ELSEVIER

Biochemical Pharmacology 61 (2001) 7–13

Biochemical Pharmacology

Inhibitory effects of ruthenium red on inositol 1,4,5-trisphosphate-induced responses in rat megakaryocytes

Masahiro Ikeda[a,*], Yoshio Maruyama[b]

[a]*Department of Veterinary Pharmacology, Faculty of Agriculture, Miyazaki University, 1-1 Gakuenki-banadai-nishi Miyazaki 889-2192, Japan*
[b]*Department of Physiology, Tohoku University School of Medicine, Sendai, Miyagi 980-8575, Japan*

Received 3 November 1999; accepted 31 May 2000

Abstract

The effects of ruthenium red (RR) on inositol 1,4,5-trisphosphate ($InsP_3$)-induced responses were studied in rat bone marrow megakaryocytes with the patch-clamp whole-cell recording technique in combination with fura-2 microfluorometry. Internal application of $InsP_3$ (100 μM) increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and activated the Ca^{2+}-dependent K^+ current. Administering $InsP_3$ together with RR (100–500 μM) inhibited $InsP_3$-induced responses (both Ca^{2+} and current responses) in a dose-dependent fashion. Pretreatment of megakaryocytes with extracellular RR (50 μM) also inhibited $InsP_3$-induced responses. Intracellular and extracellular application of RR reduced ADP-induced increases in $[Ca^{2+}]_i$. In contrast, in isolated single pancreatic acinar cells, RR had no effect on $InsP_3$-induced responses. Taken together, these results suggest that the site of the inhibitory action of RR is at the $InsP_3$ receptor, or its closely associated proteins. In addition, we have shown that RR is a useful pharmacological tool with which to examine the $InsP_3$-mediated responses of megakaryocytes.

Keywords: Ruthenium red; Megakaryocyte; Inositol 1,4,5-trisphosphate; Platelet; Patch-clamp whole-cell recording; Fura-2 microfluorometry

1. Introduction

Two classes of intracellular Ca^{2+} release channels, the $InsP_3$ receptor and the RyR, are located mainly on ER/SR membranes. These channels play an important role in the regulation of intracellular Ca^{2+} signaling [1–5]. Some agents that were thought to act specifically on one class of release mechanism have been used to characterize Ca^{2+} signaling [3]. However, recent observations indicate that the situation is more complex. Caffeine, which is known to activate RyR Ca^{2+} release, has been shown to inhibit IICR [6–8]. Bezprozvanny *et al*. have demonstrated that heparin, a potent competitive inhibitor of $InsP_3$ binding to the $InsP_3$ receptor, activates RyR Ca^{2+} release [9]. These data suggest that the traditional tools used for characterizing intracellular Ca^{2+} release channels need to be re-evaluated.

Isolated ER/SR membranes from various types of cells have been used to study intracellular Ca^{2+} release mechanisms. While these preparations are very useful, it has been pointed out that the results obtained are not always consistent with those obtained using cells and/or tissues [10, 11]. Although we can provide no precise reason for this discrepancy, several workers have suggested that it may be due to the modifications of proteins that may occur during the purification procedures [10, 11]. Therefore, the mechanisms underlying the intracellular Ca^{2+} release observed using the purified ER/SR membranes also require further investigation at the cell and/or tissue Ca^{2+} level.

RR was originally thought to be a specific inhibitor of RyR Ca^{2+} release among the two classes of intracellular Ca^{2+} release mechanisms [3, 12–15]. However, Adunyah and Dean have reported that RR inhibits IICR in purified platelet membranes [16]. Vites and Pappano have also shown that RR selectively prevents $InsP_3$-induced atrium contractures [11]. Although these data suggest that RR acts on IICR in certain types of cells, including platelets and atrial cells, the effects of RR on IICR at a cellular Ca^{2+} level remain to be elucidated.

† Corresponding author. Tel.: +81-985-58-7268; fax: +81-985-2284.
E-mail: a0d302u@cc.miyazaki-u.ac.jp (M. Ikeda).

Abbreviations: ER/SR, endoplasmic reticulum/sarcoplasmic reticulum; $InsP_3$, inositol 1,4,5-trisphosphate; IICR, InsP3-induced Ca^{2+} release; $[Ca^{2+}]$, intracellular Ca^{2+} concentration; PS, pipette solution; PSS, physiological salt solution; RR, ruthenium red; and RyR, ryanodine receptor.

0006-2952/01/$ – see front matter
PII: PII S0006-2952(99)00519-0

Several studies have indicated that the features of megakaryocytes and their descendent platelets are similar, in terms of their responses to agonists and their ion channel distributions [17–20]. It has been shown that megakaryocytes contain the $InsP_3$ receptor and lack functional RyR Ca^{2+} release [7, 20]. Therefore, we examined the inhibitory effects of RR on $InsP_3$-induced responses in rat megakaryocytes, using the patch-clamp whole-cell recording technique in combination with fura-2 microfluorometry.

