoplasmic reticulum vesicles are reacted with 1 μM ryanodine at pH 6.3 (▲) and 7.0 (▼) for the times given on the abscissa. The logarithm of the normalized amplitude of calcium release from vesicles treated with and without ryanodine A_R/A_0 in salt solution of high ionic strength are plotted on the ordinate. A_R and A_0 are the release amplitudes observed in the presence and absence of ryanodine, respectively.

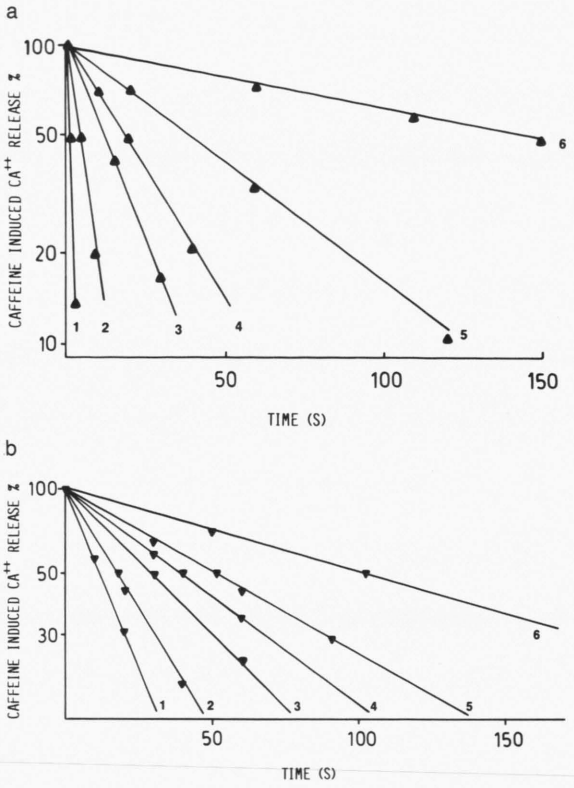

Fig. 2. The dependence on time of the inactivation of caffeine induced calcium release by different ryanodine concentrations. a) Treatment at pH 6.3 with ryanodine 100 μM (1); 30 μM (2); 10 μM (3); 5 μM (4); 3 μM (5); 1 μM (6). b) Treatment at pH 7.0 with ryanodine 1 μM (1); 0.6 μM (2); 0.4 μM (3); 0.3 μM (4); 0.2 μM (5); 0.1 μM (6). Ryanodine treatment was performed as described in Materials and Methods for the times given on the abscissa. The logarithms of the normalized release amplitudes are plotted on the ordinates.

and pH 7.0, respectively. At this concentration of the drug inactivation takes a few seconds at pH 7.0 and requires several minutes at pH 6.3 indicating a pronounced effect of the pH of the incubation medium on the rate of inactivation. The linear relations between the logarithms of the release amplitudes and the reaction time indicate that the interaction of ryanodine with its target is dominated by a single reaction step. The time course of inactivation of the caffeine induced calcium release effected at different ryanodine concentrations is illustrated by plotting the logarithm of the declining release amplitude *versus* time in Fig. 2a and 2b. Inactivation rates as well as the reaction mode do not noticeably change when the vesicles were incubated in high salt media not supplemented with additional calcium. In these media containing 10 mg/ml vesicular protein, a free calcium concentration of 0.1 mM was registered with a calcium sensitive electrode at a total calcium content of 30 nmol/mg protein determined by atomic absorption measurements. When the half-times of inactivation derived from the time course shown in Fig. 2a and 2b are plotted *versus* the reciprocal value of the applied concentration linear graphs are obtained (Fig. 3a, 3b). This linear relationship is consistent with the formation of a one-to-one drug receptor complex dominated by a single set of rate constants. The following evaluation is based on the assumption that the drug interacts with its targets in a two step reaction sequence. The initially formed drug target complex is assumed to be in a rapid equilibrium with its components. Subsequently the drug target complex is transformed into the final caffeine insensitive complex. The latter reaction is governed by the rate constants k_{+1} and k_{-1}.

$$E + R \overset{K_o}{\longleftrightarrow} ER \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E^{+}R.$$

