Biphasic modulation of ryanodine binding to sarcoplasmic reticulum vesicles of skeletal muscle by Zn^{2+} ions

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With the use of a [^{3}H]ryanodine binding assay, the modulation of skeletal muscle ryanodine receptor (RyR1) by Zn^{2+} was investigated. In the presence of 100 μM free Ca^{2+} concentration ($[Ca^{2+}]_f$) as activator, the equilibrium [^{3}H]ryanodine binding to heavy sarcoplasmic reticulum vesicles was biphasically modulated by Zn^{2+}. The binding was increased by a free Zn^{2+} concentration ($[Zn^{2+}]_f$) of less than 1 μM; a peak binding, approx. 140 % of the control (without added Zn^{2+}) was obtained at 0.3 μM $[Zn^{2+}]_f$. An inhibitory effect of Zn^{2+} became obvious with a $[Zn^{2+}]_f$ of more than 1 μM; the $[Zn^{2+}]_f$ for producing half inhibition was 2.7 ± 0.5 μM (mean $\pm$ S.D.). Scatchard analysis indicated that the increase in the binding induced by low $[Zn^{2+}]_f$ was due to a decrease in K_d, whereas both an increase in K_d and a possible decrease in B_{max} were responsible for the decrease in binding induced by high $[Zn^{2+}]_f$. The binding in the presence of micromolar $[Zn^{2+}]_f$ showed a biphasic time course. In the presence of 3 μM $[Zn^{2+}]_f$, after reaching a peak with an increased rate of initial binding, the binding gradually declined. The decline phase could be prevented by decreasing $[Zn^{2+}]_f$ to 0.5 μM or by adding 2 mM dithiothreitol, a thiol-reducing agent. The $[Ca^{2+}]_f$ dependence of binding was changed significantly by Zn^{2+}, whereas Ca^{2+} had no clear effect on the $[Zn^{2+}]_f$ dependence of binding. Moreover, some interactions were found in the effects between Zn^{2+} and other RyR1 modulators. It is indicated that Zn^{2+} can modulate the activation sites and inactivation sites for Ca^{2+} on RyR1. The physiological significance of the effects of Zn^{2+} on ryanodine binding is discussed.

Key words: binding assay, Ca^{2+}, caffeine, dithiothreitol, ryanodine receptor.

INTRODUCTION

Despite years of study, the mechanism underlying Ca^{2+} release gated by ryanodine receptors/Ca^{2+} release channels in skeletal muscle cells (RyR1s) remains elusive [1]. It has been shown that the function of RyR1s can be regulated by various endogenous and exogenous factors, including several univalent and bivalent ions [2–5]. Among the bivalent cations, the effects of Ca^{2+} and Mg^{2+} are well known [3,4].

Zn^{2+} ions are known to be an essential catalytic or structural element of many proteins involved in the metabolic regulation and gene expression of mammalian cells [6,7]. It has been reported that, similarly to some heavy metals such as Cu^{2+}, Hg^{2+} and Cd^{2+}, Zn^{2+} can induce Ca^{2+} release from sarcoplasmic reticulum (SR) vesicles of skeletal muscle [8], suggesting an effect of Zn^{2+} on RyR1s. However, we observed recently (X. Y. Cheng, K. Y. Chen, X. H. Zhang and P. H. Zhu, unpublished work) that the contraction induced by 5 mM caffeine was depressed in small bundles of rat soleus muscles perfused with saline containing 20 μM $[Zn^{2+}]_f$. Only when the preparations were perfused with 10 μM $[Zn^{2+}]_f$ could a potentiation of contraction by caffeine be revealed in some of them.

As a convenient method, a [^{3}H]ryanodine binding assay has been used to investigate the effect of various agents on the function of RyR1s [2–5]. A close correlation between ryanodine binding and the gating state of RyR1s has been established for many ligands of RyR1s [2], although there are a few exceptions [4,8]. A ligand that can increase the binding usually causes an opening of RyR1s. An advantage of the [^{3}H]ryanodine binding assay is that it permits the assessment of the effect of various ionic conditions [3,9]. To have more understanding about the effect of Zn^{2+} on Ca^{2+} release from SR, the effect of Zn^{2+}on ryanodine binding to SR vesicles of rabbit skeletal muscle was investigated in the present study.

MATERIALS AND METHODS

Materials

[^{3}H]Ryanodine was purchased from DuPont NEN. Unlabelled ryanodine, EDTA, EGTA, BSA, PMSF, leupeptin, aprotinin, benzamide, pepstatin, dithiothreitol (DTT), Hepes, Na-Pipes and K-Pipes were all obtained from Sigma. Tris was a product of Boehringer Mannheim. Ruthenium Red (RR) was from Merck. All other chemicals were of analytical grade.

Membrane preparations

Heavy SR (HSR), light SR (LSR) and transverse tubule (TT) membrane vesicles were prepared as described previously [10]. However, instead of a linear sucrose gradient, a 20 %/35 %/40 % (w/v) sucrose step gradient was used to fractionate the KCl-extracted membranes. The membrane vesicles located at the 35 %/40 % and 20 %/35 % interfaces were designated HSR and TT respectively, whereas the fraction between them was designated LSR [11]. The protein concentration of the membrane vesicles was determined by the method of Bradford [12], with BSA as standard. All membrane vesicles were suspended in storage medium [0.3 M sucrose/5 mM K-Pipes (pH 7.0)], then frozen quickly and stored at -70 °C.

Equilibrium [^{3}H]ryanodine binding assays

Unless indicated otherwise, [^{3}H]ryanodine binding assays were performed as described elsewhere [5], with some modifications. The samples of membrane vesicles (0.25 mg/ml) were incubated

Abbreviations used: DTT, dithiothreitol; HSR, heavy sarcoplasmic reticulum; LSR, light sarcoplasmic reticulum; RR, Ruthenium Red; RyR1, skeletal muscle ryanodine receptor; SR, sarcoplasmic reticulum; TT, transverse tubule.