2. Materials and methods

2.1. Cell preparation

Male Wistar rats (6–10 weeks old) were purchased from Japan SLC Inc. and used in all of the experiments. Animals were stunned and then bled to death quickly and painlessly. The cell preparation procedures performed are similar to those described previously [7, 21]. Briefly, the femoral bones were removed and the bone marrow flushed out with PSS and dispersed by repetitive pipetting. After the removal of large pieces of tissue, cells were washed twice by gentle centrifugation for a short time and then resuspended in a PSS. This isolation procedure was completed within 15 min. The megakaryocytes were used within 1.5 hr of isolation. Single acinar cells were freshly isolated from the pancreas of rats using serial treatment with collagenase (200 U/mL, Wako Pure Chemicals) and trypsin (1.5 mg/mL, Sigma type XI). The acinar cells were used within 1 hr of isolation. The PSS had the following composition (in mM): 140 NaCl, 4 KCl, 2 $MgCl_2$, 1 $CaCl_2$, and 5 HEPES. The pH was adjusted to 7.2 using NaOH. The Ca-free PSS was made by eliminating $CaCl_2$ and adding 2 or 5 mM EGTA.

2.2. Whole-cell injection and microfluorometry

We monitored the $[Ca^{2+}]_i$ of cells using fura-2 microfluorometry combined with patch-clamp whole-cell recordings at room temperature (around 25°), described in our previous paper [7, 17]. Briefly, $InsP_3$ (synthetic, Dojindo Laboratories) and/or RR (Sigma) were passively loaded into the cells using a tight seal whole-cell configuration (EPC-7, List). In order to adjust the similar intracellular condition in both megakaryocytes and acinar cells, we used a common PS as an intrapipette solution. The PS contained (in mM): 140 K glutamate, 4 KCl, 2 $MgCl_2$, 0.2 EGTA, and 5 HEPES. The pH was adjusted to 7.2 using KOH. To monitor $[Ca^{2+}]_i$ and Ca^{2+}-dependent K^+ currents simultaneously, the holding potential was set at -40 mV for the megakaryocytes [7, 18]. In preliminary experiments with the PS and PSS, we only observed small Ca^{2+}-dependent currents (both monovalent and Cl^- currents) of acinar cells at a holding potential of -40 mV, because the Cl^- equilibrium potential was near -80 mV under these conditions. Therefore, we used a holding potential of 40 mV in the acinar cells to monitor $[Ca^{2+}]_i$ and Ca^{2+}-dependent currents simultaneously [21]. When filled with a PS, the pipette resistance ranged from 2 to 6 MΩ. The access resistance between cells and pipettes was around 6 MΩ for the megakaryocytes and around 15 MΩ for the acinar cells. The input capacitance of the cells was 60–180 pF for the megakaryocytes and 7–9 pF for the acinar cells. The current responses were monitored on a chart recorder (WR7700, Graphtec) or a pulse code modulated data recorder (RD101T, Teac) through a low-pass filter of 10–20 Hz. The cells were loaded with 10 μM fura-2/acetoxymethyl ester (Dojindo Laboratories) for 15 min at 37°. The loaded cells were then excited alternately at 340 and 380 nm, and the resulting fluorescence was measured at 510 nm (OSP-3, Olympus). The $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescence intensity at the two excitation wavelengths (F340/F380).

2.3. Drug application

Extracellular ADP (Oriental Yeast) was puff-applied to isolated single megakaryocytes from a nearby pipette of 15–20 μm in diameter. Each application was achieved by bringing the pipette to the setting point (within 30 μm of the cell) from the outside of the bathing solution. The puff pressure was adjusted to achieve rapid and effective ADP application. To study the effect of externally applied RR on the $InsP_3$- and ADP-induced responses, two bathing chambers (test and control) were placed on the stage of an inverted microscope (IMT-2, Olympus). One chamber contained a batch of cells immersed in RR dissolved in PSS (test chamber), and the other contained the batch of cells immersed in the PSS alone (control chamber). All other chemicals and reagents used in these experiments were purchased from either Sigma or Wako Pure Chemicals.

2.4. Data analysis

Data are expressed as means ± SE. Statistical comparisons of means between two groups were performed using Student's *t*-test; Dunnett's method was used to test comparisons of means between three groups.

3. Results

3.1. Effect of RR on fura-2 fluorescence

RR is a polyvalent cation [11, 22], so this agent may be able to bind to the anionic fura-2. Hence, we studied the effects of RR on the ratio method using fura-2 *in vitro*. Figure 1A shows that RR decreases both fluorescence intensities, F340 and F380, at two excitation wavelengths, 340 nm and 380 nm, in a pCa 7 solution. These deteriorations were RR concentration-dependent. In contrast, the F340/F380 ratio was fairly constant in various concentrations of RR.