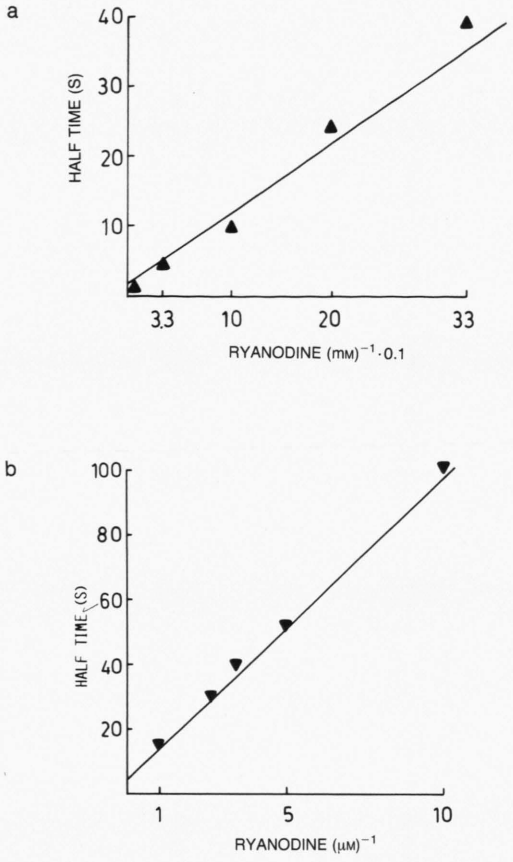

Fig. 3. Dependence on the ryanodine concentration of the half times of calcium release inactivation. The reciprocal concentrations of ryanodine in the respective high salt incubation media are plotted on the abscissa. Incubation was performed at pH 6.3 (a) and pH 7.0 (b). Calcium release was induced by the addition of 10 mM caffeine.

For the time constant of this sequence the assumed scheme yields the expression:

$$\frac{1}{T} = \frac{k_{+1} \cdot R}{K_o + R} + k_{-1}. \qquad (1)$$

The following results support the assumption that under the applied conditions $k_{-1} \ll k_{+1} \cdot R/K_o + R$ yielding the simplified relation:

$$T = \frac{K_o}{k_{+1} \cdot R} + \frac{1}{k_{+1}} \cdot \qquad (2)$$

Thus by plotting T *versus* the reciprocal drug concentration one obtains the dissociation constant of the drug receptor complex as well as the rate constant k_{+1}.

Apparent dissociation constants of the ryanodine protein complex as high as 40 μM at pH 6.3 and 2 μM at pH 7.0 are obtained from Fig. 3, indicating an astonishingly pronounced pH-dependence of the first binding step (Table). The thus derived ryanodine complex appears to be less stable than the complex which is observed in binding experiments performed with radioactively labeled ryanodine [9, 12, 15] and also to be less stable than the ryanodine complex responsible for the suppression of the calcium releasing action of caffeine as previously reported [8].

If we disregard the possibility that drug binding sites and the sites where the drug affects calcium release are unrelated, we must assume that blockage of the caffeine induced calcium release as well as high affinity ryanodine binding occurs subsequent to the formation of the initial formed drug complex of lower affinity. In the present case the analysis of the sequential reaction is fascilitated by the fact that at all drug concentrations the observed time constants prove to be time independent. The rate constant k_{+1} for the second reaction step results from the evaluation of the described rate measurements (Table). To obtain k_{-1}, the dissociation constant of the second ryanodine complex must be determined which requires complete achievement of reaction equilibrium; *i.e.*, one has to wait until the effect of the drug at different concentrations has become time independent. This attempt is limited by the stability of the enzyme since prolonged incubation leads also in the absence of the drug to a progressive loss of the ability of the vesicles to actively store calcium and of the caffeine induced calcium release. This difficulty was overcome by the addition of 0.3 M sucrose to the KCl containing incubation medium allowing incubation times up to 6 h at room temperature. Long incubation times are especially required at low ryanodine concentration in incubation media of pH 6.3. Fig. 4 illustrates that at the concentration of 10 nM added ryanodine it takes more than 4 h to reach reaction equilibrium. In contrast at pH 7.0 the equilibrium is reached in less than 30 min at the same low ryanodine concentration (*cf.* also Fig. 2). Fig. 5 illustrates the effect of increasing concentration of ryanodine in the incubation medium at pH 7.0 on the amplitude of the calcium release induced by caffeine with 10 mg vesicular protein in the KCl containing incubation medium at binding equilibrium. In the presence of 7 pmol of ryanodine in the assay the caffeine induced calcium release is half-maximally suppressed. The

