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The interaction of calcium and ryanodine with cardiac sarcoplasmic reticulum

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The binding of [^{3}H]ryanodine with cardiac sarcoplasmic reticulum vesicles depends on the calcium concentration. Binding in the absence of calcium appears to be non-specific because it shows no saturation up to 20 μM ryanodine. The apparent K_m value for calcium varied between 2 and 0.8 μM when the ryanodine concentration varied between 10 and 265 nM. The Hill coefficient for the calcium dependence of [^{3}H]ryanodine binding was near two. Scatchard analysis of ryanodine binding indicated a high-affinity site with a B_{max} of 5.2 ± 0.4 pmol/mg with a K_d of 6.8 ± 0.1 nM. Preincubation under conditions in which the high-affinity sites were saturated did not result in stimulation of the calcium uptake rate indicative of closure of the calcium channel. Stimulation of calcium uptake rate occurred only at higher concentrations of ryanodine (apparent K_m = 17 μM). This stimulation of the calcium uptake rate also required calcium in the submicromolar range. The data obtained support the hypothesis that ryanodine binding to the low-affinity site (K_m about 17 μM) is responsible for closure of the calcium release channel and the subsequent increase in the calcium uptake rate of the sarcoplasmic reticulum. Because the number of ryanodine-binding sites is much less than the number of calcium transport pumps the channel is probably distinct from the pump.

Introduction

Ryanodine is a neutral alkaloid extracted from *Ryania speciosa* that causes an irreversible contracture of skeletal muscles and a decrease in the developed tension of cardiac muscle [1]. There are several possible sites of action of ryanodine in these intact tissues, but it is now accepted that the principal site of ryanodine action is the sarcoplasmic reticulum. Recent studies have shown conclusively that ryanodine stimulates calcium uptake by cardiac sarcoplasmic reticulum vesicles [6–10] while the Ca^{2+}-ATPase activity is slightly inhibited [6,8,9]. The earlier results [2–5] showing depression of calcium uptake are probably due to the fact that the effect of ryanodine depends on the ryanodine concentration, temperature, time of preincubation [9] and the calcium concentration during preincubation [6].

Although the mechanism of ryanodine action is still unclear, considerable evidence suggests that it influences a calcium efflux pathway distinct from the Ca^{2+}-ATPase pump. Stimulation of oxalate-supported uptake is not due to a change in the passive efflux [9] or to effects on oxalate entry or precipitation, as steady-state uptake is increased in the absence of oxalate [9]. Lack of any effect of ryanodine on the calcium requirement of the Ca^{2+}-ATPase suggests that the coupling ratio of the pump is also unchanged [9,17]. Ryanodine increases the capacity of cardiac sarcoplasmic reticulum vesicles for calcium oxalate and calcium

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phosphate [10] by closing an efflux pathway present in only some of the vesicles [7,10]. Studies of [^{3}H]ryanodine binding to rabbit cardiac [13] and skeletal [13,18] sarcoplasmic reticulum suggest that the density of the ryanodine receptors is much less than the density of the Ca^{2+}-ATPase [14–16,19], suggesting that the pump and efflux pathway are distinct entities.

Both the action of ryanodine on the stimulation of oxalate-supported calcium uptake and the binding of [^{3}H]ryanodine have been reported to require calcium [6,13]. However, the maximum binding of [^{3}H]ryanodine required 100 μM calcium [13], whereas the effect on calcium uptake required 10 μM calcium [17]. Both of these values are considerably higher than the calcium concentrations believed to be necessary for the release of calcium in situ [23]. For this reason, we have re-examined the calcium requirement for [^{3}H]ryanodine binding and its effect on calcium uptake. The results indicate that occupancy of the high-affinity ryanodine-binding site is not attended by closure of the efflux pathway. Complete closure of the calcium efflux pathway requires nearly 50 μM ryanodine. The calcium requirement for [^{3}H]ryanodine binding was found to be in the micromolar and sub-micromolar range, with a Hill coefficient in excess of 2.0, and with an apparent affinity which changed with the concentration of ryanodine used in the binding assay.

