Effect of Zn^{2+} ions on ryanodine binding to sarcoplasmic reticulum of striated muscles in the presence of pyrithione[1]

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KEY WORDS zinc ion; pyrithione; radioligand binding; ryanodine receptor; skeletal muscle; myocardium

ABSTRACT

AIM: To explore whether the differential effects of Zn^{2+} on ryanodine binding to the sarcoplasmic reticulum (SR) of skeletal and cardiac muscles resulted from different permeability of the SR to Zn^{2+}. **METHODS:** [^{3}H]ryanodine binding assays were performed to examine the effect of Zn^{2+} on ryanodine binding to the SR in the presence of pyrithione sodium (PyNa), a specific Zn^{2+} ionophore. **RESULTS:** As a control, PyNa up to 50 μmol/L did not induce any effect on ryanodine binding to the SR of cardiac muscle. But PyNa 1-100 μmol/L increased ryanodine binding in skeletal muscle with maximum binding (222.2 %±20.9 % of the control) and inhibited ryanodine binding to 50 % of the control at about 500 μmol/L. In the presence of PyNa 10 and 50 μmol/L the dose-dependence of the effect of Zn^{2+} in cardiac muscle was still monophasic and not changed by PyNa, while the biphasic effect of Zn^{2+} in skeletal muscle became monophasic. **CONCLUSION:** Different permeability of the SR to Zn^{2+} may account for the differential effects of Zn^{2+}on ryanodine binding in skeletal and cardiac muscles. PyNa is not a strictly specific Zn^{2+} ionophore.

INTRODUCTION

Our previous study showed that ryanodine receptors/calcium release channels (RyRs) in the sarcoplasmic reticulum (SR) of skeletal and cardiac muscles were differentially modulated by Zn^{2+} [1,2]. In skeletal muscle, this modulation was biphasic. The ryanodine binding was increased by a free Zn^{2+} concentration ($[Zn^{2+}]_{free}$) of less than 1 μmol/L; a peak binding was obtained at $[Zn^{2+}]_{free}$ 0.3 μmol/L. An inhibitory effect appeared with $[Zn^{2+}]_{free}$ of more than 1 μmol/L[1]. In contrast, only an inhibitory effect of Zn^{2+} was shown with the RyRs of cardiac muscle[2].

Several important questions remained with regard to the Zn^{2+} effect. One was the mechanism underlying the differential effects of Zn^{2+}. At least, the following two possibilities might be mentioned. First, the effect of Zn^{2+} on RyRs was isoform specific, since different isoforms of RyRs were expressed in skeletal and cardiac muscle cells[3]. Second, if both sites of RyRs localized at the *cis* (cytoplasmic) side and *trans* (luminal) side of the SR could be affected by Zn^{2+}, the differential effects might be explained by different permeability of the SR to Zn^{2+}.

Pyrithione sodium (PyNa), as a specific Zn^{2+} ionophore, was used in many studies[4-7]. To test the second possibility, the effects of Zn^{2+} on ryanodine binding to the SR of skeletal and cardiac muscles were in-

[1] Project supported by the National Basic Research Program of China (G1999054001).
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Received 2004-03-29 Accepted 2004-06-14

vestigated in the presence of PyNa.

MATERIALS AND METHODS

Materials [^{3}H]Ryanodine was purchased from DuPont NEN. Pyrithione sodium (PyNa), unlabeled ryanodine, egtazic acid, bovine serum albumin (BSA), phenylmethylsulfonyl fluoride, leupeptin, aprotinin, benzamide, pepstatin, dithiothreitol, HEPES, and K-PIPES were all obtained from Sigma. Tris was a product of Boehringer. All of other chemicals were of analytical grade.

Membrane preparations The SR vesicles of skeletal muscle were prepared by a method described previously[8], with modifications. Instead of linear sucrose gradient, 20 %/35 %/40 % sucrose step gradient was used to fractionate KCl-extracted membrane[1]. The membrane vesicles located at the 35 %/40 % interface were designated as SR[9]. The method for preparing the SR vesicles of cardiac muscle was as previously described[2]. The protein concentration of the vesicles was determined by the method of Bradford, with BSA as standard[10]. The vesicles were suspended in a storage medium (sucrose 0.3 mol/L, K-PIPES 5 mmol/L, pH 7.0), quickly frozen and stored at -70 ºC.