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at 34 °C for 4.5 h in binding buffer containing 250 mM KCl, 15 mM NaCl, 1 nM [^{3}H]ryanodine, 0.1 mM EGTA, 14 nM ryanodine and 25 mM Hepes, pH 7.10. Depending on the purpose of the experiment, various RyR1 modulators were added to the binding buffer at different times. In the medium simultaneously containing Ca^{2+}, Zn^{2+} and usually 0.1 mM EGTA, the total concentrations of Ca^{2+} and Zn^{2+} ($[Ca^{2+}]_t$ and $[Zn^{2+}]_t$) necessary for obtaining the desired free concentrations ($[Ca^{2+}]_f$ and $[Zn^{2+}]_f$ respectively) were determined by a computer program WinMaxc [13]. Because the affinity between EGTA and Zn^{2+} or Ca^{2+} is very high and the affinity for Zn^{2+} is about 100-fold higher than that for Ca^{2+} [14], the calculated $[Ca^{2+}]_t$ and $[Zn^{2+}]_t$ might not be very reliable. EGTA was nevertheless chosen as a chelator for buffering $[Ca^{2+}]_f$ between 0.1 μM and 1 mM and $[Zn^{2+}]_f$ between 0.1 μM and 10 μM respectively. There were two reasons for using EGTA: this chelator is most commonly used in binding experiments, and EGTA at a concentration of up to 1 mM did not clearly affect the binding if $[Ca^{2+}]_f$ and pH were kept constant (results not shown). The binding reaction was quenched by rapid filtration through a Whatman GHF/B glass-fibre filter. The filter was washed four times with 3 ml of ice-cold wash buffer [250 mM KCl/15 mM NaCl/20 mM Tris/HCl (pH 7.00)] and then shaken overnight with 3 ml of scintillation liquid (DuPont). The bound [^{3}H]ryanodine was determined with a scintillation counter (Beckman, LS 6000IC). Non-specific ryanodine binding was measured in the presence of 4 mM EGTA. To determine the total activity, incubation medium was mixed directly with scintillation liquid, without filtering and washing.

Time course of [^{3}H]ryanodine binding

HSR (0.5 mg/ml) was added to buffer A [250 mM KCl/15 mM NaCl/25 mM Hepes (pH 7.10)]. The composition of buffer B was similar to that of conventional binding buffer but the concentrations of [^{3}H]ryanodine, ryanodine and EGTA were doubled. To determine the time course of [^{3}H]ryanodine binding, 100 μl of buffer A was mixed with 100 μl of buffer B to start the binding at various times from 0 to 270 min. The binding reactions were quenched simultaneously by rapid filtration.

Scatchard analysis

To obtain the binding data for Scatchard analysis, 0–36 nM ryanodine was added to binding buffer containing 0.5 nM [^{3}H]ryanodine. The Scatchard analysis was based on a one-site model [5]. From the plot of the ratio of bound to free ryanodine (B/F) against B, K_d (the equilibrium binding constant) and B_{max} (the maximal number of ryanodine-binding sites) were estimated from the equation $B/F = (B_{max} - B)/K_d$.

Hill analysis

The binding data in the presence of various $[Zn^{2+}]_f$ were fitted to the Hill equation: $B = B_{max}\{1 - [Zn^{2+}]_f{}^h/(K_{d/ZnI} + [Zn^{2+}]_f{}^h)\}$, where B is the amount of bound ryanodine (pmol/mg), B_{max} is the maximal binding in the absence of inhibitor, $K_{d/ZnI}$ is the apparent affinity of the inhibitory site for Zn^{2+} and h is the pseudo-Hill coefficient. The Hill coefficient and seed values for the non-linear curve fit were obtained directly from a linear regression of logit–log plots of $\ln[(B_{max} - B)/B)$ against $\ln([Zn^{2+}]_f)$; $IC_{50/ZnI}$, the $[Zn^{2+}]_f$ for producing half inhibition, was calculated from the relationship $IC_{50/ZnI} = (K_{d/ZnI})^{1/h}$.

Measurement of $[Ca^{2+}]_f$

With an electrometer (FD223; WPI), $[Ca^{2+}]_f$ was checked by a Ca^{2+}-selective electrode on the basis of ETH 1001, a Ca^{2+} ionophore [15]. Before measurement, the Ca^{2+}-selective electrode was calibrated in a series of calibration solutions with pCa values between 3 and 7. The performance of the Ca^{2+}-selective electrode was not obviously changed by the presence of Zn^{2+} up to 10 μM (results not shown). The Ca^{2+}-selective electrodes usually showed a Nernstian behaviour between pCa 3 and pCa 6.

RESULTS

Biphasic effect of Zn^{2+} on ryanodine equilibrium binding

First, the effect of Zn^{2+} on ryanodine equilibrium binding to HSR was investigated in the presence of 100 μM $[Ca^{2+}]_f$ as activator. As shown in Figure 1, the binding was significantly increased by a $[Zn^{2+}]_f$ of less than approx. 1 μM. A peak binding to HSR, approx. 140 % of the control, was obtained at 0.3 μM $[Zn^{2+}]_f$ (Figure 1B). However, a depressive effect of Zn^{2+} became obvious with a $[Zn^{2+}]_f$ of more than 1 μM. In fact, the binding was completely depressed at 10 μM $[Zn^{2+}]_f$. Similar results were observed in eight other experiments, clearly indicating that the equilibrium ryanodine binding is biphasically modulated by Zn^{2+} ions in the presence of 100 μM $[Ca^{2+}]_f$.

In comparison with HSR, the binding to LSR or TT was significantly lower but was also modulated biphasically by Zn^{2+} (Figure 1). It is suggested that Zn^{2+} ions directly affect either

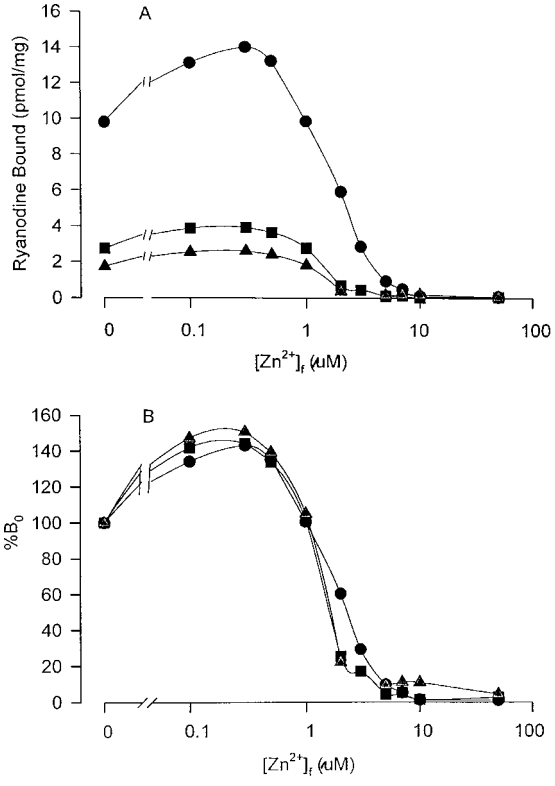

Figure 1 Biphasic effect of Zn^{2+} on [^{3}H]ryanodine binding to HSR (●), LSR (■) and TT (▲) membranes in the presence of 100 μM $[Ca^{2+}]_f$

(**A**) Binding data, showing averages for representative experiments performed in duplicate; (**B**) binding data normalized by B_0, the binding in the absence of Zn^{2+}. The samples of membrane vesicles (0.25 mg/ml) were incubated at 34 °C for 4.5 h in binding buffer containing 250 mM KCl, 15 mM NaCl, 1 nM [^{3}H]ryanodine, 0.1 mM EGTA, 14 nM ryanodine and 25 mM Hepes, pH 7.10. $[Ca^{2+}]_f$ and $[Zn^{2+}]_f$ were determined by the WinMaxc program [13]. $[Ca^{2+}]_f$ was checked by a Ca^{2+}-selective electrode. Non-specific ryanodine binding was obtained in the presence of 4 mM EGTA.