Methods

Sarcoplasmic reticulum preparation

Vesicles of cardiac sarcoplasmic reticulum were isolated from minced canine ventricles as described previously [24] except that in the overnight spin the vesicles were placed on a discontinuous sucrose gradient containing 10 ml 25% sucrose, 1 M KCl, 10 mM imidazole buffer (pH 7.0) overlaying 5 ml 33% sucrose, 1 M KCl, 10 mM imidazole. The sarcoplasmic reticulum vesicles collected after centrifugation between 27 and 33% sucrose were diluted 1:1 with 10 mM imidazole buffer and spun at 40 000 × g for 2 h. The resulting pellet was resuspended in 30% sucrose, 20 mM Tris-HCl buffer (pH 7.0) and frozen at −20 °C until used. The protein concentration of the preparation was estimated by the method of Lowry et al. [25] using bovine serum albumin as a standard.

[^{3}H]Ryanodine binding

[9,21-^{3}H]Ryanodine was prepared by New England Nuclear. The purity of the [^{3}H]ryanodine was checked in our laboratory by HPLC using a Vydac C_{18} column (4.6 mm × 25 cm) eluted with 70% 10 mM KH_2PO_4 buffer (pH 5.0) with 25% acetonitrile. HPLC of commercial ryanodine supplied by S.P. Penick showed two major peaks corresponding to ryanodine and 9,21-didehydroryanodine [26], eluting at 8.0 min and 7.2 min, respectively, at a flow of 1 ml/min. The [^{3}H]ryanodine co-purified with the ryanodine. The concentrations of the [^{3}H]ryanodine solutions used in the binding studies were determined by the absorbance of stock solutions using the absorbance coefficient $1.79 \cdot 10^4$ M^{-1} at 271 nm. This absorbance coefficient differs from some published values ($1.51 \cdot 10^4$ [27]) but is close to others ($1.74 \cdot 10^4$, calculated from data in Ref. 28). Preliminary studies showed that the maximum absorption was shifted from 267 nm in ethanol to 271 nm in water and the extinction coefficient was slightly higher in water.

The binding of [^{3}H]ryanodine was conducted at 37 °C in a bath containing 166.6 mM KCl, 33.3 mM imidazole buffer (pH 7.0), 16.7 mM NaN_3. The concentrations of ryanodine, calcium and EGTA (ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) were varied as described for the individual experiments. The amount of bound [^{3}H]ryanodine was estimated by filtering 0.05–0.1 ml of the bath through 0.45 μm Millipore filters, washing with 4 ml wash solution (166.6 mM KCl, 33.3 mM imidazole buffer (pH 7.0), 16.7 mM NaN_3, kept on ice), and counting the filters by liquid scintillation spectrometry. Preliminary experiments showed that the amount of radioactivity on the filters was linear with the protein concentration of the bath from 0.25 to 2.0 mg/ml and was linear with the volume of the bath filtered from 0.025 to 0.20 ml. The measured pH of the reaction baths was 7.03–7.04. The rate and extent of [^{3}H]ryanodine binding was found to depend on both the ryanodine concentration and calcium concentration. At 105 nM ryanodine and 0.3 mM added calcium, the binding was complete in 15

min and was unchanged throughout 120 min of incubation. For all studies, a time course of [^{3}H]ryanodine binding was performed to assure that binding equilibrium was attained.

Oxalate-supported calcium uptake and Ca^{2+}-ATPase rate

The oxalate-supported calcium uptake rate was determined by Millipore filtration using 0.45 μm filters. The reaction bath was maintained at 37° C and contained 100 mm KCl, 20 mM imidazole buffer (pH 7.0), 10 mM NaN_3, 5 mM Na_2-ATP, 5 mM $MgCl_2$, 10 mM potassium oxalate, 0.2 mM added calcium, 0.05 mg cardiac sarcoplasmic reticulum protein per ml, and 0.3 μCi ^{45}Ca/ml. The rate of calcium uptake was calculated from the linear regression of the calcium uptake of six samples obtained at various times after beginning the uptake reaction by the addition of ATP, magnesium and calcium (at 37° C) to an otherwise complete reaction. The inorganic phosphate liberated from ATP by the cardiac sarcoplasmic reticulum Ca^{2+}-ATPase was measured from the filtrates of the same reaction baths used to determine calcium uptake. The inorganic phosphate was measured colorimetrically [29] and the Ca^{2+}-ATPase rate was calculated from the linear regression of six values obtained at the various times. In all cases the oxalate-supported calcium uptake and liberation of P_i from ATP was linear with time, with correlation coefficeints in excess of 0.99.