[^{3}H]Ryanodine binding assays Unless otherwise indicated, [^{3}H]ryanodine binding assays were carried out as described elsewhere[11]. The SR vesicles of skeletal or cardiac muscle (0.25 g/L) were incubated at 34 ºC for 4.5 h in binding buffer containing KCl 250 mmol/L, NaCl 15 mmol/L, [^{3}H]ryanodine 1 nmol/L, ryanodine 14 nmol/L, HEPES 25 mmol/L, and egtazic acid 100 μmol/L. Free Ca^{2+} concentration ($[Ca^{2+}]_{free}$) was 100 μmol/L (for skeletal muscle) or 50 μmol/L (for cardiac muscle), and pH was 7.10. In the medium containing various $[Zn^{2+}]_{free}$ (for observing the effect of Zn^{2+}) or various $[Ca^{2+}]_{free}$ (for observing the Ca^{2+}dependence of ryanodine binding), the total Ca^{2+} ($[Ca^{2+}]_{total}$) and total Zn^{2+} ($[Zn^{2+}]_{total}$) necessary for obtaining desired $[Ca^{2+}]_{free}$ and $[Zn^{2+}]_{free}$ were determined by the computer program WinMaxc [12]. The binding reaction was stopped by a rapid filtration through Whatman GHF/B glass fiber filter. The filter was washed four times with 3 mL of ice cold wash buffer (KCl 250 mmol/L, NaCl 15 mmol/L, and Tris 20 mmol/L, pH 7.0), and then shaken with 3 mL scintillation liquid (Du Pont) over night. The radio-activity of the bound [^{3}H]ryanodine was determined by a scintillation counter (Beckman LS 6000IC). Nonspecific ryanodine binding was measured in the presence of ryanodine 1 μmol/L. To determine the total activity, the incubation medium was directly mixed with scintillation liquid, without filtering and washing.

Scatchard analysis Ryanodine 0-36 nmol/L was added into the binding buffer containing [^{3}H]ryanodine 0.5 nmol/L. Scatchard analysis was based on one-site model[11]. 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) can be estimated from the equation: $B/F=(B_{max}-B)/K_d$.

Statistical analysis Data were represented as mean±SD. Statistical analysis was performed by *t*-test. $P<0.05$ was considered as statistically significant.

RESULTS

Effect of PyNa on ryanodine binding in the absence of Zn^{2+} Ryanodine binding to the SR of skeletal muscle was increased by PyNa in a dose-dependent manner, while PyNa up to 50 μmol/L did not change ryanodine binding to the SR of cardiac muscle (Fig 1A). Similar results were obtained in another experiment.

With further increasing the concentration of PyNa a biphasic change of ryanodine binding appeared in skeletal muscle. Ryanodine binding increased with increasing PyNa. A maximum binding, 222.2 %±20.9 % (n=8) of the control, was obtained at PyNa 100 μmol/L. At PyNa 500 μmol/L ryanodine binding was reduced to about 50 % of the control (Fig 1B). The binding buffer contained total EGTA 100 μmol/L, which has greater affinity for Zn^{2+} than for Ca^{2+}. Therefore, $[Zn^{2+}]_{free}$ in the binding buffer should be negligible, even if trace Zn^{2+} contamination was present. Therefore, the biphasic effect of PyNa on ryanodine binding to the SR of skeletal muscle was independent of Zn^{2+}.

The increase of ryanodine binding induced by PyNa 50 μmol/L derived from both a decrease in K_d ($P<0.01$) and an increase in B_{max} ($P<0.01$). However, the inhibitory effect of PyNa 500 μmol/L was induced only by a reduction in B_{max} ($P<0.01$, Fig 2).