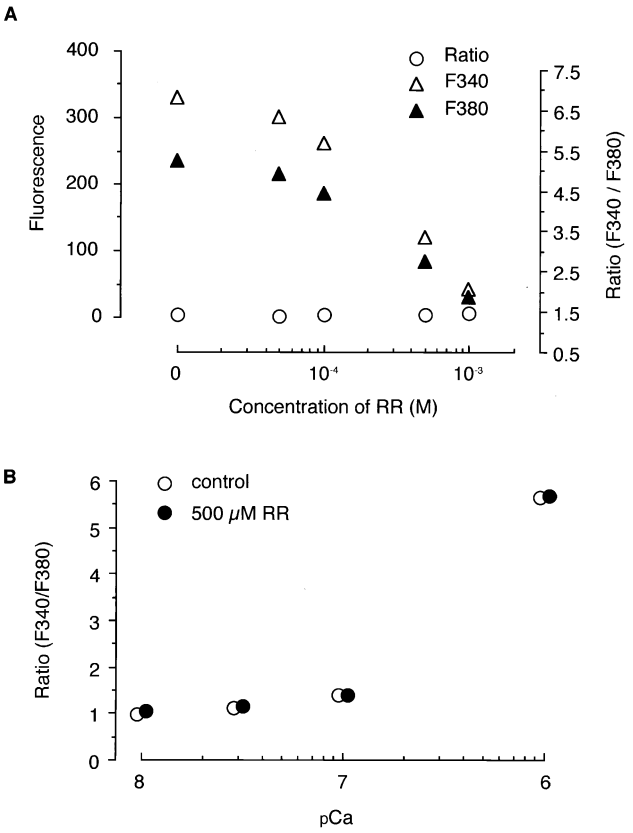

Fig. 1. Effects of RR on fura-2 fluorescence intensities, F340 and F380, and its ratio (F340/F380). A Ca–EGTA solution containing 5 μM fura-2 in the absence or presence of various concentrations of RR (0.05–1 mM) was excited by alternating excitation beams of 340 nm and 380 nm, and the resulting emissions were collected at 510 nm at room temperature (around 25°). The Ca–EGTA solution contained 75.19 mM KCl, 60.17 mM KOH, 20 mM PIPES (piperazine-*N*-*N*'-bis(2-ethanesulfonic acid)), 1.44 mM $MgCl_2$, 10 mM EGTA, pH 7.20, and around 10^{-7} M free Ca^{2+} (A). The ratio (F340/F380) in the absence or presence of 500 μM RR was plotted against calculated pCas in a solution comprised of 147 mM KCl, 19.7 mM HEPES/HEPESNa-KOH, 1 mM $MgCl_2$, 1 mM EGTA, 5 μM fura-2, pH 7.20, and various concentrations of free Ca^{2+} at room temperature (B). The method used for calculating pCa was that of Oiki and Okada [23].

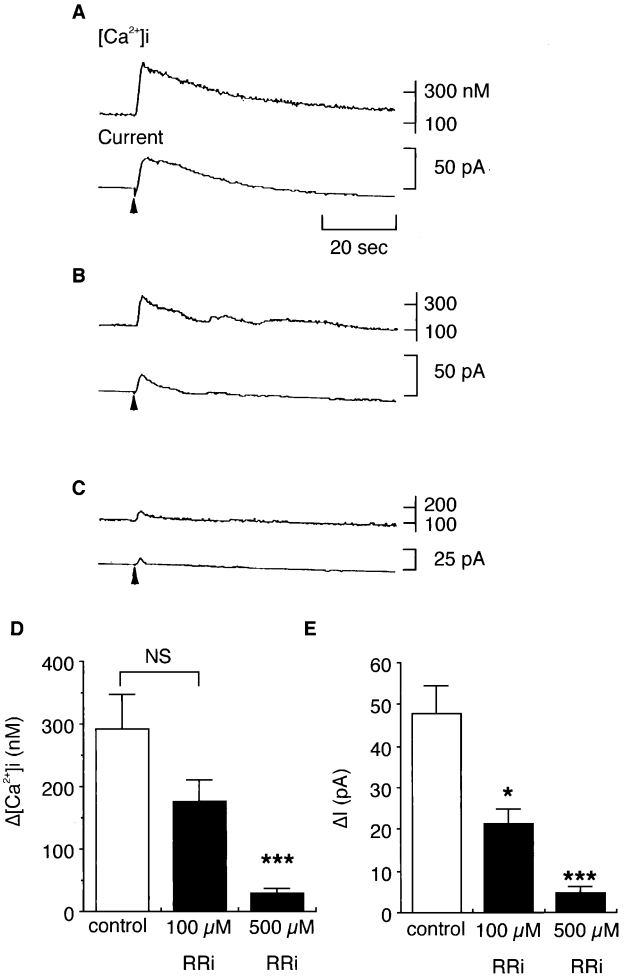

Fig. 2. Effects of intracellularly applied RR on the changes in fura-2 signals (upper trace) and membrane currents (lower trace) induced by $InsP_3$. Megakaryocytes were dialysed with PS containing 100 μM $InsP_3$ (A), 100 μM $InsP_3$ + 100 μM RR (B), or 100 μM $InsP_3$ + 500 μM RR (C). Whole-cell dialyses were established at the points indicated by the arrowheads. D and E, effects of intracellularly applied RR on the maximum changes in $InsP_3$-induced responses. Bar graphs show the maximum deviations of the $[Ca^{2+}]_i$ (D) and membrane current (E) from each basal level after whole-cell dialysis with 100 μM $InsP_3$ (control; N = 10), 100 μM $InsP_3$ + 100 μM RR (100 μMRRi; N = 4), or 100 μM $InsP_3$ + 500 μM RR (500 μMRRi; N = 8). Values are presented as means ± SE. NS, not significant. $^*P < 0.05$ and $^{***}P < 0.001$ vs. control (Dunnett's method).

Next, we checked the effect of RR on the pCa–ratio relationship *in vitro*. RR (500 μM) had no effect on the pCa–ratio relationship at Ca^{2+} concentrations of 0.01 and 1 μM, which may have physiological relevance (Fig. 1B). Furthermore, we compared the $K_d \cdot \beta$ value in the absence of RR to that in the presence of RR, because the Ca^{2+} concentration that gives $(R_{min} + R_{max})/2$ is $K_d \cdot \beta$ but not K_d: where K_d stands for the apparent dissociation constant of fura-2 for Ca^{2+}, R_{max} and R_{min} are F340/F380 ratio values in the presence of an excess amount and a virtual absence of Ca^{2+}, respectively, and β refers to the ratio of the fluorescence intensity coefficient at 380 nm in the virtual absence of Ca^{2+} to that in the presence of an excess amount of Ca^{2+}. The $K_d \cdot \beta$ value in the presence of RR was 2.0 μM, similar to that in the absence of RR (2.1 μM). On the basis of these data, we concluded that it would be feasible to use the fluorescence ratio method using fura-2 to measure the $[Ca^{2+}]_i$ in the presence of RR.

