Inhibition of smooth-muscle myosin-light-chain phosphatase by Ruthenium Red

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Ruthenium Red (RuR) is widely used as an inhibitor of ryanodine receptor Ca^{2+} release channels, but has additional effects such as the induction of Ca^{2+} sensitization of contraction of permeabilized smooth muscles. To address the mechanism underlying this process, we examined the effects of RuR on contractility in permeabilized guinea-pig ileum and on the activity of myosin-light-chain phosphatase (MP). RuR increased the force at submaximal $[\text{Ca}^{2+}]$ (pCa 6.3) approx. 4-fold. This effect was not observed after thiophosphorylation of MP. RuR also seemed capable of preventing the thiophosphorylation of MP, suggesting a direct interaction of RuR with MP. Consistent with this possibility, smooth-muscle MP was inhibited by RuR in a concentration-dependent manner (IC_{50} 23 μM). Exogenous calmodulin significantly increased RuR-induced contraction at pCa

6.3 but had little effect on contraction induced by microcystin at this $[\text{Ca}^{2+}]$. Ca^{2+} -independent contraction was induced by RuR (EC_{50} 843 μM) and by microcystin (EC_{50} 59 nM) but the maximal force induced by RuR was smaller than that induced by microcystin. The addition of 300 μM RuR enhanced the contraction induced by 30 nM microcystin but markedly decreased that induced by 1 μM microcystin. Such a dual action of RuR on microcystin-induced effects was not observed in experiments using purified MP. We conclude that the RuR-induced Ca^{2+} sensitization of smooth-muscle contraction is due to the direct inhibition of MP by RuR.

Key words: Ca^{2+} sensitization, phosphatase inhibition, smooth-muscle contraction.

INTRODUCTION

Smooth-muscle contraction is activated by Ca^{2+} /calmodulin (CaM)-dependent myosin-light-chain kinase (MLCK)-catalysed phosphorylation of the 20 kDa light chains (LC_{20}) of myosin [1,2]. Myosin-light-chain phosphatase (MP) also has a key role in the regulation of smooth-muscle contractility because the ratio of phosphorylated to unphosphorylated myosin, the major determinant of force development, depends on the relative activities of MLCK and MP [1,2]. Consequently, the regulation of MP has been the focus of much attention [3]. Inhibition of MP can be effected by signalling pathways acting via arachidonic acid [4,5], protein kinase C and the protein kinase C (PKC)-potentiated inhibitory protein of type 1 phosphatase (CPI-17) [6–9], or RhoA and Rho-associated kinase [10–15], leading to Ca^{2+} sensitization. In contrast, NO and NO-generating agents, acting via the activation of soluble guanylate cyclase, the production of cGMP and the activation of cGMP-dependent protein kinase, trigger the activation of MP and Ca^{2+} desensitization [16–18].

Ruthenium Red (RuR) $\{[(\text{NH}_3)_5\text{Ru}-\text{O}-\text{Ru}(\text{NH}_3)_4-\text{O}-\text{Ru}(\text{NH}_3)_3]\text{Cl}_6\}$ has been widely used as a pharmacological tool to inhibit Ca^{2+} -induced Ca^{2+} release via blockade of the ryanodine receptor Ca^{2+} release channel in the sarcoplasmic reticulum (SR) of several types of muscle, including smooth muscle [19–21]. However, several additional effects of RuR have been reported. RuR inhibits the plasma membrane Ca^{2+} pump [22], Ca^{2+} uptake into mitochondria [23], voltage-dependent L-type Ca^{2+} channels and large-conductance Ca^{2+} -dependent K^+ channels [24]. It also

decreases the Ca^{2+} sensitivity of Ca^{2+} uptake into cardiac SR [25] and interacts with Ca^{2+} -binding proteins such as calsequestrin, the SR Ca^{2+} -ATPase and EF-hand-containing Ca^{2+} -binding proteins, including CaM and troponin C [26]. RuR inhibits Ca^{2+} binding to CaM and has thereby been proposed to suppress smooth-muscle contractility via the inhibition of MLCK [27].