Ryanodine binding was increased at PyNa 50 μmol/L and decreased at PyNa 500 μmol/L at almost all concentrations of $[Ca^{2+}]_{free}$, respectively. The Ca^{2+} dependence of ryanodine binding was not altered by PyNa 50 μmol/L, but at PyNa 500 μmol/L free Ca^{2+} seemed had less effects on ryanodine binding (Fig 3). In cardiac muscle PyNa also did not change the effect of free Ca^{2+} on ryanodine binding (data not shown).

Effect of PyNa on ryanodine binding in the presence of Zn^{2+} As seen in Fig 1, in the absence of

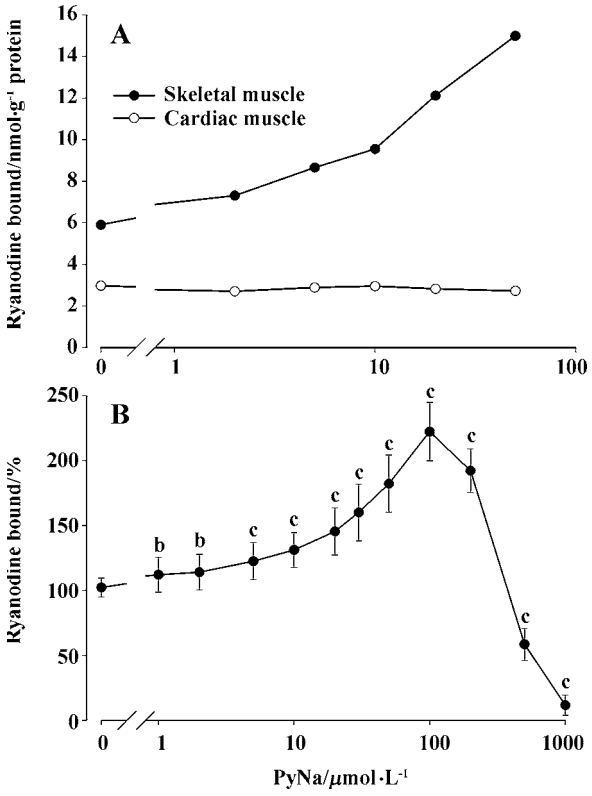

Fig 1. Effects of pyrithione sodium (PyNa) on [^{3}H]ryanodine binding to the SR of skeletal and cardiac muscles. (A) Effect of PyNa 1-100 μmol/L on [^{3}H]ryanodine binding in skeletal and cardiac muscles. Binding data were averages in duplicate. Similar results were obtained in another experiment. (B) Biphasic effect of PyNa 1-1000 μmol/L on [^{3}H]ryanodine binding in skeletal muscle. The data were expressed as percentage of ryanodine binding in the absence of PyNa (n=8). Mean±SD. $^b P<0.05$, $^c P<0.01$ *vs* control.