Calculation of free calcium concentration

The free calcium concentration was calculated using the SPICE2 electronic network simulation program described elsewhere [30–32]. The apparent association constant under our conditions of incubation (pH 7.04, 166.6 mM KCl, 33.3 mM imidazole buffer, 16.7 mM NaN_3) was calculated to be $3.32 \cdot 10^6$ M^{-1}. In these calculations, the first and second dissociations of EGTA were assumed to be complete at our conditions of near-neutral pH. The association constants for the individual reactions were obtained by interpolation using van't Hoff plots where the data were available [33–35].

The accuracy of our calculated free calcium concentrations depends on the accuracy of our solutions of calcium and EGTA as well as the value for the apparent association constant. To more accurately assess the free calcium concentration, we titrated our calcium solutions against the EGTA solutions using a calcium-sensitive electrode (Corning) connected to a digital pH meter. The calcium and EGTA solutions were originally prepared gravimetrically from $CaCO_3$ and the free acid of EGTA. Taking the $CaCO_3$ solutions as accurate, the EGTA solutions were found by titration to be 95.3% of their nominal value. The pH during the titration was buffered using 0.2 Mops (4-morpholinepropanesulfonic acid) and changed only 0.1 pH units during the course of the titration. Correction of the nominal EGTA concentration based on this titration resulted in only slight differences in the values of K_m and h for calcium (see Table I). Any errors introduced by this correction would tend to overestimate K_m and underestimate h.

Ryanodine

Ryanodine used in these studies was isolated from the commercial preparation obtained from S.P. Penick Corporation. As described above, this contains a mixture of two major components, previously identified as ryanodine and 9,21-didehydroryanodine [26]. The ryanodine fraction was purified from the mixture using preparative HPLC eluting a 2.2×25 cm C_{18} reverse phase column (VYDAC) with 25% acetonitrile in water.

Results

The time course of [^{3}H]ryanodine binding and its calcium requirement are shown in Fig. 1. In this case, binding of ryanodine was essentially complete within 25 min, but the level of binding was markedly dependent on the calcium cocnentration. The data of Fig. 1 were obtained with 53 nM [^{3}H]ryanodine. The time required to reach binding equilibrium was shorter with higher concentrations of ryanodine. The calcium requirement for [^{3}H]ryanodine binding was investigated at three concentrations of ryanodine, with time courses studied to assure binding equilibrium. The results for one cardiac sarcoplasmic reticulum preparation are shown in Fig. 2. These results indicated that the equilibrium level of binding varies

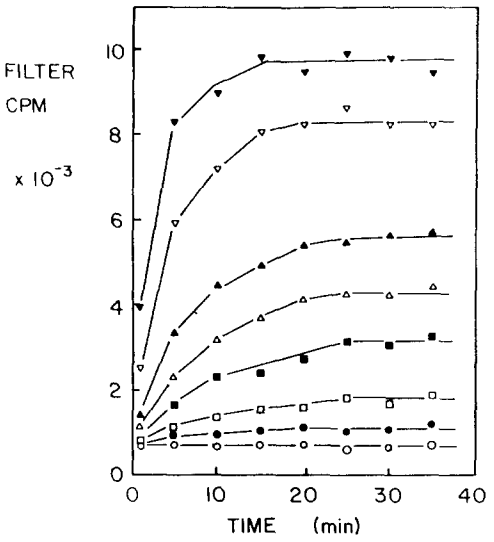

Fig. 1. The time course of [^{3}H]ryanodine binding to cardiac sarcoplasmic reticulum vesicles. Conditions for binding are described in Methods. Each binding reaction bath contained 0.5 mg/ml sarcoplasmic reticulum protein, 0.95 mM EGTA, 52.5 nM [^{3}H]ryanodine and varying amounts of added calcium in order to achieve different free calcium concentrations as determined by the SPICE2 simulation program described in Methods. The pH of the reaction bath was 7.02. Free calcium concentrations investigated were 1.0 nM (○), 0.282 μM (●), 0.436 μM (□), 0.597 μM (■), 0.939 μM (△), 1.33 μM (▲), 2.25 μM (▽), and 18.9 μM (▼).