We were therefore surprised to find that, in spite of the demonstrated inhibition of MLCK by RuR via blockade of Ca^{2+} binding to CaM [27], the application of RuR to permeabilized strips prepared from several types of smooth muscle markedly increased the Ca^{2+} sensitivity of the contractile system, concomitant with an increase in LC_{20} phosphorylation [28]. The features of RuR-induced Ca^{2+} sensitization in permeabilized smooth-muscle strips, e.g. a decrease in the rate of relaxation in the presence of RuR upon changing $[\text{Ca}^{2+}]$ from pCa 5.0 to more than 8.0, suggested the inhibition of MP as a possible mechanism, but no direct evidence was obtained.

The present study was therefore undertaken to elucidate the mechanism of RuR-induced Ca^{2+} sensitization of the smooth-muscle contractile system, with the use of permeabilized muscle preparations and purified MP.

MATERIALS AND METHODS

Tension measurement

Male Hartley guinea-pigs, weighing 300–350 g, were stunned by a blow on the head and immediately exsanguinated. The terminal portion of the ileum was removed and longitudinal muscle strips

Abbreviations used: ATP[S], adenosine 5'-[γ -thio]triphosphate; CaM, calmodulin; CPI-17, protein-kinase-C-potentiated inhibitory protein of type 1 phosphatase; LC_{20} , 20 kDa light chains of smooth-muscle myosin; ML-9, 1-(5-chloronaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine; MLCK, myosin-light-chain kinase; MOPSO, 2-hydroxy-3-morpholinopropanesulphonic acid; MP, myosin-light-chain phosphatase; MYPT, myosin-targeting subunit of MP; PKC, protein kinase C; PP1c δ , catalytic subunit of the δ isoform of type 1 protein phosphatase; R2G, relaxing solution containing 2 mM EGTA; RuR, Ruthenium Red; SR, sarcoplasmic reticulum.

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Figure 1 Effects of RuR on the contraction of permeabilized ileal longitudinal smooth muscle of the guinea-pig

(A) Ca^{2+} sensitization of contraction induced by RuR. Application of $300 \mu\text{M}$ RuR markedly enhanced the tonic contraction of permeabilized ileal smooth muscle elicited at pCa 6.3. SR function was destroyed by treatment with $10 \mu\text{M}$ A23187. R2G indicates relaxing solution containing 2 mM EGTA. The second application of pCa 6.3 solution induced a contraction comparable to the first one. (B) Abolition of RuR-induced Ca^{2+} sensitization after the inhibition of MP activity by thiophosphorylation. Permeabilized ileal smooth muscle was treated with 1 mM ATP[S] (ATP[S]) in the presence of the MLCK inhibitor ML-9 to prevent thiophosphorylation of LC_{20} (panel a). After washout, MP remained irreversibly thiophosphorylated [15,35]. The subsequent contraction at pCa 6.3 was larger than the first one, presumably due to the inhibition of MP by thiophosphorylation of MYPT. Under these conditions, the Ca^{2+} sensitizing effect of RuR was not observed, whereas further contraction was induced by pCa 4.5 solution (panel b). (C) Cumulative data obtained from experiments similar to those shown in (A) and (B). The amplitude of contraction at pCa 6.3 just before the application of $300 \mu\text{M}$ RuR was taken as 100% in each preparation. Open and filled bars indicate the amplitude of contraction before and after the application of RuR respectively. Left two columns: the application of RuR markedly enhanced the contraction (* $P < 0.05$ compared with 100%, $n = 5$). Right two columns: after treatment with ATP[S] and ML-9, contraction at pCa 6.3 was unaffected by $300 \mu\text{M}$ RuR ($P > 0.05$ compared with 100%, $n = 7$).