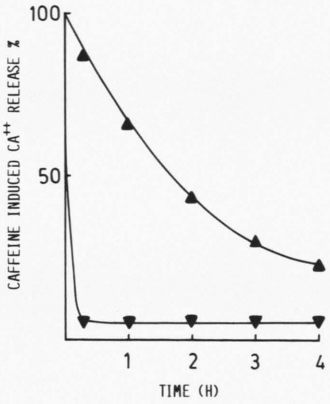

Fig. 4. Effect of pH on the time course of calcium release inactivation by 10 nM ryanodine. Calcium release was induced with 10 mM caffeine; the normalized release amplitude is plotted on the ordinate. Incubation in high salt medium was performed at pH 6.3 (▲) and pH 7.0 (▼), respectively, for the times given on the abscissa.

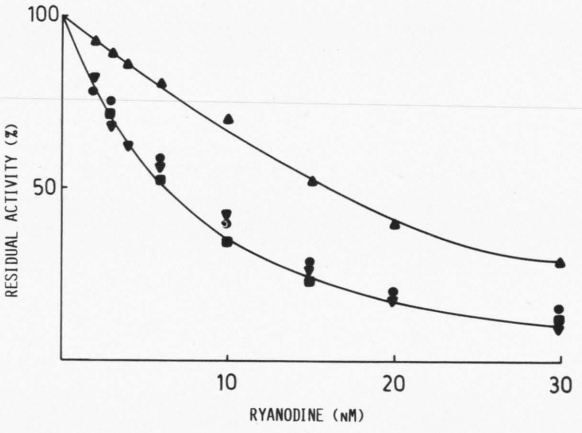

Fig. 5. Dependence on the concentration of added ryanodine on the amplitude of caffeine induced calcium release, the residual calcium level and the initial calcium uptake at binding equilibrium. At pH 6.3 the vesicles were treated with ryanodine for 4–6 h. At pH 7.0 the treatment was shortened to 1–2 h. Caffeine induced calcium release at pH 7.0 (▼) and pH 6.3 (▲) was measured as described in Materials and Methods and the normalized amplitudes are plotted on the ordinate. The residual calcium level at pH 7.0 (■) was determined in the uptake medium after 10–15 min and its ryanodine sensitive fraction is plotted on the ordinate

$$(Ca_R - Ca_{Rmax}) : (Ca_0 - Ca_{Rmax}).$$

Ca_0, Ca_R, Ca_{Rmax} are the respective calcium concentrations in the absence of ryanodine, in the presence of the specified ryanodine concentration and in the presence of maximally effective ryanodine concentration. Under control conditions the level of total calcium is reduced to 2.4 ± 0.06 μM, $n = 6$. At maximal effective ryanodine concentrations (~ 10 μM) residual calcium drops to 0.6 ± 0.1 μM, $n = 4$. The initial calcium uptake (●) at pH 7.0 was monitored by terminating calcium uptake after one minute. Initial uptake of the ryanodine preparation was related to the uptake of the untreated preparation. The ryanodine sensitive fraction is plotted on the ordinate

$$(V_R - V_{Rmax}) : (V_0 - V_{Rmax}).$$

V_0, V_R, V_{Rmax} are the one minute calcium uptake increments in the absence of ryanodine, in the presence of the specified ryanodine concentration, and in the presence of a maximally effective ryanodine concentration [8]. Residual calcium concentrations are given as means ± SEM.

shown titration curves can be evaluated for the receptor's affinity for ryanodine and the number of activity related ryanodine binding sites by a plotting procedure proposed by Gutfreund [11]. The relation between degree of inhibition (I) and total concentration of ryanodine (R) can be applied if the drug interacts with a single site.

$$\frac{1}{1-I} = \frac{1}{K_d} \cdot \frac{[R_{total}]}{I} - \frac{n_i}{K_d} \cdot \qquad (3)$$

The slope of the linear relation yields the dissociation constant (K_d) of the ryanodine target complex and from the intersection of the line with the abscissa, concentration of binding sites (n_i) can be derived. As shown in Fig. 6, the transformation of the titration curves can well be approximated by straight lines. The derived dissociation constant of 3 nM well agrees with the lowest dissociation constant obtained in binding experiments using radioactive ryanodine [9, 12].