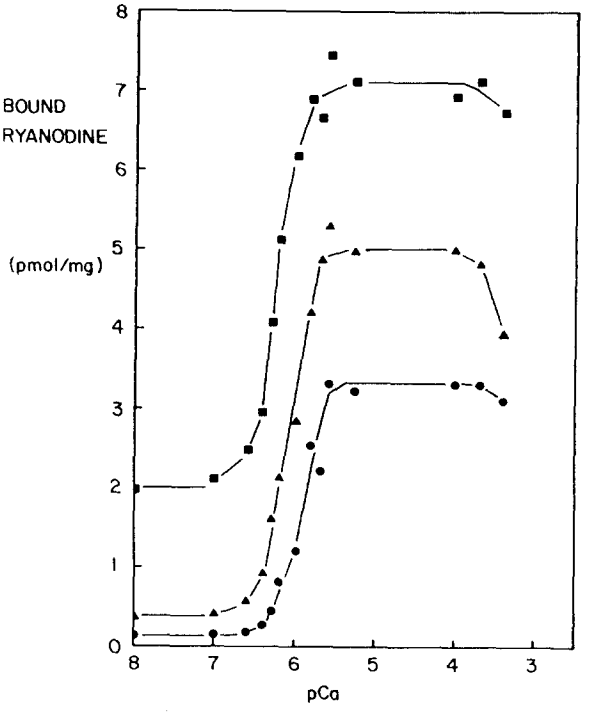

Fig. 2. Calcium dependence of the extent of [^{3}H]ryanodine binding to cardiac sarcoplasmic reticulum vesicles. [^{3}H]Ryanodine binding was measured by Millipore filtration as described in Methods at various free calcium concentrations at preincubation times at which maximal equilibrium binding was assured. Binding conditions were identical to those in Fig. 1. [^{3}H]Ryanodine concentrations studied were 10.6 nM (●), 52.5 nM (▲), and 264.6 nM (■).

with the calcium concentration and the ryanodine concentration. The amount of binding at no added calcium ($pCa < 8.0$) also increased with increasing concentrations of ryanodine. In addition, the calcium concentration required for half-maximal [^{3}H]ryanodine binding shifted to lower values with increases in ryanodine concentration.

To quantitate the calcium requirement for ryanodine binding, a Hill-type analysis of the data of Fig. 2 was performed. In this case, the fractional saturation, Y, was defined as B/B_{max}, where B is the equilibrium binding level at a specified calcium and ryanodine concentration and B_{max} is the maximum equilibrium binding at a specified ryanodine concentration. In all cases, the binding at $pCa < 8.0$ was subtracted from B and B_{max}, so only the calcium-dependent [^{3}H]ryanodine binding was investigated. The Hill plots were linear and parallel for ryanodine concentrations from 11 to 265 nM and the apparent K_m for calcium shifted to lower concentrations with higher concentrations of ryanodine. The average Hill coefficient and K_m for three analyses on separate cardiac sarcoplasmic reticulum preparations are given in Table I).