Table 1 Hill analysis of the effect of Zn^{2+} on [^{3}H]ryanodine binding to HSR in the presence of various $[Ca^{2+}]_f$ values and RyR1 modulators

Values are means ± S.D. $^*P < 0.05$, $^{**}P < 0.01$ compared with the control (without RyR modulators).

Composition of medium	$IC_{50/ZnI}$ (μM)	Hill coefficient	$K_{d/ZnI}$ (μM)	Number of experiments
30 μM Ca^{2+}	3.6 ± 0.7	1.7 ± 0.3	8.8 ± 2.2	6
100 μM Ca^{2+}	2.7 ± 0.5	2.0 ± 0.4	7.5 ± 2.1	20
+ 10 mM caffeine	1.6 ± 0.4*	2.0 ± 0.4	2.4 ± 0.7**	10
+ 1 mM Mg^{2+}	5.7 ± 0.1**	2.7 ± 0.2*	127.6 ± 55.7**	6
+ 2 mM AMP	2.8	1.7	5.5	2
+ 2 mM DTT	260	0.5	21.4	2
1 mM Ca^{2+}	3.1 ± 0.3	1.9 ± 0.2	8.7 ± 3.2	4

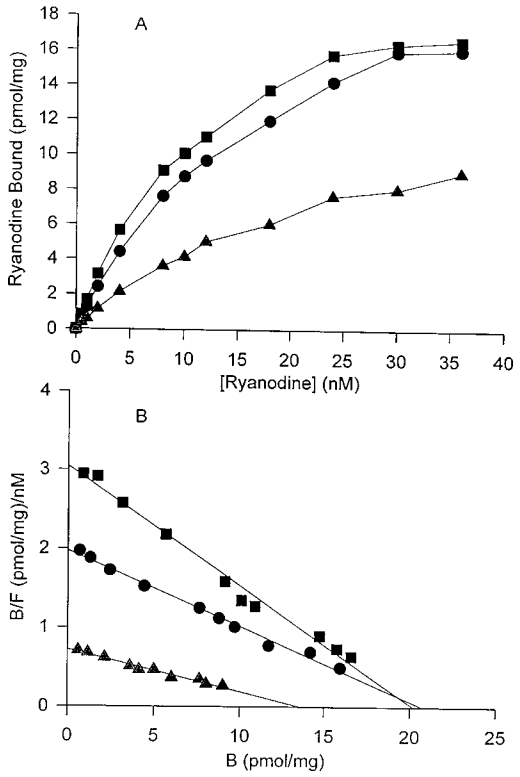

Figure 2 Scatchard analysis of the effect of Zn^{2+} on [^{3}H]ryanodine binding to HSR in the presence of 100 μM $[Ca^{2+}]_f$

(**A**) Binding data, showing averages for representative experiments performed in duplicate; (**B**) the corresponding Scatchard plots. Equilibrium bindings were obtained in the presence of 0.5 nM [^{3}H]ryanodine and various concentrations of ryanodine (0–36 nM). The Scatchard analysis was based on a one-site model [5]; the ratio of bound to free ryanodine (B/F) was plotted against B. Similar results were obtained in another eight experiments. Symbols: ●, 0 μM Zn^{2+}; ■, 0.5 μM Zn^{2+}; ▲, 3 μM Zn^{2+}.

Table 2 Scatchard analysis of the effect of Zn^{2+} on [^{3}H]ryanodine binding to HSR at various $[Ca^{2+}]_f$ values and in the presence of RyR modulators

Values are means ± S.D. $^*P < 0.05$, $^{**}P < 0.01$ compared with the control (in the absence of Zn^{2+}).

Composition of medium	K_d (nM)	B_{max} (pmol/mg)	Number of experiments
30μM Ca^{2+}			
+ 0 μM Zn^{2+}	24.1	26.6	2
+ 0.5 μM Zn^{2+}	13.5 ± 2.9	25.6 ± 0.7	4
+ 3.0 μM Zn^{2+}	38.7 ± 0.6	16.8 ± 2.1	4
100 μM Ca^{2+}			
+ 0 μM Zn^{2+}	13.3 ± 4.1	20.4 ± 4.0	12
+ 10 mM caffeine	11.4	30.6	2
+ 0.5 μM Zn^{2+}	8.6 ± 1.1**	22.1 ± 2.6	12
+ 10 mM caffeine	7.1	29.1	2
+ 3.0μM Zn^{2+}	24.1 ± 4.4**	17.3 ± 6	12
1 mM Ca^{2+}			
+ 0 μM Zn^{2+}	33.8	15.8	2
+ 0.5 μM Zn^{2+}	27.8 ± 1.3	15.8 ± 1.8	4
+ 3.0 μM Zn^{2+}	32.3 ± 1.4	11.0 ± 4.5	4

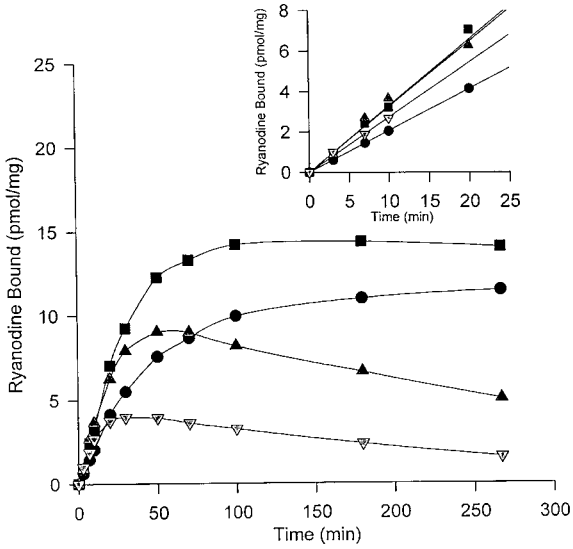

Figure 3 Effect of Zn^{2+} on the time course of [^{3}H]ryanodine binding to HSR in the presence of 100 μM $[Ca^{2+}]_f$

HSR (0.5 mg/ml) was added to buffer A [250 mM KCl/15 mM NaCl/25 mM Hepes]. The composition of buffer B was similar to that of conventional binding buffer except that the concentrations of [^{3}H]ryanodine, ryanodine and EGTA were doubled. Buffer A (100 μl) was mixed with 100 μl of buffer B at various times from 0 to 270 min to start the binding. The binding reaction was quenched by rapid filtration at the same time. The results are means for duplicate determinations. The inset represents the increase in the initial binding rate induced by Zn^{2+}. Symbols: ●, 0 μM Zn^{2+}; ■, 0.5 μM Zn^{2+}; ▲, 3 μM Zn^{2+}; ▼, 7 μM Zn^{2+}.