Yet the number of binding sites of 0.7 pmol/mg obtained for our preparations appears to be quite small. In order to check the evaluation procedure, we have reduced the protein concentration in the incubation media to 5 mg/ml in some experiments. In agreement with equation (3) the number of binding sites per ml is reduced by 50% corresponding to the same content of sites per mg protein (not shown).

Surprisingly the number of ryanodine binding sites derived from the inactivation of caffeine induced calcium release proved to strongly depend on medium pH. From the inhibition at pH 6.3 depicted in Fig. 6, the Gutfreund representation [11] of the data shows that the affinity of the drug for the membranes remains unchanged while the number of binding sites increases 5-fold to 2.5 pmol per mg. This value approaches the lower margin of the figures observed in various binding experiments [9, 12]. On account of the known concentration of bound ryanodine, the

titration curves in Fig. 5 can be transformed into the relation between the effect of bound ryanodine on calcium release and the free concentration of ryanodine (Fig. 7). It emphasizes the pH invariance of the ryanodine effect already indicated by the results shown in Fig. 6.

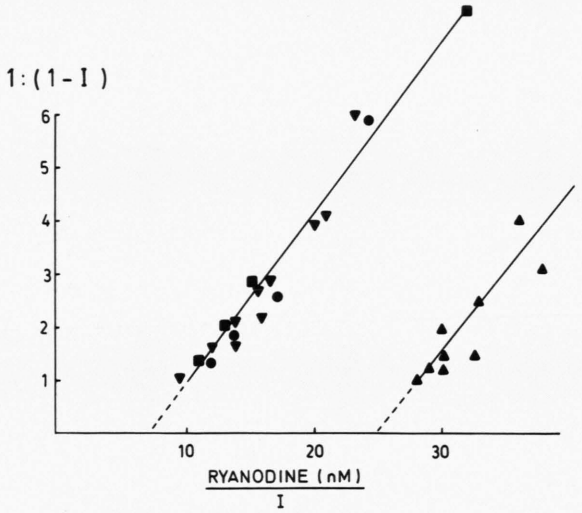

Fig. 6. The effect of ryanodine on caffeine induced calcium release, residual calcium level, and initial calcium uptake at binding equilibrium represented according to Gutfreund [11] for the evaluation of the dissociation constant of the ryanodine membrane complex and the number of ryanodine binding sites. The data presented in Fig. 5 are plotted by applying Eqn. (3). 1/1-*I* is plotted on the ordinate. *I* is the respective extent of the effect of ryanodine normalized to one. The ratio ryanodine added/*I* is plotted on the abscissa. (▼) Caffeine induced calcium release, pH 7.0; (▲) caffeine induced calcium release, pH 6.3; (●) initial calcium uptake; (■) residual calcium level.

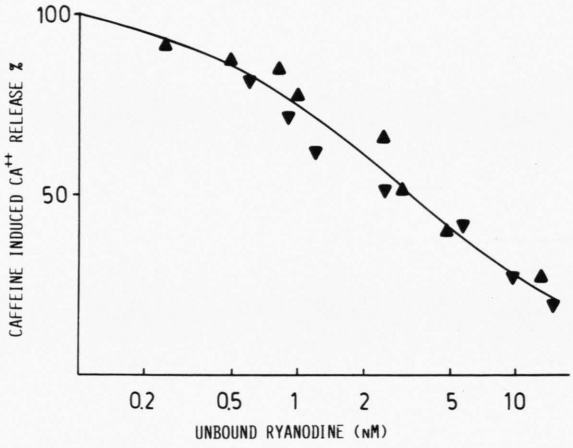

Fig. 7. Independence of the relation between unbound ryanodine and caffeine induced calcium release on the pH of the incubation medium. Binding equilibrium was achieved at pH 6.3 (▲) and pH 7.0 (▼) by incubating the vesicles in high salt media for 4–6 and 1–2 h respectively. Unbound concentrations of ryanodine were obtained as a difference of the total ryanodine added to the incubation medium and the apparent amount of ryanodine bound to the vesicular membranes deduced from the results shown in Fig. 6.