The relationship between [^{3}H]ryanodine binding and the free [^{3}H]ryanodine concentration is shown in Fig. 3. The binding in the absence of calcium was linear with concentration and showed no evidence of saturation up to 20 μM ryanodine. Therefore, this binding was attributed to nonspecific binding. The binding in the presence of 64 μM calcium showed a saturable site below 100 nM ryanodine. A Scatchard plot for the calcium-dependent [^{3}H]ryanodine binding is shown in Fig. 4. The results suggest that there is only one site below 100 nM ryanodine, with perhaps a second site becoming visible at much higher concentrations. The second site may also be visible in Fig. 3, where the slopes of the line above 100 nM ryano-

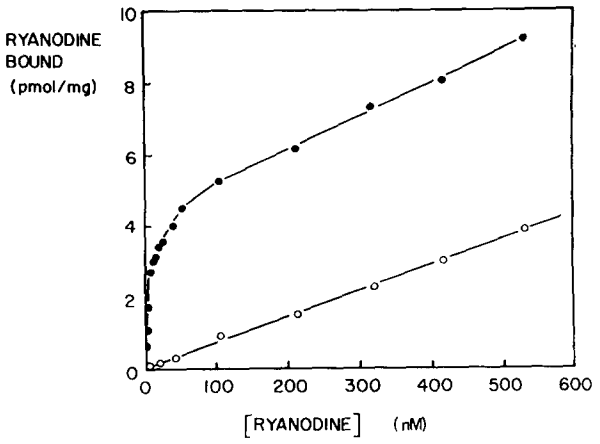

Fig. 3. Relationship between bound [^{3}H]ryanodine and free ryanodine concentration in the absence (○) and presence (●) of optimal free calcium (pCa = 4.19). Binding reaction conditions are as described in Methods except that the baths contained 0.95 mM EGTA without any added calcium (the "0 μM" calcium condition) or 0.95 mM EGTA and 1.013 mM $CaCl_2$ (p*Ca* of 4.19). The free ryanodine concentration was obtained by subtracting the total amount of [^{3}H]ryanodine bound to the cardiac sarcoplasmic reticulum vesicles from the [^{3}H]ryanodine added to the bath. Nonspecific [^{3}H]ryanodine binding to cardiac sarcoplasmic reticulum vesicles is illustrated by the binding in the absence of optimal free calcium (○). Total [^{3}H]ryanodine binding is illustrated by the binding curve obtained in the presence of optimal calcium.

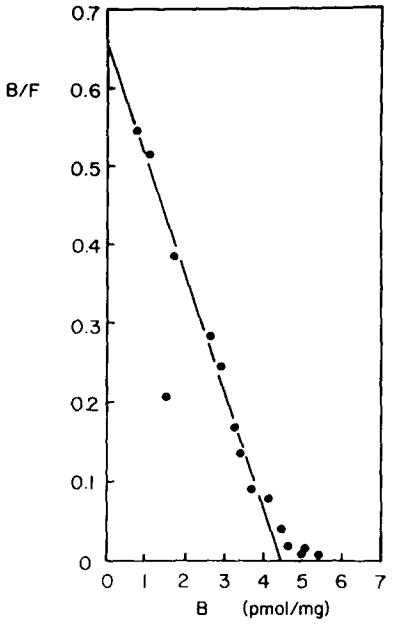

Fig. 4. Scatchard plot of [^{3}H]ryanodine binding. Specific binding of [^{3}H]ryanodine was determined as the total [^{3}H]ryanodine bound in the presence of optimal calcium (64.7 μM calcium) minus non-specific binding obtained in the absence of calcium. The ordinate axis is the specifically bound [^{3}H]ryanodine in pmol per mg sarcoplasmic reticulum protein divided by the free ryanodine concentration in nM. The abscissa is the specifically bound [^{3}H]ryanodine in pmol per mg protein. The affinity of the ryanodine receptor was estimated from the slope of the line shown, while the number of binding sites was estimated from the intercept of the line with the abscissa.

dine is significantly greater than the slope of the binding curve obtained in the absence of calcium. The average number of binding sites for three separate preparations of cardiac sarcoplasmic reticulum vesicles was 5.2 ± 4 pmol/mg, while the K_d averaged 6.8 ± 0.1 nM.