RyR1 or closely associated proteins. In the following experiments we examined only the effects on the binding to HSR.

From the Hill analysis, the Hill coefficient for the inhibitory effect of Zn^{2+} was 2.0 ± 0.4 (mean ± S.D., $n = 20$) (Table 1), suggesting the presence of two inhibitory sites. $IC_{50/ZnI}$ was 2.7 ± 0.5 μM ($n = 20$) (Table 1). The result shown in Figure 2 is a representative Scatchard analysis of 12 experiments performed in the presence of 100 μM $[Ca^{2+}]_f$. In this case K_d and B_{max} for the control were 10.5 nM and 20.8 pmol/mg protein respectively. With 0.5 μM $[Zn^{2+}]_f$, K_d decreased to 6.7 nM but B_{max} did not change. In contrast, 3 μM $[Zn^{2+}]_f$ (a concentration slightly higher than $IC_{50/ZnI}$) not only increased K_d to 18.9 nM but also decreased B_{max} to 13.6 pmol/mg. The results for the effects of Zn^{2+} on K_d and B_{max} are summarized in Table 2. It is indicated that the increase in the equilibrium binding induced by low $[Zn^{2+}]_f$ was due to a decrease in K_d alone, whereas the depression of the binding

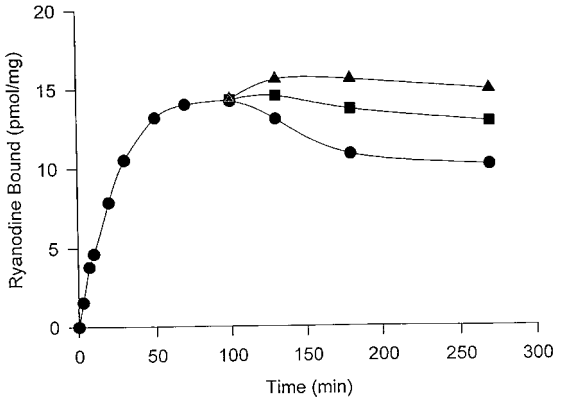

Figure 4 Effect of decreasing $[Zn^{2+}]_f$ or adding 2 mM DTT on the time course of [^{3}H]ryanodine binding

$[Ca^{2+}]_f$ was 100 μM in all experiments. Symbols: ●, 3 μM $[Zn^{2+}]_f$ was present in the binding medium throughout the incubation; ■, EGTA and Ca^{2+} were added to decrease $[Zn^{2+}]_f$ to 0.5 μM after incubation for 100 min in binding medium containing 3 μM $[Zn^{2+}]_f$; ▲, 2 mM DTT was added after incubation for 100 min in binding medium containing 3 μM $[Zn^{2+}]_f$.

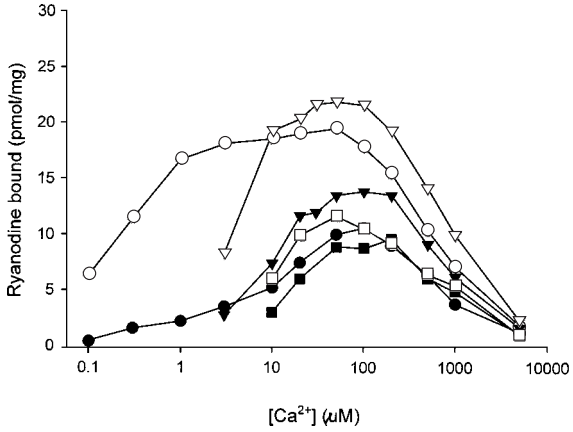

Figure 5 Effect of Zn^{2+} and caffeine on the $[Ca^{2+}]_f$ dependence of [^{3}H]ryanodine binding to HSR

The samples of membrane vesicles (0.25 mg/ml) were incubated at 34 °C for 4.5 h in binding buffer containing 250 mM KCl, 15 mM NaCl, 1 nM [^{3}H]ryanodine, 0.1 mM EGTA, 14 nM ryanodine, 25 mM Hepes and various $[Ca^{2+}]_f$ and $[Zn^{2+}]_f$ at pH 7.10, with or without 10 mM caffeine. Results are averages for representative experiments performed in duplicate. Similar results were obtained in another two experiments. Symbols: ●, control; ○, 10 mM caffeine; ▼, 0.5 μM Zn^{2+}; ▽, 0.5 μM Zn^{2+} plus 10 mM caffeine; ■, 3 μM Zn^{2+}; □, 3 μM Zn^{2+} plus 10 mM caffeine.

induced by high $[Zn^{2+}]_f$ resulted mainly from an increase in K_d and probably also from a small decrease in B_{max}.

Biphasic time course of the effect of Zn^{2+}

In the control, the binding increased monophasically with incubation and reached an equilibrium within approx. 4 h (Figure 3). In the presence of 0.5 μM $[Zn^{2+}]_f$, as expected from Figure 1, the equilibrium binding was enhanced. Although the time course was still monophasic, the initial binding rate R_0 evidently increased from 0.2 pmol/min per mg in the control to 0.3 pmol/min per mg, as illustrated in the inset of Figure 3.

At 3 or 7 μM $[Zn^{2+}]_f$, R_0 was similarly increased, but the time course became biphasic (Figure 3). In the presence of 3 μM $[Zn^{2+}]_f$, a peak binding was obtained after incubation for approx. 1 h; the binding then gradually deceased. Owing to the decline of binding at high $[Zn^{2+}]_f$, binding could not reach equilibrium within up to 4.5 h. In spite of this, the term 'equilibrium binding' is still adopted here; this should be kept in mind when evaluating the results obtained at high $[Zn^{2+}]_f$. Similar biphasic time courses were observed in another six (3 μM $[Zn^{2+}]_f$) and two (7 μM $[Zn^{2+}]_f$) experiments.

To clarify whether or not the gradual depression of the binding seen at high $[Zn^{2+}]_f$ was due to a general deterioration of the protein of RyR1s that might have occurred with prolonged incubation at high $[Zn^{2+}]_f$, we first incubated the sample in the presence of 3 μM $[Zn^{2+}]_f$ for 100 min and then decreased $[Zn^{2+}]_f$ to 0.5 μM. To decrease $[Zn^{2+}]_f$ without changing $[Ca^{2+}]_f$, certain amounts of EGTA and Ca^{2+} calculated by WinMaxc were added to the binding buffer [13]. The effect of decreasing $[Zn^{2+}]_f$ is shown in Figure 4. The time course became monophasic with the decrease in $[Zn^{2+}]_f$. To test whether this change in the time course was caused by an increase in total [EGTA], we did another experiment. Total concentrations of EGTA and Ca^{2+} were increased after incubating the sample with 3 μM $[Zn^{2+}]_f$ for 100 min but $[Zn^{2+}]_f$ and $[Ca^{2+}]_f$ remained unchanged. Under these conditions, decreased binding was seen (results not shown), indicating that the decrease in $[Zn^{2+}]_f$ was responsible for the change of the binding time course. More interestingly, the addition of 2 mM DTT after incubation with 3 μM $[Zn^{2+}]_f$ for 100 min also could abolish the decline phase (Figure 4). Taken together, these results indicate that the biphasic time course of the binding seen at high $[Zn^{2+}]_f$ might not have resulted from a general deterioration of RyR1s.