(250 μm in width, 100 μm in depth and 1.5–2 mm in length) were dissected. For tension measurements the muscles were maintained horizontally between two hooks and immersed in a pool of solution (300 μl) that was rounded by surface tension on a rotation plate 40 mm in diameter. Tension measurements were performed as described previously [29]. To prevent a change in surface tension and ionic strength of the solution by evaporation, the muscle was transferred from pool to pool at intervals of approx. 10 min. The transfer occasionally resulted in a spike-like artifact in the tension recording. The electrical signals of the tension recording were filtered with a low-pass filter at 10 Hz (–3 dB). Strips were allowed to equilibrate at a predetermined optimal resting tension of 100–200 mg for approx. 60 min before

the start of each experiment. The preparation was then repeatedly exposed to 142.9 mM K^{+} solution at intervals of approx. 30 min until the contraction was reproducible. Experiments were performed at room temperature (21–24 °C).

Smooth-muscle permeabilization

After measuring steady contractions induced by 142.9 mM K^{+} solution, strips were incubated in relaxing solution containing 2 mM EGTA (R2G) for 20 min. Permeabilization of the smooth-muscle preparations was achieved by incubation with $60 \mu\text{M}$ β -escin in a solution of pCa 6.3 at room temperature. After permeabilization, the solution was changed to R2G. The pCa of R2G was more than 8.0, assuming Ca^{2+} contamination of the solution to be less than $50 \mu\text{M}$. When the Ca^{2+} sensitivity of the contractile response was studied in detail, intracellular Ca^{2+} storage sites were depleted by treatment of permeabilized muscle strips for 20 min with $10 \mu\text{M}$ A23187 in R2G.

Assay of purified smooth-muscle MP

The effect of RuR on the activity of purified smooth-muscle MP was determined with phosphorylated chicken gizzard myosin as substrate. Myosin, MLCK, MP and CaM were prepared as described previously [30–33]. The assay was performed at 25 °C in 200 μl of reaction mixture containing 20 mM 2-hydroxy-3-morpholinopropanesulphonic acid (MOPSO)/KOH (pH 7.0 at 25 °C), an appropriate amount of KCl to adjust the ionic strength to 0.15 M, 1 mM EDTA, 0.5 mM dithiothreitol, $0.2 \mu\text{M}$ phosphorylated myosin and 0.16 nM MP, with various concentrations of RuR and/or microcystin-LR (a potent myosin phosphatase inhibitor). Purified MP was preincubated with drugs in the reaction mixture for 2 min (see Figure 3) or 20 min (see Figure 7) at 25 °C. Reactions were started by the addition of phosphorylated myosin and stopped after 1 min by the addition of 200 μl of ice-cold 10% (w/v) trichloroacetic acid. The resulting mixture was centrifuged at 15000 rev./min (16000 g) for 5 min at 4 °C (Hitachi Himic CF15D) and the precipitate was washed twice with acetone to remove trichloroacetic acid. The washed precipitate was dried, dissolved in 12.5 μl of lysis buffer [9.5 M urea/2% (w/v) Nonidet P40/2.8% (w/v) Pharmalyte (pH 4.5–5.4)/5% (v/v) 2-mercaptoethanol] and centrifuged at 15000 rev./min (16000 g) for 2 min at room temperature (Eppendorf centrifuge 3200) to remove insoluble material. The supernatant was subjected to two-dimensional isoelectric focusing/SDS/PAGE as described previously [32]. After staining of the gel with Coomassie Brilliant Blue, the densities of the spots of phosphorylated and unphosphorylated LC_{20} were measured with a MasterScan Interpretive densitometer (Scanalytics, Billerica, MA, U.S.A.).