Since ryanodine has been postulated to both activate and inactivate the calcium channel in skeletal sarcoplasmic reticulum [20], it was of interest to determine whether occupancy of the high-affinity site was correlated with inactivation, or closure, of the calcium channel. The effect of 500 nM and 100 μM ryanodine on calcium uptake of the cardiac sarcoplasmic reticulum was investigated, while [^{3}H]ryanodine binding was simultaneously followed in parallel reactions identical in all respects except for the inclusion of [^{3}H]ryanodine. The results, shown in Fig. 5, show that 500 nm ryanodine binds to its maximal level of about 5.5 pmol/mg within 5 min of incubation and that this binding level remains constant for 20 min. However, this concentration of ryanodine produced no change in the rate of calcium uptake. Incubation of 100 μM ryanodine under the same

TABLE I

CALCIUM REQUIREMENT FOR RYANODINE BINDING

Cardiac vesicles were incubated as described in the legend to Fig. 2, and the data were analyzed as described in Results. The values given are the mean ± S.E. for $n = 3$ separate cardiac sarcoplasmic reticulum preparations.

Ryanodine concentration (nM)	Parameter from Hill plot	
	K_m (μM)	h
10.6	1.96 ± 0.21	2.02 ± 0.28
52.5	1.27 ± 0.08	1.90 ± 0.20
264.6	0.75 ± 0.05	2.22 ± 0.28

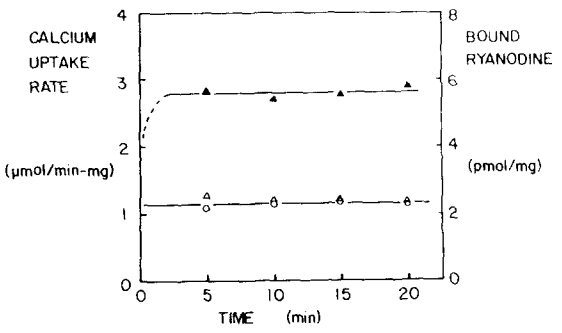

Fig. 5. Time course of specific binding of ryanodine to cardiac sarcoplasmic reticulum vesicles and its effect on oxalate-supported calcium uptake rate. Reaction conditions for ryanodine binding were as described in Methods except that 500 nM ryanodine were used. Specific binding was determined as the difference between total binding obtained in the presence of 20 μM added calcium (no EGTA present) and nonspecific binding obtained in the presence of 0.95 mM EGTA and no added calcium ("0 μM" calcium conditions). The bath protein concentration was 1 mg/ml. Ryanodine binding and oxalate-supported calcium uptake rate were determined from aliquots from identical reaction baths except that [^{3}H]ryanodine was omitted when calcium uptake rate was to be measured. Oxalate-supported calcium uptake rate was measured as described in Methods. Ryanodine binding (▲); oxalate-supported calcium uptake rate when cardiac sarcoplasmic reticulum was preincubated in the presence (△) or absence (○) of ryanodine.

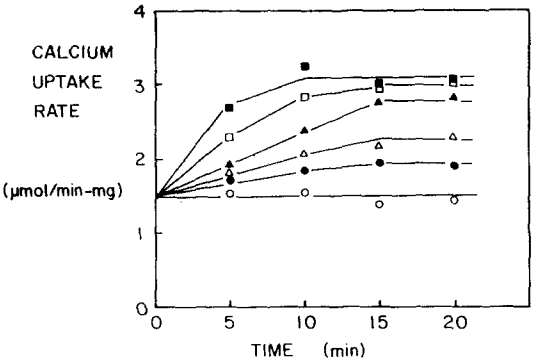

Fig. 6. Equilibrium effect of ryanodine on calcium uptake activity of cardiac sarcoplasmic reticulum vesicles. Vesicles were preincubated under conditions identical to those described in Methods for the binding of [^{3}H]ryanodine except that the baths contained 20 μM added calcium and no added EGTA. The preincubation bath contained 1 mg cardiac sarcoplasmic reticulum protein per ml. At 5-min intervals, a 100 μl aliquot was transferred to the uptake bath where the uptake reaction was initiated by ATP, magnesium and calcium as described in Methods. Ryanodine concentrations investigated were control (○), 10 μM (●), 20 μM (△), 40 μM (▲), 60 μM (□), and 100 μM (■).