Effect of Zn^{2+} on $[Ca^{2+}]_f$ dependence of the equilibrium binding

It is well known that micromolar $[Ca^{2+}]_f$ activates RyR1s by binding to its activation site (CaA) with high affinity, whereas $[Ca^{2+}]_f$ at mM concentrations has an inhibitory effect by binding to the inactivation site (CaI) with low affinity [2,3]. Consequently, Ca^{2+} ions have a biphasic effect on ryanodine binding and on the gating of RyR1s. Although the $[Zn^{2+}]_f$ necessary for increasing and decreasing ryanodine binding in the presence of 100 μM $[Ca^{2+}]_f$ was significantly lower than the corresponding $[Ca^{2+}]_f$ (Figure 1), the biphasic modulation of the binding by Zn^{2+} suggests that two kinds of Zn^{2+}-binding site, an activation site with high affinity and an inactivation site with low affinity, were involved. To determine the relationship between the binding sites for Ca^{2+} and Zn^{2+}, the effect of Zn^{2+} ions on the $[Ca^{2+}]_f$ dependence of the equilibrium binding was examined.

As shown previously [3], in the absence of Zn^{2+} the binding expressed a bell-shaped dependence on $[Ca^{2+}]_f$ and was increased significantly by 10 mM caffeine, especially at low $[Ca^{2+}]_f$ (Figure 5).

In the presence of Zn^{2+} ions, the binding was still biphasically dependent on $[Ca^{2+}]_f$. It is evident that the binding was increased in a $[Ca^{2+}]_f$-dependent manner by 0.5 μM $[Zn^{2+}]_f$ at almost all $[Ca^{2+}]_f$ values tested except 3 μM $[Ca^{2+}]_f$, whereas the depression effect of 3 μM $[Zn^{2+}]_f$ was apparent only at lower $[Ca^{2+}]_f$. Moreover, the activation phase of the binding was shifted to the right at 3 μM $[Zn^{2+}]_f$.

A distinct character of the results illustrated in Figure 5 is that the effects of caffeine and Zn^{2+} were non-linearly additive. Because 0.5 μM $[Zn^{2+}]_f$ at low $[Ca^{2+}]_f$ might have decreased the binding (Figure 5), the fact that the binding measured in the presence of 10 mM caffeine and 0.5 μM $[Zn^{2+}]_f$ at 3 μM $[Ca^{2+}]_f$ was lower than that with 10 mM caffeine alone should not be

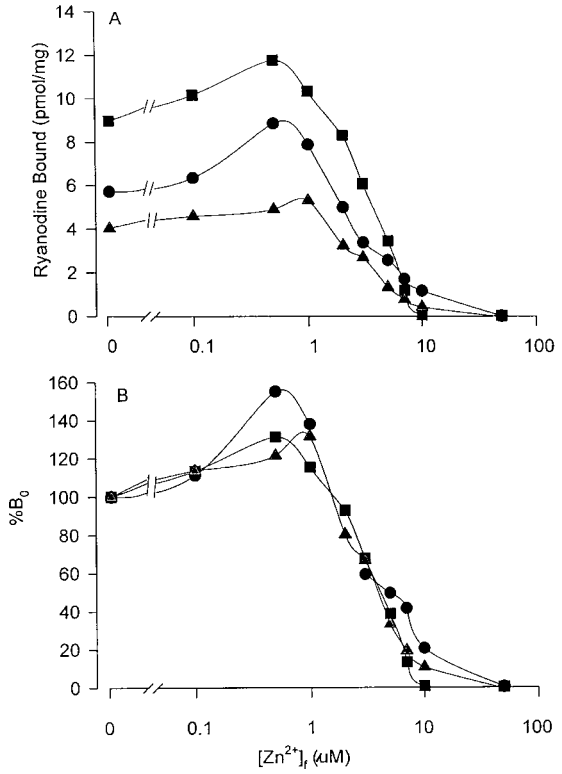

Figure 6 Effect of Ca^{2+} on the $[Zn^{2+}]_f$ dependence of [^{3}H]ryanodine binding to HSR

(**A**) Binding data, showing averages for representative experiments performed in duplicate; (**B**) binding data normalized by B_0, the binding in the absence of Zn^{2+}. The samples of membrane vesicles (0.25 mg/ml) were incubated at 34 °C for 4.5 h in binding buffer containing 250 mM KCl, 15 mM NaCl, 1 nM [^{3}H]ryanodine, 0.1 mM EGTA, 14 nM ryanodine, 25 mM Hepes and various $[Zn^{2+}]_f$ values in the presence of 30 μM (●), 100 μM (■) or 1 mM (▲) $[Ca^{2+}]_f$. Similar results were obtained in another six experiments.

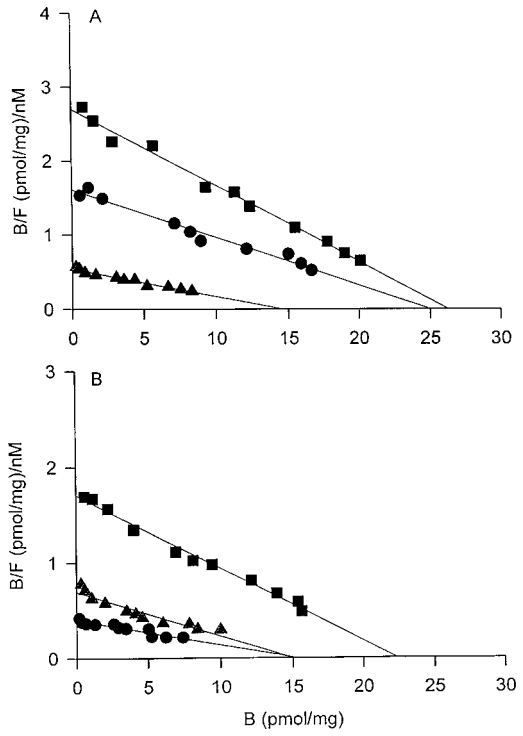

Figure 7 Scatchard analysis of the effect of Zn^{2+} on [^{3}H]ryanodine equilibrium binding to HSR at various $[Ca^{2+}]$

(**A**) Measurements made at 0.5 μM $[Zn^{2+}]_f$; (**B**) Measurements made at 3 μM $[Zn^{2+}]_f$. Equilibrium bindings were obtained in the presence of 0.5 nM [^{3}H]ryanodine and various concentrations of ryanodine (0–36 nM). The Scatchard analysis was based on a one-site model [5]; the ratio of bound to free ryanodine (*B*/*F*) was plotted against *B*. Symbols: ●, 30 μM Ca^{2+}; ■, 100 μM Ca^{2+}; ▲, 1 mM Ca^{2+}.

taken as evidence against the non-linear addition of the effects of Zn^{2+} and caffeine. However, it was still desirable to examine the interaction of the effects of Zn^{2+} and caffeine at lower $[Ca^{2+}]_f$. Unfortunately, owing to Ca^{2+} contamination in the reagents we were unable to do this experiment in the present study.