Table. Rate and dissociation constants for the formation of the functional complex between ryanodine and the calcium releasing channel of the sarcoplasmic reticulum.

Conditions	a) $E + R \underset{k'_{-1}}{\overset{k'_{+1}}{\rightleftharpoons}} ER$			b) $E + R \overset{K_o}{\longleftrightarrow} ER \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E^+R$			
	k'_{+1} [$M^{-1} \cdot s^{-1}$]	k'_{-1} [s^{-1}]	$K'_d = k'_{-1}/k'_{+1}$ [M]	K_o [M]	k_{+1} [s^{-1}]	k_{-1} [s^{-1}]	$K_d = K_o \cdot k_{-1}/k_{+1}$ [M]
pH 6.3, 20 °C	5×10^3	7×10^{-3}	1.4×10^{-6}	4×10^{-5}	0.28	2×10^{-5}	3×10^{-9}
pH 7.0, 20 °C	5×10^4	3×10^{-3}	0.6×10^{-7}	2×10^{-6}	0.14	2×10^{-4}	3×10^{-9}
pH 7.1, 37 °C Pessah *et al.* [9]	10^5	10^{-5}	10^{-10}	2×10^{-6}	0.19	8×10^{-4}	9×10^{-9}

a) The rate constants k'_{+1}, k'_{-1} for the assumed second order reaction were obtained by plotting $k_{obs} = 0.69/T$ *versus* the concentration of ryanodine [R]. The resulting K_d' is three orders of magnitude greater than the value K_d observed in this study. In contrast a value which is by a factor of 30 to small results from Pessah's *et al.* [9] data.

b) The values K_o and k_{+1} were evaluated as described in the text by plotting $T = 0.69/k_{obs}$ *versus* the reciprocal concentrations of [R]. k_{-1} was obtained from the observed value for K_d and the relation $K_d = K_o \cdot k_{-1}/k_{+1}$. Pessah's *et al.* [9] kinetic and equilibrium binding data were adapted to the two-step reaction mechanism by using $K_o = 2 \times 10^{-6}$ M for the dissociation constant of the initial complex as described in this study. The first order rate constants obtained from ours and Pessah's *et al.* measurements satisfactorily agree.

The obtained dissociation constant K_d yields together with K_o and k_{+1} on account of the relation $K_d = K_o \cdot k_{-1}/k_{+1}$ the rate for ryanodine dissociation, k_{-1}. Since K_d is not significantly dependent on pH, the pH dependence of K_o and of the forward rate constant k_{+1} is compensated by a 10-fold increase of the backward rates between pH 6.3 and pH 7.0 (Table). The dissociation constants derived from measurements performed at binding equilibrium are not at all compatible with the constants obtained from the evaluation of the kinetic data reported in Fig. 2 assuming a one step second order binding reaction. The thus obtained dissociation constants (K'_d) are 20–500-fold greater than the directly measured ones (K_d) (Table). This finding supports the proposed two step reaction sequence. Furthermore the kinetic and binding data reported by Pessah *et al.* [9] can satisfactorily be adapted by the two step reaction mechanism while the one step mechanism yields a K'_d value which is far to small (Table).

We previously reported that ryanodine affects not only the caffeine induced calcium release, but also the initial rate of calcium uptake and the final level of calcium in the uptake medium [8]. The representation of the data for the initial rate of calcium uptake and the final calcium level as proposed by Gutfreund [11] in Fig. 5 and Fig. 6 demonstrates that the three functions of the membranes are governed by the same dissociation constant and the same number of binding sites for the drug.

In contrast to this concordance of drug action on the three respective functions, calcium induced calcium release displays a different dependence on the concentration of ryanodine (Fig. 8). With rising concentrations from 2 to 30 nM calcium release steadily but less steeply declines than the other functions and the release of 40 to 50% which persists can not further be suppressed by even 100 μM ryanodine. When the ryanodine sensitive fraction of the calcium induced calcium release is plotted *versus* the added concentrations, the resulting action concentration profile could not be fitted by the Gutfreund procedure [11].