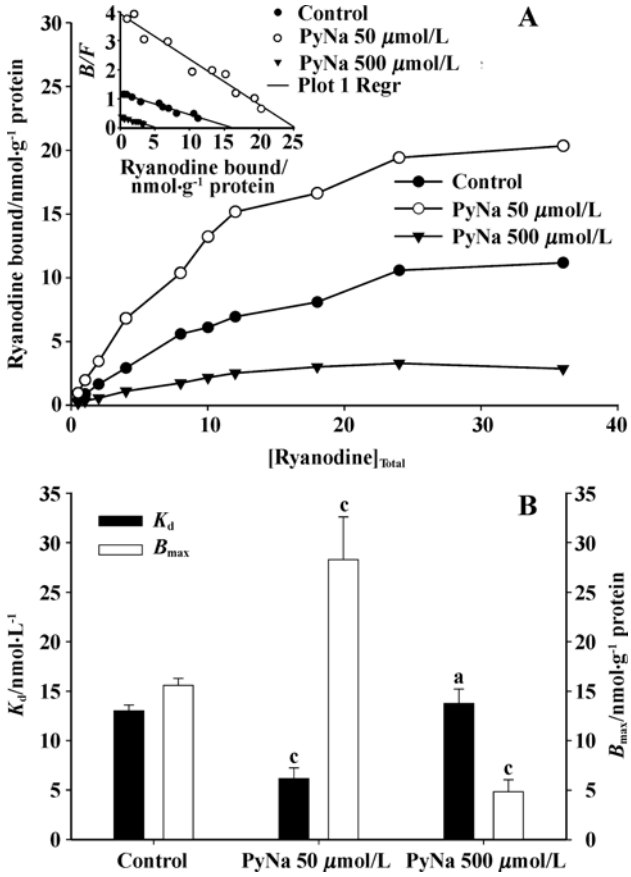

Fig 2. Scatchard analysis of the effect of pyrithione sodium (PyNa) on [^{3}H]ryanodine binding to the SR of skeletal muscle. (A) [^{3}H]ryanodine binding in the presence of PyNa 50 and 500 μmol/L. Data were averages of representative experiments performed in duplicate. (B) Summarized K_d and B_{max}. n=4 in control or 6 in PyNa 50 or 500 μmol/L. group. Mean±SD. $^a P>0.05$, $^c P<0.01$ *vs* control.

$[Zn^{2+}]_{free}$ ryanodine binding was significantly increased by PyNa 10 or 50 μmol/L. Moreover, in accordance with the previous result, Zn^{2+} had a biphasic effect on ryanoding binding to the SR of skeletal muscle in the absence of PyNa[1].

The biphasic effect of Zn^{2+} on ryanoding binding to the SR in skeletal muscle was apparently altered by PyNa (Fig 4). First, in the presence of PyNa the biphasic effect of Zn^{2+} almost became monophasic. Second, ryanoding binding to the SR was inhibited by $[Zn^{2+}]_{free}$ of around 1 μmol/L in the presence of PyNa 10 μmol/L, but in the presence of PyNa 50 μmol/L the inhibitory effects of Zn^{2+} on ryanoding binding to the SR were observed at lower concentration of $[Zn^{2+}]_{free}$.

Different from that seen in skeletal muscle, the effect of Zn^{2+} on ryanodine binding in cardiac muscle was not changed by PyNa (Fig 5).

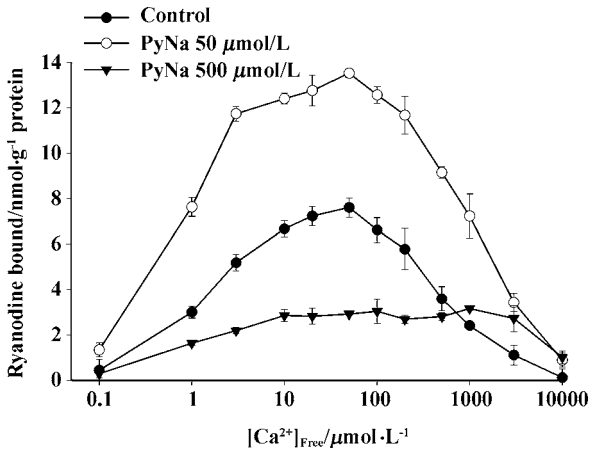

Fig 3. Effect of pyrithione sodium (PyNa) on $[Ca^{2+}]_{free}$-dependence of [^{3}H]ryanodine binding to the SR of skeletal muscle. n=6 in control or 3 in PyNa 50 or 500 μmol/L group. Mean±SD.