3.2. Effect of RR on IICR in megakaryocytes

We passively loaded 100 μM $InsP_3$ into megakaryocytes through the patch pipettes during whole-cell configuration. Upon establishment of the whole-cell dialysis, $[Ca^{2+}]_i$ rapidly developed to a peak within 2–3 sec, and then gradually decreased (upper trace of Fig. 2A). Simultaneous recordings of membrane currents showed increases of outward currents, which were mostly parallel to the increase in $[Ca^{2+}]_i$

(lower trace of Fig. 2A). In some cases, transient small inward currents (<8 pA) were observed immediately after the establishment of whole-cell dialysis (lower trace of Fig. 2A). Although the exact reason for this response is not clear, the following might be one possible explanation. We previously observed small outward currents at a holding potential of 40 mV immediately after the establishment of whole-cell dialysis in rat pancreatic acinar cells [24]. Therefore, it is conceivable that these small currents were due to membrane leakage. These $InsP_3$ responses are in good agreement with our previous observations, and we have also reported that $InsP_3$-induced responses of megakaryocytes are equally observable in the absence of external Ca^{2+}, indicating that this $InsP_3$-induced increase in $[Ca^{2+}]_i$ is due mainly to the release of Ca^{2+} from internal Ca^{2+} stores [7]. When we passively loaded 100 μM $InsP_3$ together with RR (100–500 μM) into megakaryocytes through the patch pipettes, reduced responses of $InsP_3$ were observed. Figure 2, D and E summarizes the results obtained from six batches of cells. Intracellular RR significantly reduced both the $InsP_3$-induced $[Ca^{2+}]_i$ increases and the Ca^{2+}-dependent K^+ currents in a concentration-dependent fashion.

Megakaryocytes were immersed in PSS alone or PSS containing 50 μM RR for 24–45 min and were subsequently administered with $InsP_3$ (100 μM) intracellularly. Figure 3C summarizes the results obtained from three batches of cells. Similarly, extracellular RR significantly reduced the $InsP_3$-induced $[Ca^{2+}]_i$. Although not of statistical significance, current responses in the presence of extracellular RR also tended to decrease (peak value: control; 62.9 ± 20.3 pA; N = 4, 50 μM RR; 22.4 ± 8.0 pA; N = 4). However, under this condition, externally applied RR did not affect the basal $[Ca^{2+}]_i$ level (Fig. 3C).

3.3. Effect of RR on the ADP-induced response in megakaryocytes

It has been suggested that megakaryocytes possess metabotropic purinoceptors and that stimulation of these receptors by ADP induces an increase in $[Ca^{2+}]_i$ [17, 19]. The majority of this $[Ca^{2+}]_i$ increase is thought to be mediated by IICR [17, 25]. Therefore, we tested the effect of RR on the ADP-induced $[Ca^{2+}]_i$ increase in the absence of extracellular Ca^{2+}. An addition of 1 μM ADP to megakaryocyte induced a transient increase in $[Ca^{2+}]_i$ 5 min after establishment of the whole-cell dialysis. This response was in good agreement with previous reports [20]. Internal dialysis of megakaryocytes with 500 μM RR for 5 min partially reduced the ADP-induced increase in $[Ca^{2+}]_i$ (Fig. 4, A and B). Figure 4C summarizes the results obtained from three batches of cells. Intracellular RR significantly reduced the ADP-induced $[Ca^{2+}]_i$ increase.

Megakaryocytes were immersed in PSS alone or PSS containing 50 μM RR for 30–35 min and subsequently