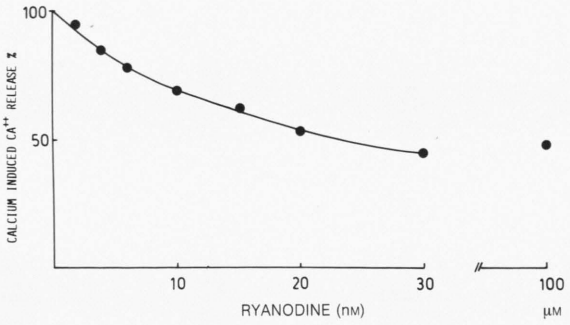

Fig. 8. Dependence on the concentration of added ryanodine of calcium induced calcium release. The normalized amplitude of the calcium release (A_r/A_0) induced by the addition of 0.5 mM EGTA + 0.45 mM Ca^{2+} is plotted on the ordinate.

Discussion

The results presented in this study show that ryanodine simultaneously affects three interrelated functions of a heavy sarcoplasmatic reticulum membrane fraction of rabbit skeletal muscle. Caffeine induced calcium release, initial calcium uptake and the concentrating ability of the isolated membranes in magnesium and ATP containing solution are equally affected by nanomolare concentrations of ryanodine when the preparations were treated in salt media of higher ionic strength prior to testing of the respective function. The caffeine induced calcium release is completely suppressed while calcium uptake of only a subfraction of the preparation, is inhibited during the initial phase of calcium storage. The ability of the vesicular preparation to concentrate calcium is strongly enhanced by ryanodine as indicated by the considerable reduction of the final calcium level determined after 15–20 min after the start of the reaction.

Yet ryanodine proved to exhibit a significantly different dose response relation for the fourth function of the sarcoplasmic reticulum membrane studied in this work, the calcium induced calcium release. While the caffeine induced calcium release is completely suppressed by 30 nM of ryanodine, the calcium induced release is maximally diminished by 50%. Furthermore the relationship between the size of the releasable fraction and the concentration of ryanodine differs from that found for the other functions. The effective concentrations of ryanodine are considerably lower than those reported by Nelson [13] for the calcium induced calcium release from actively loaded vesicles. In contrast, the calcium induced calcium release from passively loaded vesicles appears to be not affected by ryanodine at concentrations below 1 μM [14].

Since it has unambiguously been shown that ryanodine does not interfere with the function of the

calcium pump [7], the described effect must be attributed to its interaction with the calcium releasing channels which are enriched in the heavy membrane fraction. We have shown that the drug affects the functioning of these channels in the same concentration range at which high affinity binding sites are occupied by the drug. The drug appears to be firmly bound to a high molecular weight protein component which is assumed to be a constituent of the so-called feet structure of the terminal cysternae [15]. The difficulties in correlating the effect of drug binding and drug action are related to the peculiarities of the binding reaction. The enhancement of drug binding by high salt concentrations [8, 9, 15] explains why in experiments performed at low ionic strength quite high drug concentrations were required to affect the calcium releasing channel [7, 14, 16]. Comparisons between *in vivo* and *in vitro* effects of the drug are complicated by the facts that drug binding depends on ionized calcium [7, 9] present in the myoplasma at effective concentrations only during muscle activity and that physiological magnesium concentrations counteract the binding reaction [5, 9, 16]. As shown previously, the gap between binding data and functional effects of the drug can be narrowed by incubating the vesicles in appropriate media of higher ionic strength prior to the functional assay performed at lower ionic strength under quasi physiological conditions [8]. The modification of the calcium channel in the high salt media proceeds relatively slowly and could be resolved in time. The reaction rate proves to be surprisingly dependent on the pH being much slower at pH 6.3 than at pH 7.0. In a previous study we erroneously had performed the binding reaction at pH 6.3 instead of at pH 7.0 as intended. Thus the selected incubation time of 30 min was not sufficient to complete the binding reaction at low drug concentrations. An apparent dissociation constant of 0.15 μM was found which is by a factor 50 lower than that reported in this study. The great sensitivity of the binding reaction to medium pH might also explain reported differences in the dissociation constants found *in vitro* and *in vivo* studies since muscle pH can considerably change and the *in vitro* experiments were performed between pH 6.8 [16] and 7.4 [15]. The applied procedure of first treating the vesicles under defined conditions for different times followed by the registration of the respective functions, especially of caffeine induced calcium release, allowed us to show that the binding to the functional relevant sites of the membranes is consistent with a two-step reaction sequence. In the initially formed encounter complex ryanodine is bound with relatively low affinity, being, however, significantly higher at pH 7.0 than at pH 6.3.