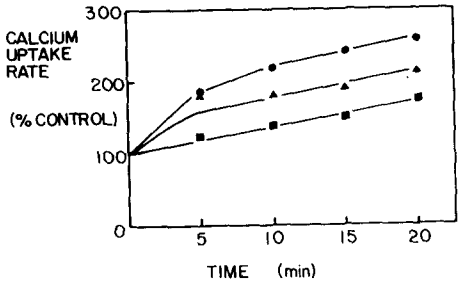

Fig. 7. Dependence of the stimulation of calcium uptake rate by ryanodine on the free calcium concentration during preincubation. Preincubation conditions were identical to those used for [^{3}H]ryanodine binding (see Methods) except that 100 μM commercially prepared ryanodine were used. The free calcium in the preincubation bath was varied by adding 20 μM calcium and various concentrations of EGTA. The total calcium in the preincubation bath was estimated by atomic absorption spectroscopy and was found to be 21.5 μM. The free calcium concentrations were calculated by the SPICE2 program as described in Methods. Oxalate-supported calcium uptake rate was estimated using an aliquot from the preincubation bath as described. Because the control uptake rates decreased sightly with time, the uptake rates are expressed in terms of per cent control where control uptakes were measured with sarcoplasmic reticulum vesicles preincubated in the absence of ryanodine at each of the preincubation calcium concentrations. The preincubation p*Ca* examined were p*Ca* 5.0 (●), 6.0 (▲), and 7.0 (■). Data points shown are the averages of two different cardiac sarcoplasmic reticulum preparations.

conditions increased the calcium uptake rate 2.6-fold (data not shown). Thus, occupancy of the high-affinity sites is not associated with closure of the channels as evidenced by an increase in the calcium uptake rate.

The stimulation of calcium uptake rate at only high concentrations of ryanodine suggests that a second low-affinity site must be operating. At 20 μM ryanodine the total binding was 189 pmol/mg, while the binding in the absence of calcium was 165 pmol/mg. Because of the large correction for non-specific binding necessary at these concentrations of ryanodine, the numbers are insufficiently precise to allow quantitative determination of the number of binding sites or their affinity. We therefore attempted to estimate the affinity of the low-affinity site by re-investigating the concentrations of ryanodine required to increase calcium uptake activity. To assure maximal effect, the ryanodine was preincubated in the presence of calcium for up to 20 min and aliquots were obtained at various times for the measurement of

oxalate-supported calcium uptake rate. The data of Fig. 6 show that the effect of various concentrations or ryanodine were maximal by 20 min of preincubation. The apparent K_m for ryanodine was near 17 μM.

Because the binding of [^{3}H]ryanodine required calcium in the micromolar and submicromolar range, but no effect was observed linking this binding to closure of the calcium channel, it was of interest to determine whether the higher ryanodine concentrations required similar low calcium concentrations in order to close the channel. The results shown in Fig. 7 indicate that the stimulation of calcium uptake rate by 100 μM ryanodine depends on the calcium concentration during preincubation, and this dependence occurs in the micromolar and submicromolar range.

Discussion

The data presented herein show that calcium influences both the binding behavior of ryanodine as well as its stimulatory effect on the calcium uptake rate of cardiac sarcoplasmic reticulum vesicles. However, these two effects of calcium are exerted in completely different domains of ryanodine concentration. The effect of calcium on [^{3}H]ryanodine binding was observed at nanomolar concentrations of ryanodine, while the effect of calcium on the stimulation of calcium uptake by ryanodine was observed at micromolar concentrations of ryanodine.

Incubation with ryanodine concentrations which saturated the high-affinity ryanodine-binding site did not increase calcium uptake by cardiac sarcoplasmic reticulum vesicles. The increase in calcium uptake by higher concentrations of ryanodine occurs by closure of a calcium efflux pathway, as discussed in the Introduction. The differences in the affinity of [^{3}H]ryanodine binding and the effect on calcium transport may be related to the observation that ryanodine may either activate or inhibit the calcium release channel of skeletal or cardiac sarcoplasmic reticulum [20] and with the observation that the early effect of high concentrations (300–500 μM) of ryanodine was an inhibition of calcium uptake [9]. In this case, occupancy of the high-affinity site may be responsible for activation of the calcium release pathway, while the slower occupancy of the low-affinity site may be related to closure of the channel. At present we have no direct evidence for binding to the low-affinity site, but we infer its existence from the effect of only high ryanodine concentrations on the oxalate-supported calcium uptake rate.