Effect of Ca^{2+} on the $[Zn^{2+}]_f$ dependence of equilibrium binding

As another approach to exploring the relationship between the binding sites for Ca^{2+} and Zn^{2+}, the effect of Ca^{2+} ions on the $[Zn^{2+}]_f$ dependence of binding was investigated. The result shown in Figure 1 represents $[Zn^{2+}]_f$ dependence in the presence of 100 μM $[Ca^{2+}]_f$. It was desirable to investigate the $[Zn^{2+}]_f$ dependence at lower and higher $[Ca^{2+}]_f$ values, at which RyR1s are partly activated and inactivated respectively. Because of the Ca^{2+} contamination just described, the low $[Ca^{2+}]_f$ used in this study was 30 μM and the high $[Ca^{2+}]_f$ was 1 mM.

It can be seen from Figure 6 that, at $[Ca^{2+}]_f$ values between 30 μM and 1 mM, the binding was still modulated biphasically by Zn^{2+} ions. The relative increase in the binding induced by low $[Zn^{2+}]_f$ and the $[Zn^{2+}]_f$ for peak binding might have been affected by high $[Ca^{2+}]_f$. The $[Zn^{2+}]_f$ for peak binding was 0.5 μM at 30 or 100 μM $[Ca^{2+}]_f$, whereas this value might have been increased to 1 μM at 1 mM $[Ca^{2+}]_f$. Otherwise, the $[Zn^{2+}]_f$ dependence was not clearly changed by $[Ca^{2+}]_f$. Similar results were observed in another six experiments.

Hill coefficients for the inhibitory effect of Zn^{2+} ions were determined by Hill plots and are summarized in Table 1. $IC_{50/ZnI}$ values at 30 μM, 100 μM and 1 mM $[Ca^{2+}]_f$ were 3.6 ± 0.7, 2.7 ± 0.5 and 3.1 ± 0.3 μM respectively. $K_{d/ZnI}$ also remained unchanged at these $[Ca^{2+}]_f$ values.

Figure 7 shows a representative Scatchard analysis at these $[Ca^{2+}]_f$ values; the results are summarized in Table 2. At 30 μM $[Ca^{2+}]_f$, K_d was decreased and increased from 24.1 nM ($n = 2$) of the control to 13.5 ± 2.9 nM ($n = 4$) and 38.7 ± 0.6 nM ($n = 4$) respectively by 0.5 and 3 μM $[Zn^{2+}]_f$. In the presence of 30 μM $[Ca^{2+}]_f$, B_{max} decreased from 26.6 pmol/mg ($n = 2$) in the control to 16.8 ± 2.1 pmol/mg by 3 μM $[Zn^{2+}]$, but not by 0.5 μM $[Zn^{2+}]$. The effect of Zn^{2+} ions on K_d and B_{max} at 30 μM $[Ca^{2+}]_f$ was generally similar to that seen with 100 μM $[Ca^{2+}]_f$. As shown in Table 2, in comparison with K_d and B_{max} assessed at 100 μM $[Ca^{2+}]_f$, 1 mM $[Ca^{2+}]_f$ itself significantly increased and decreased K_d and B_{max} respectively. As a result, the effect of 3 μM $[Zn^{2+}]_f$ on K_d and B_{max} was less evident at 1 mM $[Ca^{2+}]_f$.

Interaction of the effects of Zn^{2+} and other RyR modulators

It has been shown that the function of RyR1s can be modulated by various endogenous and exogenous factors, including caffeine, Mg^{2+} and adenine nucleotide [2–5]. It is thought that caffeine increases the apparent affinity of the activation site for Ca^{2+}, whereas Mg^{2+} inhibits ryanodine binding by competing with Ca^{2+} for the Ca^{2+} activation site [3,4]. In contrast with caffeine, AMP increases ryanodine binding mainly by decreasing the

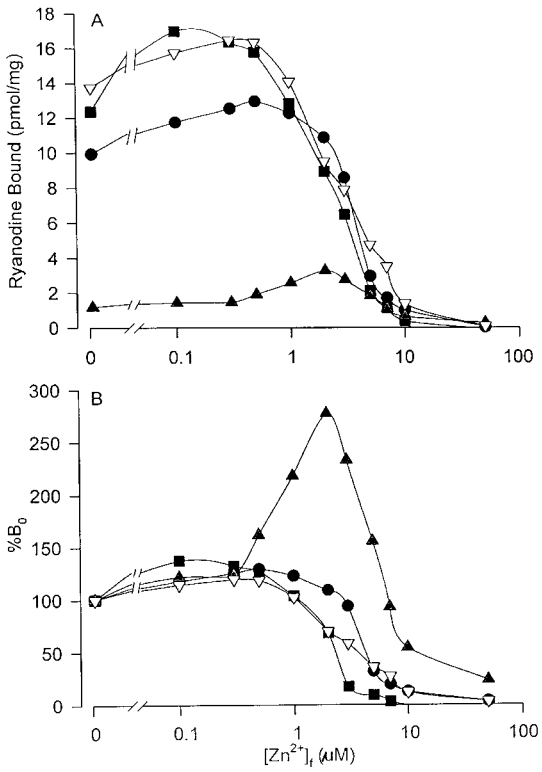

Figure 8 Effect of caffeine, Mg^{2+} and AMP on the $[Zn^{2+}]_f$ dependence of $[^3H]$ryanodine binding to HSR in the presence of 100 μM $[Ca^{2+}]_f$

(**A**) Binding data, showing averages for representative experiments performed in duplicate; (**B**) binding data normalized by B_0, the binding in the absence of Zn^{2+}. The samples of membrane vesicles (0.25 mg/ml) were incubated at 34 °C for 4.5 h in binding buffer containing 250 mM KCl, 15 mM NaCl, 1 nM $[^3H]$ryanodine, 0.1 mM EGTA, 14 nM ryanodine, 25 mM Hepes and various $[Zn^{2+}]_f$ values in the presence of different reagents. Symbols: ●, control; ■, 10 mM caffeine; ▲, 1 mM Mg^{2+}; ▽, 2 mM AMP.