The functional significance of this transiently formed complex is unknown. In a second reaction step the properties of the channels are modified. The transition of the initial to the final complex procedes in time ranges which are experimentally easily accessible. While at room temperature 20–22 °C at pH 7.0 and a concentration of 10 nM the reaction is complete in a few seconds it requires for completion hours at pH 6.3. These long lasting experiments, however, required special precaution to preserve the activity of the vesicles.

For the evaluation of the relation between drug action and the added concentration of the drug we applied Gutfreund's procedure [11] for the analysis of titration data and could establish a pH invariant ryanodine dissociation constant of the ryanodine channel complex of 3 nM. This dissociation constant is in the same range as that found in recent binding experiments with radioactive ryanodine [12] and for the negative inotropic effect which ryanodine exerts on the rabbit heart [3]. The fact that a well reproducible dissociation constant of the ryanodine complex could be found makes it unlikely that in the concentration range between 2 and 20 nM ryanodine induces an irreversible change in the membrane as it might be inferred from the extremely slow dissociation of radioactive ryanodine from the complex observed by Pessah *et al.* ([9], Table). Some kind of occlusion might be induced when the drug is applied at higher concentrations. The evident uncertainty needs clarification. In this connection it should be stressed that the agreement between binding data and the drug effects on isolated channel structure are obtained at high salt concentrations during the preincubation period. It must also be recalled that the most recent findings on the effect of ryanodine on passively loaded sarcoplasmic reticulum structures were performed in the absence of magnesium and ATP in the release assay. The conditions applied *in vitro* are thus not comparable to the *in vivo* situation [14] which makes it difficult to draw definite conclusions concerning the action of the drug *in vivo*. Yet these restrictions do not limit considerations concerning the mechanism by which the drug affects the calcium channel.

In contrast to the agreement found between dissociation constant determined in binding experiments and that deduced from the effect of the drug on various functional activities, a great discrepancy appears to exist between the number of binding sites determined by the two procedures. Evidently different classes of ryanodine binding sites exist of which the functionally relevant ones are a relatively small fraction only.

In this study the set of respective functions was tested in many cases in one and the same assay to perform the measurements under precisely identical conditions. This experimental set-up revealed that the ryanodine complex formed during the incubation in high salt solutions changes its properties in a time dependent manner in the assay medium. Similar time dependent changes induced by ryanodine during active loading have recently been reported by Nelson [13]. Properties of the ryanodine complex measured during the first minutes after being added to the assay most likely reflect the state of the channel ryanodine complex as it might exist at the end of the incubation period. Distinct inhibition of the initial calcium uptake by ryanodine indicate that the channels are opened by ryanodine. During the following 5–10 min, however, the channels are progressively shut off. The rate of calcium uptake increases and the calcium level is significantly reduced below that observed in the controls. The mechanism of this transition has been attributed to ATP dependent changes inside the vesicles [8]. The notion that these changes might be caused by the rising internal calcium concentration is difficult to investigate. Yet it cannot be the high internal calcium alone that effects channel closing, otherwise the channels should be in the closed state after passive loading which is not the case [14]. Since low concentrations of ionized calcium are required for the caffeine induced calcium release, it might be suspected that the abolition of the caffeine induced calcium release by ryanodine is caused by the more complete removal of calcium from the medium. This possibility, however, is unlikely since the caffeine induced calcium release could not be restored by the simultaneous addition of 1 μM calcium together with caffeine which is sufficient to support a caffeine induced calcium release (not shown). In contrast to caffeine, calcium, however, appears to be able to overcome at least partially the locking by ryanodine of the calcium channels in their closed state.

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