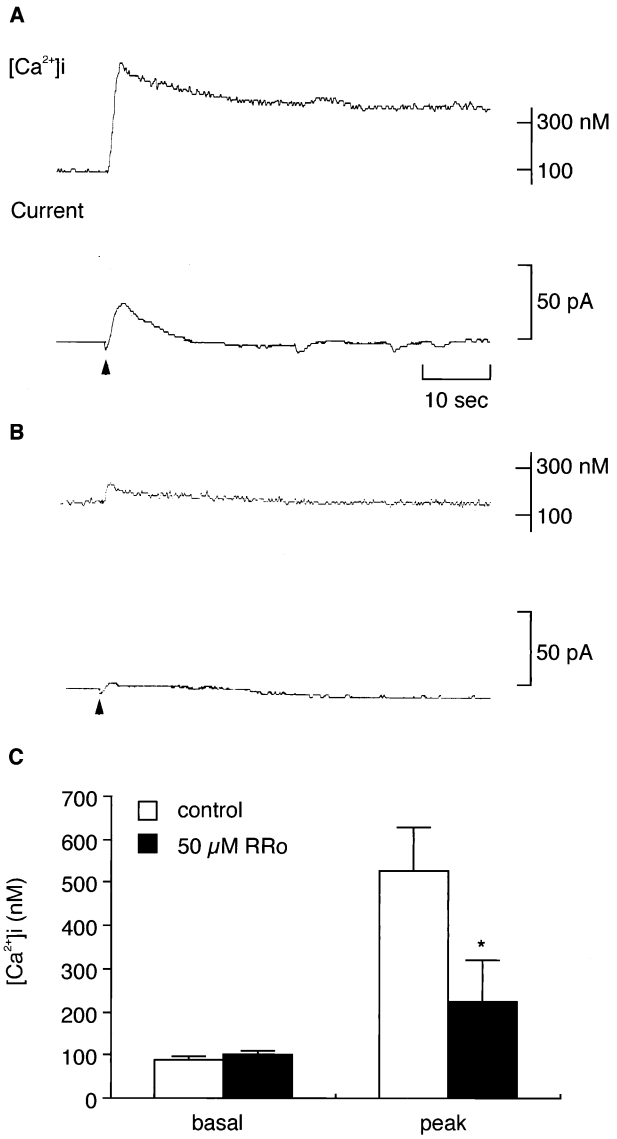

Fig. 3. Effects of extracellularly applied RR on the changes in fura-2 signals (upper trace) and membrane currents (lower trace) induced by $InsP_3$. Megakaryocytes were incubated without (A) or with 50 μM RR (B). Each whole-cell dialysis with $InsP_3$ (100 μM) was established at the points indicated by the arrowheads. C, bar graph showing the basal (before whole-cell dialysis with $InsP_3$) and peak $[Ca^{2+}]_i$ level (after whole-cell dialysis with 100 μM $InsP_3$) in the absence (open column; N = 4) or presence of extracellular 50 μM RR (solid column; N = 4). Values are presented as means ± SE. *$P < 0.05$ vs. control (Student's *t*-test).

stimulated with ADP (1 μM). Figure 4D summarizes the results obtained from three batches of cells. The ADP-induced $[Ca^{2+}]_i$ increase without whole-cell dialysis (Fig. 4D, control) was slightly larger than that with whole-cell dialysis (Fig. 4C, control). Reduced ADP responses with whole-cell dialysis appeared to be due to intracellular Ca^{2+} chelation, because we used a PS which contained 0.2 mM EGTA. In the presence of extracellular RR, the ADP-induced $[Ca^{2+}]_i$ increase was also partially but significantly inhibited (Fig. 4D).

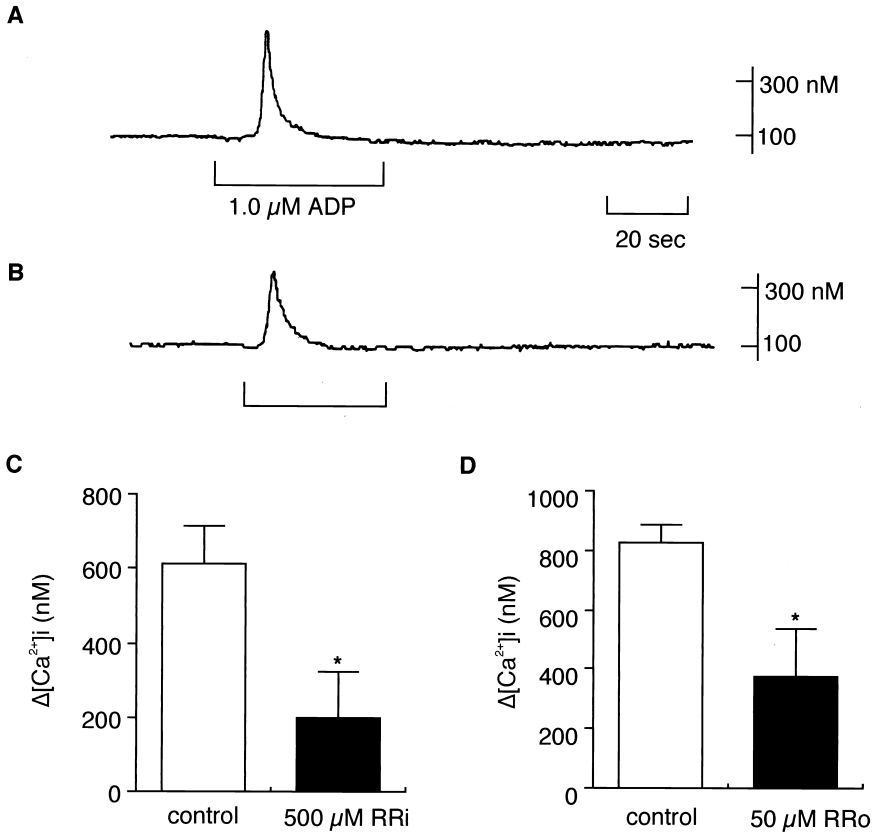

Fig. 4. Effect of RR on the ADP-induced response. A and B, ADP-induced $[Ca^{2+}]_i$ responses recorded after whole-cell dialysis without (A) or with 500 μM RR (B). The bar below each record indicates ADP (1 μM) application. C, bar graph showing the maximum deviations of $[Ca^{2+}]_i$ in response to ADP from each basal level after whole-cell dialysis without (open column; N = 5) or with RR (solid column; N = 5). D, bar graph showing the maximum deviations of $[Ca^{2+}]_i$ in response to ADP from each basal level in the absence (open column; N = 5) or presence of extracellular 50 μM RR (solid column; N = 5). Values are presented as means ± SE. *$P < 0.05$ vs. control (Student's *t*-test).