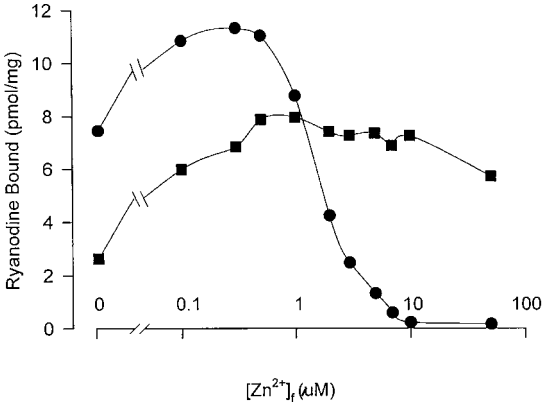

Figure 9 Effect of DTT on the $[Zn^{2+}]_f$ dependence of $[^3H]$ryanodine binding to HSR in the presence of 100 μM $[Ca^{2+}]_f$

The samples of membrane vesicles (0.25 mg/ml) were incubated at 34 °C for 4.5 h in binding buffer containing 250 mM KCl, 15 mM NaCl, 1 nM $[^3H]$ryanodine, 0.1 mM EGTA, 14 nM ryanodine, 25 mM Hepes and various $[Zn^{2+}]_f$ values in the absence (●) or the presence (■) of 2 mM DTT. The binding data are averages for representative experiments performed in duplicate.

apparent affinity of the inactivation sites for Ca^{2+} ions [3]. To characterize the binding sites for Zn^{2+} and explore the mechanism underlying the effect of Zn^{2+}, the effect of Zn^{2+} ions was investigated in the presence of various RyR1 modulators. In these experiments, $[Ca^{2+}]_f$ was kept at 100 μM.

The effect of caffeine on the $[Ca^{2+}]_f$ dependence of the binding, in the absence or presence of Zn^{2+}, is illustrated in Figure 5. As described above, the effects of caffeine and Zn^{2+} were non-linearly additive. The results shown in Figure 8 indicate that the binding in the presence of 10 mM caffeine was still biphasically modulated by Zn^{2+}. However, the $[Zn^{2+}]_f$ for peak binding was significantly decreased from 0.5 μM in the control to 0.1 μM (Figure 8) ($n = 4$). From the Hill analysis, $IC_{50/ZnI}$ and $K_{d/ZnI}$ were evidently decreased from 2.7 ± 0.5 and 7.5 ± 2.1 μM ($n = 20$) respectively in the control to 1.6 ± 0.4 and 2.4 ± 0.7 μM ($n = 10$) by 10 mM caffeine. However, the Hill coefficient remained unchanged (Table 1).

With the addition of 1 mM Mg^{2+}, the binding was significantly decreased; however, Zn^{2+} ions could still modulate the binding biphasically. In contrast with caffeine, 1 mM Mg^{2+} caused a obvious rightwards shift of $[Zn^{2+}]_f$ dependence (Figure 8). The $[Zn^{2+}]_f$ for peak binding was increased from 0.5 μM in the control to approx. 2 μM. In addition, the relative increase in binding induced by Zn^{2+} ions was most distinctive in the presence of Mg^{2+} (Figure 8B). The Hill analysis indicated that, in the presence of 1 mM Mg^{2+}, $IC_{50/ZnI}$ and $K_{d/ZnI}$ were increased by 1.6-fold and 17-fold respectively (Table 1).

In the absence of Zn^{2+}, 2 mM AMP caused an increase in binding. However, a biphasic $[Zn^{2+}]_f$ dependence of the binding was still present in the presence of 2 mM AMP (Figure 8). In contrast with the effect of caffeine, AMP had no clear effect on $[Zn^{2+}]_f$ for peak binding, and $IC_{50/ZnI}$ and $K_{d/ZnI}$ might not have been changed by AMP (Table 1).

The interaction between RR and Zn^{2+} was also examined. In comparison with the effect of 1 mM Mg^{2+}, binding was depressed more potently by 1 μM RR. Although binding in the presence of 1 μM RR became very low, the effect of Zn^{2+} still might have been biphasic (see Figure 10).

The results shown in Figure 4 indicated that the biphasic time course of the binding seen at high $[Zn^{2+}]_f$ became monophasic with the addition of 2 mM DTT. To ascertain more about the interaction between Zn^{2+} and DTT, the effect of DTT was investigated in the presence of various $[Zn^{2+}]_f$ values. In the absence of Zn^{2+}, the binding was evidently depressed by DTT (Figure 9), as shown previously [5]. However, in the presence of Zn^{2+} the effect of DTT was $[Zn^{2+}]_f$-dependent (Figure 9, $n = 2$). At $[Zn^{2+}]_f$ values less than 1 μM, which was able to increase binding (Figure 1), binding was decreased by the addition of DTT (Figure 9). However, at $[Zn^{2+}]_f$ values higher than 1 μM, the effect of DTT was interestingly reversed. More experiments were performed to compare the effect of DTT in the presence of 0.5 and 3 μM $[Zn^{2+}]_f$. The results shown in Figure 10 provide more evidence for the $[Zn^{2+}]_f$ dependence of the effect of DTT. Another finding on the effect of DTT was that the effect of either 0.5 or 3 μM $[Zn^{2+}]_f$ on $[Ca^{2+}]_f$ dependence, as represented in Figure 5, could be antagonized by 2 mM DTT (results not shown). Because Zn^{2+} might bind to DTT, $[Zn^{2+}]_f$ might have been decreased by added DTT. Because the apparent dissociation constant between Zn^{2+} and DTT is not available, we could not calculate $[Zn^{2+}]_f$ and $[Ca^{2+}]_f$ in the binding medium containing 0.1 mM EGTA and 2 mM DTT. Until $[Zn^{2+}]_f$ is measured directly, we do not know to what extent the mutually antagonistic effects of Zn^{2+} and DTT can be accounted for by the association between Zn^{2+} and DTT.

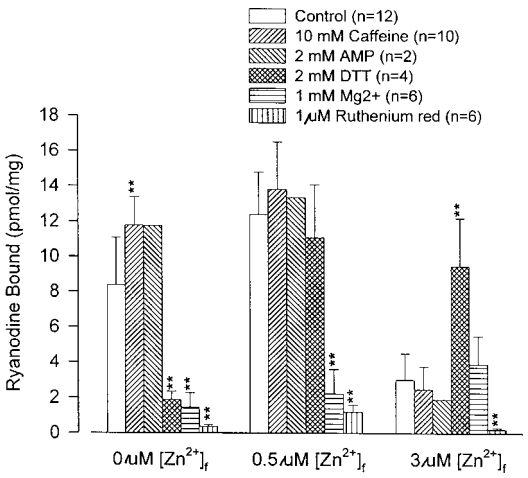

Figure 10 Effects of various RyR1 modulators on [^{3}H]ryanodine binding to HSR in the absence and in the presence of Zn^{2+}

Specific [^{3}H]ryanodine equilibrium binding was determined as described in the legend to Figure 1. $[Ca^{2+}]_f$ was 100 μM. The columns with error bars show means $\pm$ S.D. $^*P < 0.05$, $^{**}P < 0.01$ compared with the control (without RyR1 modulators).