Solutions

The composition of the standard Hepes-buffered solution was 137.0 mM NaCl/5.9 mM KCl/1.2 mM CaCl_2 /14.0 mM glucose/10.0 mM Hepes. The pH of all Hepes-buffered solutions was adjusted to 7.4 with NaOH. A high $[\text{K}^{+}]$ solution was prepared by replacing NaCl with equimolar KCl. Ca^{2+} -free solutions were prepared by replacing Ca^{2+} with 2 mM EGTA. The apparent stability constant of CaEGTA at 23 °C and pH 7.0 was 106.35 M^{-1} and the method for the calculation of pCa (computer program SP 6802) was originally described by Horiuchi [34]. The ionic strength of the solution was maintained at 0.2 M by adjusting the concentration of potassium methanesulphonate. The pH was adjusted to 7.0 with KOH at 23 °C.

Statistics

Results are expressed as means $\pm$ S.E.M. Statistical significance was examined by using a paired Student *t* test. The symbols * and ** indicate $P < 0.05$ and $P < 0.01$ respectively against the control.

Materials

The following compounds were obtained from the sources shown in parentheses: RuR, dithiothreitol, trichloroacetic acid, urea, 2-mercaptoethanol and Coomassie Brilliant Blue (Wako Pure Chemical Industries), microcystin-LR (Research Biochemicals International), 1-(5-chloronaphthalene-1-sulphonyl)-1*H*-hexahydro-1,4-diazepine (ML-9), adenosine 5'-[γ -thio]triphosphate (ATP[S]), β -escin and Nonidet P40 (Sigma-Aldrich Co.), EGTA, EDTA and MOPSO (Dojindo), A23187 (Molecular Probes) and Pharmalyte (Amersham Pharmacia Biotech).

RESULTS

Effects of RuR and ATP[S] on permeabilized smooth muscle

Figure 1(A) shows the typical Ca^{2+} sensitization induced by 300 μM RuR in a permeabilized strip of the longitudinal layer of guinea-pig ileum in pCa 6.3 solution, a $[\text{Ca}^{2+}]$ sufficient to activate a submaximal contraction. This preparation was treated with β -escin to permeabilize the plasma membrane and with the Ca^{2+} ionophore A23187 to destroy SR function. The tonic contraction in pCa 6.3 solution was enhanced by the addition of 300 μM RuR, as described previously [28]. The Ca^{2+} sensitizing effect of RuR was reversed on washout. Cumulative data show an average approx. 4-fold increase in force in response to RuR (Figure 1C).

Treatment of permeabilized smooth-muscle strips with ATP[S] resulted in the thiophosphorylation of proteins, including the myosin-targeting subunit of MP (MYPT), resulting in decreased MP activity and increased sensitivity of the contractile apparatus to Ca^{2+} [35,36]. In Figure 1(B) a permeabilized muscle strip was treated with 1 mM ATP[S] in the presence of 300 μM ML-9, which inhibits MLCK and therefore prevents LC_{20} thiophosphorylation. Under these conditions, MYPT but not LC_{20} was expected to be thiophosphorylated, as shown by

Buus et al. [36]. This thiophosphorylation inhibits MP activity. Accordingly, the contractile response to the second trial of pCa 6.3 after the ATP[S] treatment was significantly larger than that to the first trial before thiophosphorylation (Figure 1B). Without ATP[S] treatment the amplitude of the contraction in the second trial at pCa 6.3 was comparable to that of the first trial ($108.4 \pm 13.8\%$; $n = 5$, $P > 0.05$). After treatment with ATP[S], the contraction in pCa 6.3 solution was not enhanced by 300 μM RuR (Figures 1Bb and 1C, $130.2 \pm 17.5\%$ of that just before the application of RuR; $n = 5$, $P > 0.05$).