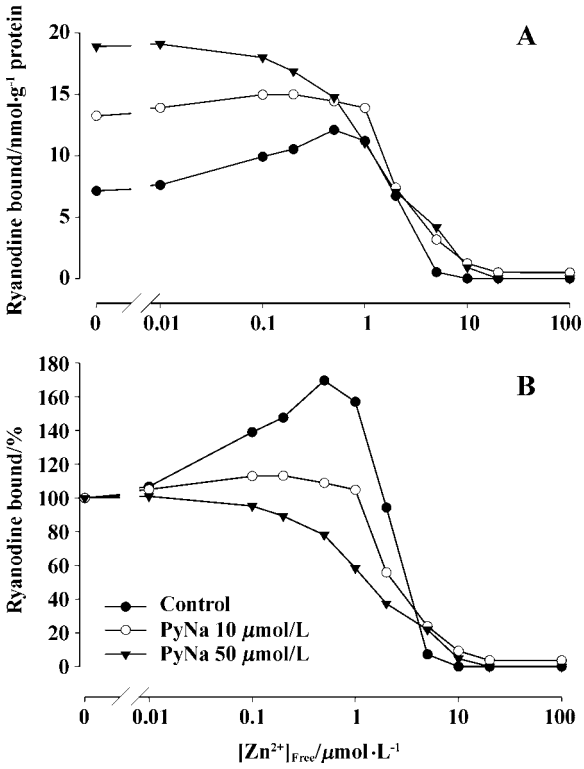

Fig 4. Effect of pyrithione sodium (PyNa) on [^{3}H]ryanodine binding to the SR of skeletal muscle in the presence of Zn^{2+}. Binding data were averages in duplicate. (A) [^{3}H] ryanodine binding was expressed as absolute value (nmol/g protein); (B) [^{3}H]ryanodine binding was expressed as percentage to corresponding baseline in the absence of free Zn^{2+}.

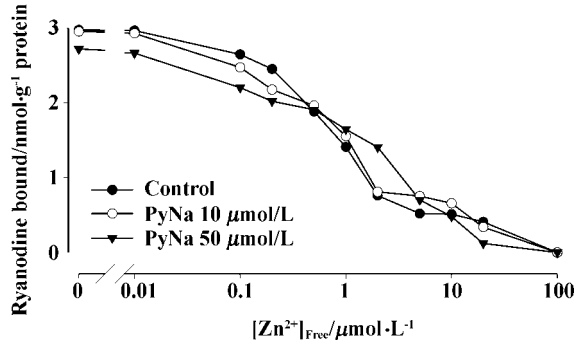

Fig 5. Effect of pyrithione sodium (PyNa) on [^{3}H]ryanodine binding to the SR of cardiac muscle in the presence of Zn^{2+}. Binding data are averages in duplicate. Similar results were obtained in another experiment.

DISCUSSION

The main finding of the present study was that the biphasic effect of Zn^{2+} in skeletal muscle became monophasic in the presence of PyNa. The PyNa-induced change of the Zn^{2+} effect suggested the presence of Zn^{2+} inactivating sites in the *trans* side of RyRs of skeletal muscle. That PyNa abolished the difference in the effects of Zn^{2+} ions between skeletal and cardiac muscles indicated that the SR isolated from skeletal and cardiac muscles might have different permeability to Zn^{2+}. The different permeability may account for the differential effects of Zn^{2+} seen previously[1,2]. But, whether this difference in Zn^{2+} permeability was intrinsic or a result of the preparation procedure remains to be investigated.

However, this conclusion was complicated by the effect of PyNa itself on ryanodine binding to the SR of skeletal muscle. We observed that PyNa biphasically changed ryanodine binding to the SR in the absence of exogenous Zn^{2+}. Although the mechanism underlying this effect was unknown, the following arguments could exclude the possibility of Ca^{2+} entry into the SR lumen. First, pyrithione, as a specific Zn^{2+} ionophore was used in many studies[4-7]. Second, Ca^{2+}-dependence of ryanodine binding was not changed by PyNa (Fig 3). Third, A23187, a specific Ca^{2+} ionophore, affected ryanodine binding in a way significantly different from that of PyNa. A detectable increase of ryanodine binding occurred at A23187 50 μmol/L, and ryanodine binding was not decreased even after A23187 500 μmol/L treatment(unpublished result).

Although pyrithione had a biphasic effect on ryanodine binding to the SR of skeletal muscle, no apparent effect was found with the SR of cardiac muscle. Since different isoforms of RyRs were expressed in skeletal and cardiac muscles cells[3], it would be interesting to investigate whether or not the effect of pyrithione on RyRs was isoform specific.

In conclusion, although the possibility of isoform specificity of Zn^{2+}effect on RyR could not be excluded, different permeability of the SR to Zn^{2+} ions might account for the differential effects of Zn^{2+} on ryanodine binding in skeletal and cardiac muscles. Besides, this study clearly indicated that the effect of pyrithione, as a Zn^{2+} ionophore, was not completely specific. It should be cautious to explain the result when PyNa was present.

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