The results on the effects of all these modulators in the absence and presence of Zn^{2+} ions are summarized in Figure 10.

DISCUSSION

In the present study, several distinct features are found in the effects of Zn^{2+} ions on the binding of ryanodine to HSR. First of all, with the use of 100 μM $[Ca^{2+}]_f$ as activator, the equilibrium binding to HSR was biphasically modulated by Zn^{2+}. The binding was significantly increased by $[Zn^{2+}]_f$ values lower than 1 μM, while a depressive effect of Zn^{2+} became obvious at higher $[Zn^{2+}]_f$ values (Figure 1). Although both Zn^{2+} and Ca^{2+} can modulate the binding biphasically, the $[Zn^{2+}]_f$ values for producing activation and inactivation of RyR1s were much lower than the corresponding $[Ca^{2+}]_f$ values. This biphasic effect of Zn^{2+} was still present at $[Ca^{2+}]_f$ values between 30 μM and 1 mM (Figure 6) and in the presence of various RyR1 modulators such as caffeine, Mg^{2+} and AMP (Figure 8). In addition, a minimum $[Ca^{2+}]_f$ seems to be essential for Zn^{2+} to have this biphasic effect (Figure 5).

Owing to the biphasic $[Ca^{2+}]_f$ dependence of ryanodine binding, it is generally proposed that activation sites (CaA) and inactivation sites (CaI) are involved in the effect of Ca^{2+} [2,3]. Micromolar $[Ca^{2+}]_f$ activates RyR1s by binding to CaA, whereas millimolar $[Ca^{2+}]_f$ has an inhibitory effect by binding to CaI. The biphasic modulation of the binding by Zn^{2+} ions indicates that two kinds of binding site, an activation site (ZnA) with high affinity and an inactivation site (ZnI) with low affinity, might be involved. If ZnA and ZnI exist, interactions between ZnA and CaA might be indicated by the following results. First, caffeine has its effect on binding mainly through increasing the apparent affinity of CaA for Ca^{2+}. The fact that 10 mM caffeine decreased the $[Zn^{2+}]_f$ for peak binding (Figure 8) suggests that caffeine has an effect on ZnA directly or through a possible interaction between ZnA and CaA. This suggestion is supported by the Mg^{2+}-induced increase in $[Zn^{2+}]_f$ for peak binding (Figure 8) and the 3 μM $[Zn^{2+}]_f$-induced rightwards shift of the activation phase of the $[Ca^{2+}]_f$ dependence of binding (Figure 5). Although an interaction between ZnA and CaA might be present, the result shown in Figure 6 indicates that ZnA and CaA are not the same site, because the $[Zn^{2+}]_f$ dependence of the binding was not clearly changed by $[Ca^{2+}]_f$. For similar reasons it is unlikely that Zn^{2+} and Ca^{2+} bind to the same site(s) for the inactivation of RyR1s.

As the second distinct feature of the effect of Zn^{2+}, the present study found that binding in the presence of high $[Zn^{2+}]_f$ showed a biphasic time course (Figures 3 and 4). Although an increase in the initial binding rate was seen at either low or high $[Zn^{2+}]_f$ values, the biphasic time course was present only at high $[Zn^{2+}]_f$ values, indicating that a slow change in the conformation of RyR1, causing a gradual decrease in the binding, occurred at high $[Zn^{2+}]_f$ values.

Recent studies have shown that Zn^{2+} ions are important for the stability of protein structure in various cells [6,7,16,17]. Although a long polypeptide can fold into an appropriate structure autonomously, the binding of Zn^{2+} ions is necessary for stabilizing the folded conformation of short polypeptides [7]. In the latter case, Zn^{2+} ions stabilize the conformation by cross-linking with the side chains of cysteine and histidine residues and forming a tetrahedral structure [7]. Because RyR1s are proteins of large molecular mass, Zn^{2+} ions might not be essential for them to maintain functional conformations. However, our recent study (H. Wang, X.-Y. Cheng, K.-Y. Chen, R.-H. Xia and P.-H. Zhu, unpublished work) found that ryanodine binding could be depressed by EGTA and other chelators of bivalent cations such as *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN). The IC_{50} values of EGTA and TPEN were 3.4 mM and 44 μM respectively. The potency of these chelators in depressing the binding was consistent with their affinities for Zn^{2+}. Moreover, the effect of EGTA could be prevented by added Zn^{2+} (results not shown). The presence of intrinsic Zn^{2+} associated with RyR1s is suggested. The intrinsic Zn^{2+} might be essential for function of RyRs. It has been shown that RyR1s can be modulated by multiple classes of thiol groups [18]. Thus, as another mechanism, Zn^{2+} might have its effect through binding to thiol groups on RyR1s. The mutually antagonistic effects between Zn^{2+} and DTT observed in this study might be taken as evidence. However, to prove the involvement of a thiol group in the effect of Zn^{2+}, it is essential to estimate quantitatively the effect of Zn^{2+} on thiol groups on purified RyR1s. In addition, diamide, a thiol-oxidizing agent, can activate the channel of RyR1 and enhance [^{3}H]ryanodine binding [18]; it would be interesting to observe how these effects are changed by Zn^{2+}.

It has been shown previously that 20 μM $[Zn^{2+}]_f$ can release 50 % of Ca^{2+} from SR vesicles [8]. The medium of that study contained 5 mM $[Mg^{2+}]_f$ and 200 μM $[Ca^{2+}]_f$. However, the present study indicated that no binding was found in the presence of 10 μM $[Zn^{2+}]_f$ (Figures 1 and 6). As shown in Figures 3 and 4, the initial binding rate was increased by Zn^{2+}; the binding in the presence of high $[Zn^{2+}]_f$ showed a biphasic time course. It is therefore likely that the initial activation of RyR1s produced by Zn^{2+} is responsible for the release of Ca^{2+} from SR vesicles induced by 20 μM $[Zn^{2+}]_f$ [8]. It has been observed that the contraction induced by caffeine was depressed in guinea-pig taenia caeci by Zn^{2+} [19]. Consistent with the effect of Zn^{2+} in smooth muscle was the observation that a depression of the contraction due to caffeine could be found in small bundles of rat soleus muscles perfused with medium containing 20–100 μM $[Zn^{2+}]_f$. However, when the preparations were perfused with 10 μM $[Zn^{2+}]_f$, a potentiation of the contraction caused by caffeine occurred in some preparations (results not shown), indicating that the effect of Zn^{2+} on caffeine contraction might also be biphasic. To substantiate this biphasic effect, it will necessary to examine the effect of lower $[Zn^{2+}]_f$.

A wide range of resting $[Zn^{2+}]_f$ and its regulation have been shown in various cells [20]. However, to our knowledge, no results are available for skeletal muscle fibres. To establish the physiological significance of the present findings it will be important to determine the endogenous Zn^{2+} level and its regulation and to identify the pathway(s) of Zn^{2+} entry into skeletal muscle fibres.

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