3.4. Effect of RR on IICR in pancreatic acinar cells

A detailed description of $InsP_3$-induced responses in pancreatic acinar cells was reported by us previously [24]. Therefore, we used pancreatic acinar cells to examine the effect of RR on the $InsP_3$-induced response of another cell type. When introduced into the cells via whole-cell dialysis, $InsP_3$ induced transient $[Ca^{2+}]_i$ increases (Fig. 5) and Ca^{2+}-dependent currents (peak value: 179.1 ± 69.6 pA; N = 5). Administering RR (500 μM) together with $InsP_3$ (50 μM) resulted in a failure to inhibit both the $InsP_3$-induced $[Ca^{2+}]_i$ (Fig. 5) and current responses (peak value: 145.7 ± 52.5 pA; N = 5).

4. Discussion

Although RR is known to be an inhibitor of RyR Ca^{2+} release [3, 12–15], we clearly showed that RR inhibited the $InsP_3$-mediated responses of the megakaryocytes. The possible mechanisms for this inhibition include: i) involvement of RyR Ca^{2+} release in the IICR of megakaryocytes; ii) a depletion of Ca^{2+} in the intracellular Ca^{2+} stores; and iii) a direct action of RR on the $InsP_3$ receptor or its closely associated proteins. We have reported that the IICR of megakaryocytes was inhibited by caffeine, a potentiator of RyR Ca^{2+} release, and was not affected by procaine, which is known to be an inhibitor of RyR Ca^{2+} release [7]. Uneyama *et al*. have also shown that ryanodine, a modulator of RyR Ca^{2+} release, has no effect on the ATP-induced Ca^{2+} response, which is probably a result of the liberation of Ca^{2+} from intracellular stores [20]. Thus, functional RyR Ca^{2+} release does not appear to occur in megakaryocytes. In the present study, externally applied RR did not change the basal $[Ca^{2+}]_i$ level, suggesting that RR has little effect on the Ca^{2+} content in the intracellular Ca^{2+} stores. Furthermore, a concomitant acute administration of RR with $InsP_3$ fully inhibited the $InsP_3$-mediated responses. Taking all these observations together, it is likely that the inhibitory effects of RR on $InsP_3$-induced responses are mediated by mechanism 3.

Recently, Sienaert *et al*. have shown that RR binds to the fusion proteins that contain a fragment of type 1 $InsP_3$ receptor [26]. Although further studies, including a study of the binding of RR to the native $InsP_3$ receptor and other types of $InsP_3$ receptors, are needed, these data may support

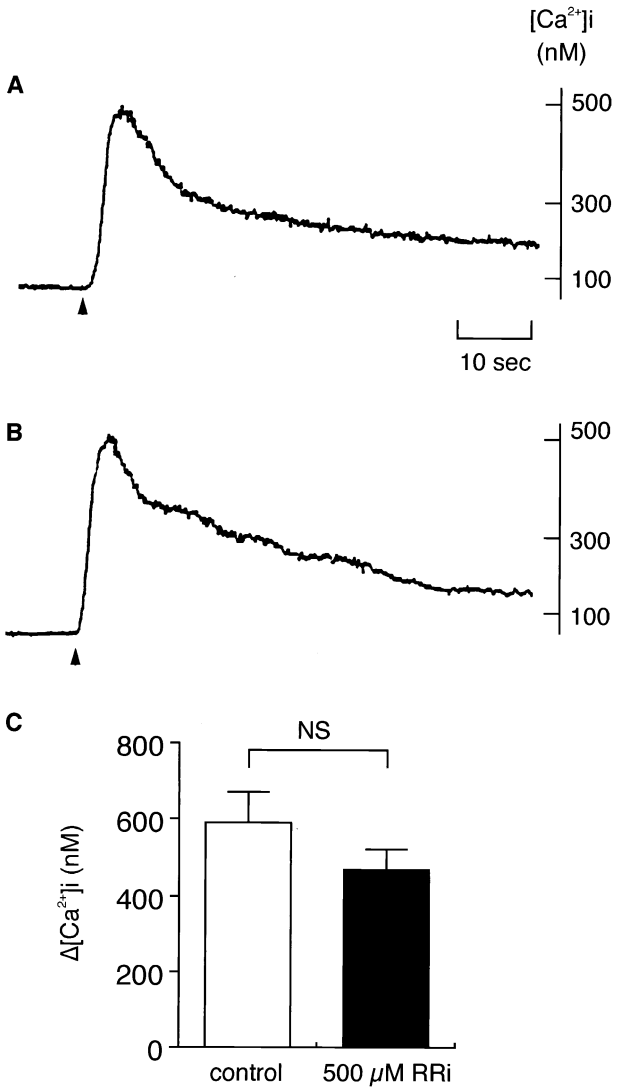

Fig. 5. Effects of intracellularly applied RR on the changes in fura-2 signals induced by $InsP_3$ in rat pancreatic acinar cells. Acinar cells were dialysed with pipette solution containing 50 μM $InsP_3$ (A) or 50 μM $InsP_3$ + 500 μM RR (B). Whole-cell dialyses were established at the points indicated by the arrowheads. C, bar graph showing the maximum deviations of $[Ca^{2+}]_i$ from each basal level after whole-cell dialysis with 50 μM $InsP_3$ (open column; N = 5) or 50 μM $InsP_3$ + 500 μM RR (solid column; N = 5). Values are presented as means ± SE. NS, not significant.

the above-mentioned hypothesis. In addition, the same group has also revealed that the pattern of RR binding to an isolated subdomain of the $InsP_3$ receptor is in part similar to that observed with $^{45}Ca^{2+}$ binding [26, 27]. These data suggest that the inhibitory effect of RR on IICR is mediated by the binding of RR to the Ca^{2+}-regulating sites of the $InsP_3$ receptor, because IICR has been reported to be modulated by both cytosolic and luminal Ca^{2+} [13, 27–29].