RuR prevents the inhibition of MP induced by thiophosphorylation

Figure 2 shows the effect of RuR on the ATP[S]-induced enhancement of contraction. Thiophosphorylation in the absence of RuR induced Ca^{2+} sensitization as expected (Figure 2A, upper panel, and 2B), but when the pretreatment with ATP[S] and ML-9 was performed in the presence of 300 μM RuR, the amplitude of the subsequent contraction in pCa 6.3 solution was not significantly different from that in the first trial at pCa 6.3 (Figures 2A, lower panel, and 2B). Experiments were always performed in a paired fashion in the absence and the presence of RuR. Taking the amplitude of the maximum contraction at pCa 5.0 as 100% in each preparation, that in pCa 6.3 solution after ATP[S] treatment was $47.7 \pm 4.2\%$ and $27.9 \pm 5.3\%$ in the absence and the presence respectively of 300 μM RuR ($n = 5$, $P < 0.05$). These findings strongly suggest that RuR directly inhibits MP and that thiophosphorylation of MP by ATP[S] is prevented by RuR.

Effect of RuR on purified smooth-muscle MP activity

To address the possibility of direct inhibition of MP by RuR, the activity of purified smooth-muscle MP was assayed in the absence and presence of RuR. The dephosphorylation of phosphorylated myosin by purified MP was quantified by two-dimensional gel electrophoresis. The activity of purified MP was 4.2 $\mu\text{mol/min}$ per mg. Figure 3(A) shows the separation of unphosphorylated, monophosphorylated and diphosphorylated LC_{20} after incubation with MP in the presence of 6.8 (Figure 3Aa) or 68 μM RuR (Figure 3Ab), corresponding to MP activities of 3.2 and

Figure 2 Inhibition of thiophosphorylation of MP by RuR

(A) Upper panel: the protocol was the same as that shown in Figure 1(B). Treatment of permeabilized ileal smooth muscle with ML-9 and ATP[S] (ATP[S]) markedly enhanced the contractile response to a subsequent application of pCa 6.3 solution. Lower panel: treatment with ML-9 and ATP[S] was performed in the presence of 300 μM RuR. In this case, no increase in contractile response to the second application of pCa 6.3 solution was observed. (B) Cumulative data from experiments similar to those shown in (A). The maximal contraction at pCa 5.0 was taken as 100% in each preparation. Open and solid columns indicate the relative amplitudes of contraction at pCa 6.3 in preparations without or with RuR treatment between the first and second trials of pCa 6.3 solution, respectively ($n = 5$). ## $P < 0.01$ compared with the first pCa 6.3 contraction; * $P < 0.05$ compared with the second pCa 6.3 contraction (after incubation with RuR).

Figure 3 Inhibitory effect of RuR on purified smooth-muscle MP activity

(A) Purified chicken gizzard MP was preincubated with $6.8 \mu\text{M}$ (a) or $68 \mu\text{M}$ RuR (b) at 25°C for 2 min. Reactions were started by the addition of phosphorylated myosin, terminated after 1 min of incubation by the addition of ice-cold 10% (w/v) trichloroacetic acid and subjected to two-dimensional isoelectric focusing (IEF)/SDS/PAGE to separate unphosphorylated and phosphorylated LC₂₀. Myosin, myosin-P and myosin-2P indicate unphosphorylated, mono-phosphorylated and diphosphorylated LC₂₀ respectively. (B) Concentration-dependent inhibition of purified MP by RuR. Specific activity is expressed as μmol of dephosphorylated myosin/min per mg of MP. A value of 100% corresponds to $4.2 \mu\text{mol}/\text{min}$ per mg. An IC₅₀ of approx. $23 \mu\text{M}$ was determined.

$0.9 \mu\text{mol}/\text{min}$ per mg respectively. The specific activity of MP was inhibited by RuR in a concentration-dependent manner, as shown in Figure 3(B). The data were well fitted by a Hill plot with a coefficient of 1.1, suggesting 1:1 stoichiometry of RuR binding to MP. The IC₅₀ was calculated to be approx. $23 \mu\text{M}$.