Because of the difficulty in reliably determining a small specific binding of [^{3}H]ryanodine in the presence of considerable non-specific binding, the apparent K_m for the low-affinity site was estimated by the effect of ryanodine on the stimulation of calcium uptake. Earlier reports [9] have shown that the apparent K_m measured in this way depends upon the time of preincubation prior to measurement of the calcium uptake rate. Thus, it was necessary to assure that sufficient time had elapsed to allow a maximal effect. The apparent K_m obtained in this way was 17 μM, but this is probably not a true measure of the affinity of the ryanodine receptor for ryanodine. The apparent K_m determined by this procedure probably overestimates the true K_m for binding to the low-affinity site because a single open channel may be sufficient to completely inhibit calcium uptake. The number of channels per vesicle is relevant to this point. The number of channels and pump units per vesicle can be calculated from the average size of the vesicle ($6 \cdot 10^{-6}$ cm [10]), the enclosed volume (1.4 μl/mg [36]), the maximum level of phosphorylated intermediate (1.2 nmol/mg [14–16]), and the number of ryanodine binding sites (5.2 pmol/mg, present work). These values allow one to calculate that there are about 450 pump units and only two ryanodine binding sites per vesicle. Since previous estimates suggest that only about 43% of the vesicles is sensitive to ryanodine [9], it follows that the ryanodine-sensitive vesicles have perhaps four or five ryanodine binding sites per vesicle. If we identify these sites with the channel, then it appears that these are perhaps 100-fold less numerous than the calcium pump units and thus are distinct from the pump. If the opening of a single channel is sufficient to completely inhibit calcium uptake activity by the vesicle, then stimulation of calcium uptake by ryanodine would not be observed until all four or five channels became closed. This would require higher concentrations of ryanodine than those necessary to close a single channel. The effect of

ryanodine on the stimulation of calcium uptake rate shows positive cooperativity when analyzed by a Hill plot, but this cooperativity may reflect the multiple channels per vesicle rather than a true cooperativity in ryanodine binding and closure of the calcium channel. Thus, the true K_m for binding and closure of the channel may be considerably less than the apparent K_m obtained from Fig. 6.

Although there is considerable evidence that ryanodine acts on a calcium efflux pathway distinct from the pump, some evidence suggests that the calcium pump may be involved with calcium release. Ruthenium red and caffeine affect the sarcoplasmic reticulum Ca^{2+}-ATPase at the same concentrations that they affect calcium release [11,21,22] and a conformational change in the Ca^{2+}-ATPase appears as an early event during calcium release [12]. However, more recent studies have shown that mercurials can completely inhibit calcium uptake activity mediated by the pump, while calcium release in response to caffeine or calcium is only slightly modified [37]. These same investigators reported that calcium releasing activity of heavy skeletal sarcoplasmic reticulum vesicles could be abolished without concomitant abolition of Ca^{2+}-ATPase activity [38]. Preliminary attempts to isolate the calcium channel were unable to separate the [^{3}H]ryanodine binding from either calsequestrin or the Ca^{2+}-ATPase [39], while more recent efforts indicated the Ca^{2+}-ATPase helped maintain receptor density [40]. Our data suggest that the calcium release channel is distinct from the pump because the density of [^{3}H]ryanodine binding sites is so far below the density of the Ca^{2+}-ATPase. This observation was valid for both the high-affinity and low-affinity sites. This conclusion does not rule out the possibility that the ryanodine receptor confers channel-like behavior to the Ca^{2+}-ATPase, which presumably would no longer work like a calcium transport ATPase. The proportion of Ca^{2+}-ATPase pump units so modified could be as little as 1% and thus escape detection by assays of ATPase activity.

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