In the present study, RR did not affect the IICR of pancreatic acinar cells. Other groups have also reported similar observations. van de Put *et al*. have shown that RR does not change the affinity of $InsP_3$ receptor for $InsP_3$ in permeabilized rabbit pancreatic acinar cells [30]. Thorn *et al*. have observed that RR, when infused together with $InsP_3$, fails to inhibit $InsP_3$-induced initial Ca^{2+} spikes in mouse pancreatic acinar cells [31]. Therefore, it is likely that RR cannot directly affect the $InsP_3$ receptor of pancreatic acinar cells. In contrast, the IICR of the megakaryocytes was sensitive to RR. One possible mechanism for this discrepancy is thought to be the different diffusion rate of RR, which is a polyvalent cation, into the cells, because the holding potential of megakaryocytes (−40 mV) was different from that of acinar cells (40 mV). However, in the above-mentioned report by Thorn *et al*., pancreatic acinar cell was held at a holding potential of −30 mV [31]. In addition, in our preliminary experiments, when the patch pipette contained mainly KCl (140 mM), enabling us to observe the Ca^{2+}-dependent currents at a holding potential of −40 mV in pancreatic acinar cells, RR (50 μM) had little effect on $InsP_3$-induced Ca^{2+}-dependent currents in pancreatic acinar cells. Thus, this discrepancy does not seem to be due to the use of a different holding potential.

Recent examinations of the distribution of $InsP_3$ receptor subtypes have indicated that 12-*o*-tetradecanoylphorbol-13-acetate-differentiated CMK cells, which have properties of mature megakaryocytes, contain type 1 and 2 $InsP_3$ receptors [32], and all subtypes of $InsP_3$ receptor (types 1, 2, and 3) are present in pancreatic acinar cells [33]. These data suggest that the different sensitivity of IICR to RR between megakaryocytes and acinar cells may be due to the differential distribution of $InsP_3$ receptor subtypes. However, a quantitative analysis of $InsP_3$ receptor subtypes in connection with functions has not been fully undertaken. Furthermore, it has been demonstrated that functional $InsP_3$ receptors are tetramers [5]. In order to explore this point, further function-related quantitative analyses of the tissue distribution of $InsP_3$ receptor subtypes and the pharmacological characterization of both heterotetrameric and homotetrameric $InsP_3$ receptor complexes need to be performed.

van de Put *et al*. have observed that RR causes the rapid release of Ca^{2+} from the $InsP_3$-sensitive store in permeabilized rabbit pancreatic acinar cells [30]. However, Thorn *et al*. [31] and our group have observed that administering $InsP_3$ together with RR did not affect the initial IICR of pancreatic acinar cells. The reason for this discrepancy is still not clear. However, one possible explanation may be that IICR precedes the RR-induced Ca^{2+} release when $InsP_3$ and RR are administered together to the cells using whole-cell patch-clamp dialysis, thereby masking the RR-induced Ca^{2+} release with IICR. In fact, van de Put *et al*. have also observed that $InsP_3$ releases stored Ca^{2+} at a faster rate than does RR [30].

In the present study, we clearly demonstrated that a cell-permeable RR was able to inhibit $InsP_3$-induced $[Ca^{2+}]_i$ elevation in megakaryocytes. This observation is in good agreement with results obtained using purified platelet membranes [16]. Furthermore, we also showed that RR inhibited agonist-induced $[Ca^{2+}]_i$ elevation using ADP. Thus, RR will be a useful pharmacological tool with which

we may acquire a better understanding of the mechanism of IICR in megakaryocytes/platelets.

Acknowledgment

The authors thank Dr. Kiyoshi Kurokawa (Tokai University) for many helpful suggestions during the course of experiments. The present work was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture.