Effect of CaM on microcystin- or RuR-induced Ca²⁺ sensitization

It has been reported that RuR inhibits Ca²⁺ binding to CaM [27]. It was therefore unexpected to find that RuR induced Ca²⁺ sensitization in permeabilized smooth muscle [28]. We tested the hypothesis that the inhibition of Ca²⁺ binding to CaM by RuR might partly inhibit Ca²⁺ sensitization triggered by RuR-mediated inhibition of MP. The application of 10 nM microcystin, a potent type 1 and 2A protein phosphatase inhibitor [37], or $100 \mu\text{M}$ RuR enhanced the tonic contraction in pCa 6.3 solution to almost the same extent ($30.4 \pm 3.9\%$ and $38.7 \pm 7.0\%$ of the control contraction at pCa 5.0 respectively; $n = 7$, $P > 0.05$; Figure 4A). The addition of $1 \mu\text{M}$ CaM enhanced these contractions but the enhancement in the presence of RuR was significantly larger than that in the presence of microcystin: to $71.2 \pm 7.4\%$ compared with $44.1 \pm 4.7\%$ of the control contraction at pCa 5.0 respectively ($n = 7$, $P < 0.05$; Figure 4B). It

Figure 4 Effects of exogenously added CaM on microcystin- or RuR-induced Ca²⁺ sensitization

(A) Submaximal contractions induced by pCa 6.3 solutions were enhanced by 10 nM microcystin (a) or $100 \mu\text{M}$ RuR (b) to comparable extents. The addition of $1 \mu\text{M}$ CaM further increased the contraction in both cases but was more pronounced in the presence of RuR than in the presence of microcystin. (B) Cumulative data obtained from experiments similar to those shown in (A). The amplitude of maximal contraction at pCa 5.0 was taken as 100% in each preparation. Open and filled bars correspond to the protocols depicted in (Aa) and (Ab) respectively. 'Drug' refers to microcystin (open bars) and RuR (filled bars) (* $P < 0.05$, $n = 7$).

can therefore be concluded that the effect of exogenous CaM was significantly greater in RuR-treated than in microcystin-treated smooth-muscle strips.

Ca²⁺-independent contraction induced by microcystin and RuR

It has been reported that the application of microcystin induces a tonic contraction of permeabilized smooth muscle in Ca²⁺-free solution [38,39]. Figure 5(A) shows the effects of microcystin (Figure 5Aa) and RuR (Figure 5Ab) on the contractility of permeabilized ileal smooth muscle in R2G solution. The cumulative addition of microcystin or RuR induced tonic contractions in a concentration-dependent manner. Figure 5(B) summarizes

Figure 5 Microcystin- and RuR-induced Ca^{2+} -independent contraction

(A) Microcystin induced a concentration-dependent tonic contraction of permeabilized ileal smooth muscle in R2G solution (a). RuR also induced a concentration-dependent contraction (b). (B) Cumulative data showing the relationships between microcystin (○) or RuR (●) concentration and developed tension ($n = 5$). The maximum contraction at pCa 5.0 was taken as 100% in each preparation. EC_{50} values of 59.2 ± 9.4 nM and 843.1 ± 42.1 μ M were determined for microcystin and RuR respectively. The maximum contraction induced by RuR was significantly smaller than that due to microcystin (* $P < 0.05$).

Figure 6 Dual effect of RuR on microcystin-induced contraction

(A) The application of either 30 nM microcystin or 300 μ M RuR induced only a small or no contraction in R2G solution. The addition of 300 μ M RuR to 30 nM microcystin resulted in a substantial contraction (a). Cumulative data are shown in (b). The maximum contraction at pCa 5.0 was taken as 100% in each preparation. The relative amplitude of contraction in the presence of both 300 μ M RuR and 30 nM microcystin was significantly larger than those induced by each compound alone ($n = 5$, ** $P < 0.01$). (B) The tonic contraction induced by 1 μ M microcystin was markedly suppressed by the addition of 300 μ M RuR (a). Cumulative data are shown in (b). The relative amplitude of contraction in the presence of both 1 μ M microcystin and 300 μ M RuR was significantly smaller than that induced by microcystin alone ($n = 5$, ** $P < 0.01$).