References

[1] Berridge MJ. Inositol trisphosphate and calcium signalling. Nature 1993;361:315–325.
[2] Clapham DE. Calcium signaling. Cell 1995;80:259–268.
[3] Ehrlich BE, Kaftan E, Bezprozvannaya S, Bezprozvanny I. The pharmacology of intracellular Ca^{2+}-release channels. Trends Pharmacol Sci 1994;15:145–149.
[4] McPherson PS, Campbell KP. The ryanodine receptor/Ca^{2+} release channel. J Biol Chem 1993;268:13765–13768.
[5] Wilcox RA, Primorse WU, Nahorski SR, Challiss RA. New developments in the molecular pharmacology of the *myo*-inositol 1,4,5-trisphosphate receptor. Trends Pharmacol Sci 1998;19:467–475.
[6] Brown GR, Sayers LG, Kirk CJ, Michell RH, Michelangeli F. The opening of the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} channel in rat cerebellum is inhibited by caffeine. Biochem J 1992;282:309–312.
[7] Ikeda M, Kurokawa K, Maruyama Y. Ca^{2+} spike initiation from sensitized inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores in megakaryocytes. Pflügers Arch 1994;427:355–364.
[8] Parker I, Ivorra I. Caffeine inhibits inositol trisphosphate-mediated liberation of intracellular calcium in *Xenopus* oocytes. J Physiol 1991;433:229–240.
[9] Bezprozvanny IB, Ondrias K, Kaftan E, Stoyanovsky DA, Ehrlich BE. Activation of the calcium release channel (ryanodine receptor) by heparin and other polyanions is calcium dependent. Mol Biol Cell 1993;4:347–352.
[10] Ehrlich BE, Watras J. Inositol 1,4,5-trisphosphate activates a channel from smooth muscle sarcoplasmic reticulum. Nature 1988;336:583–586.
[11] Vites AM, Pappano AJ. Ruthenium red selectively prevents Ins(1,4,5)P_3–but not caffeine-gated calcium release in avian atrium. Am J Physiol 1992;262:H268–H277.
[12] Fleischer S, Inui M. Biochemistry and biophysics of excitation–contraction coupling. Annu Rev Biophys Biophys Chem 1989;18:333–364.
[13] Iino M, Endo M. Calcium-dependent immediate feedback control of inositol 1,4,5-triphosphate-induced Ca^{2+} release. Nature 1992;360:76–78.
[14] Khodakhah K, Armstrong CM. Inositol trisphosphate and ryanodine receptors share a common functional Ca^{2+} pool in cerebellar Purkinje neurons. Biophys J 1997;73:3349–3357.
[15] Phillippe M, Basa A. The effects of ruthenium red, an inhibitor of calcium-induced calcium release, on phasic myometrial contractions. Biochem Biophys Res Commun 1996;221:656–661.
[16] Adunyah SE, Dean WL. Effects of sulfhydryl reagents and other inhibitors on Ca^{2+} transport and inositol trisphosphate-induced Ca^{2+} release from human platelet membranes. J Biol Chem 1986;261:13071–13075.
[17] Ikeda M, Kurokawa K, Maruyama Y. Cyclic nucleotide-dependent regulation of agonist-induced calcium increases in mouse megakaryocytes. J Physiol 1992;447:711–728.
[18] Kawa K. Voltage-gated calcium and potassium currents in megakaryocytes dissociated from guinea-pig bone marrow. J Physiol 1990;431:187–206.
[19] Kawa K, Guinea-pig megakaryocytes can respond to external ADP by activating Ca^{2+}-dependent potassium conductance. J Physiol 1990;431:207–224.
[20] Uneyama H, Uneyama C, Akaike N. Intracellular mechanisms of cytoplasmic Ca^{2+} oscillation in rat megakaryocyte. J Biol Chem 1993;268:168–174.
[21] Maruyama Y. Agonist-induced changes in cell membrane capacitance and conductance in dialysed pancreatic acinar cells of rats. J Physiol 1988;406:299–313.
[22] Luft JH. Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. Anat Rec 1971;171:347–368.
[23] Oiki S, Okada Y. New method for evaluation of purity and apparent stability constant of EGTA in physiological Ca–EGTA buffer solutions. J Physiol Soc Jpn 1987;49:317.
[24] Maruyama Y. Activation and desensitization mechanisms of muscarinic current response in single pancreatic acinar cells of rats. J Physiol 1989;417:343–359.
[25] Somasundaram B, Mahaut-Smith MP. A novel monovalent cation channel activated by inositol trisphosphate in the plasma membrane of rat megakaryocytes. J Biol Chem 1995;270:16638–16644.
[26] Sienaert I, De Smedt H, Parys JB, Missiaen L, Vanlingen S, Sipma H, Casteels R. Characterization of a cytosolic and a luminal Ca^{2+} binding site in the type I inositol 1,4,5-trisphosphate receptor. J Biol Chem 1996;271:27005–27012.
[27] Sienaert I, Missiaen L, De Smedt H, Parys JB, Sipma H, Casteels R. Molecular and functional evidence for multiple Ca^{2+}-binding domains in the type 1 inositol 1,4,5-trisphosphate receptor. J Biol Chem 1997;272:25899–25906.
[28] Iino M. Biphasic Ca^{2+} dependence of inositol 1,4,5-trisphosphate-induced Ca release in smooth muscle cells of the guinea pig taenia caeci. J Gen Physiol 1990;95:1103–1122.
[29] Missiaen L, Taylor CW, Berridge MJ. Spontaneous calcium release from inositol trisphosphate-sensitive calcium stores. Nature 1991;352:241–244.
[30] van de Put FH, Hoenderop JG, De Pont JJ, Willems PH. Ruthenium red selectively depletes inositol 1,4,5-trisphosphate-sensitive calcium stores in permeabilized rabbit pancreatic acinar cells. J Membr Biol 1993;135:153–163.
[31] Thorn P, Gerasimenko O, Petersen OH. Cyclic ADP-ribose regulation of ryanodine receptors involved in agonist evoked cytosolic Ca^{2+} oscillations in pancreatic acinar cells. EMBO J 1994;13:2038–2043.
[32] Sugiyama T, Yamamoto-Hino M, Miyawaki A, Furuichi T, Mikoshiba K, Hasegawa M. Subtypes of inositol 1,4,5-trisphosphate receptor in human hematopoietic cell lines: Dynamic aspects of their cell-type specific expression. FEBS Lett 1994;349:191–196.
[33] Yule DI, Ernst SA, Ohnishi H, Wojcikiewicz RJ. Evidence that zymogen granules are not a physiologically relevant calcium pool. Defining the distribution of inositol 1,4,5-trisphosphate receptors in pancreatic acinar cells. J Biol Chem 1997;272:9093–9098.