Figure 7 Effect of microcystin on the activity of purified smooth-muscle MP in the absence and presence of RuR

The concentration-dependent inhibition of MP activity by microcystin in the absence (○) or presence of RuR (300 μ M) (●) was determined as shown in Figure 3, except that the reaction mixture without phosphorylated myosin was preincubated for 20 min at 25 °C before reactions were started. The IC_{50} for microcystin was approx. 0.2 nM. Circles on the ordinate indicate the activities in the absence of microcystin and in the absence and the presence of RuR. MP activity was abolished in the presence of 300 μ M RuR and was unaffected by the addition of microcystin. The results are means of duplicate measurements.

the concentration–response relationships of microcystin- and RuR-induced contractions. The amplitude of contraction in pCa 5.0 solution was taken as 100 % in each preparation. The EC_{50} of microcystin was approx. 10^{-4} that of RuR (59.2 ± 9.4 nM compared with 843.1 ± 42.1 μ M respectively; $n = 5$, $P < 0.01$). The maximum contraction induced by RuR was significantly smaller than that induced by microcystin (48.2 ± 8.2 % compared with 70.7 ± 5.1 % respectively of the initial pCa 5.0-induced contraction; $n = 5$, $P < 0.05$; Figure 5B).

Dual action of RuR on microcystin-induced contraction

Ca^{2+} -independent contractions shown in Figure 5 were hardly observed after the application of a low concentration of microcystin (less than 10 nM) or RuR (less than 300 μ M). A single application of 30 nM microcystin or 300 μ M RuR induced only a very small tonic contraction in Ca^{2+} -free solution (R2G, pCa > 8): 4.2 ± 3.1 % and 4.7 ± 2.8 % of the control contraction at pCa 5.0 ($n = 5$; Figure 6A). When 300 μ M RuR was added in the presence of 30 nM microcystin, however, substantial contraction was induced (25.1 ± 2.9 % of the control contraction at pCa 5.0; $n = 5$, $P < 0.01$ compared with microcystin or RuR alone; Figure 6A). We therefore conclude that microcystin and RuR act in a synergistic fashion with regard to MP inhibition.

In contrast, the same concentration of RuR (300 μ M) markedly inhibited the contraction induced by 1 μ M microcystin (from 86.0 ± 8.8 % to 22.7 ± 3.0 % of the control contraction at pCa 5.0; $n = 5$, $P < 0.01$; Figure 6B).

Effects of RuR on purified smooth-muscle MP activity in the presence of microcystin

The possibility that 300 μ M RuR might antagonize the inhibitory effect on MP of high concentrations of microcystin was examined (Figure 7). The activity of purified smooth-muscle MP was decreased by microcystin in a concentration-dependent manner with an IC_{50} of approx. 0.2 nM, as seen previously. MP was completely inhibited by 300 μ M RuR (see Figure 3) and did not decrease the inhibition due to 0.01–1 nM microcystin. Inhibition

of the phosphatase by microcystin was therefore not antagonized by a high concentration of RuR.

DISCUSSION

The present study demonstrates for the first time that RuR directly inhibits the activity of MP to enhance LC_{20} phosphorylation and increase the Ca^{2+} sensitivity of contraction in permeabilized smooth muscle. We previously showed that RuR induces Ca^{2+} sensitization [28]. In the present study we elucidated the mechanism whereby RuR enhances the Ca^{2+} sensitivity of contraction, which involves direct inhibition of MP.

The IC_{50} of RuR for the inhibition of purified MP was 23 μ M under the present experimental conditions, indicating that MP can be inhibited by RuR at slightly lower concentrations than those required for Ca^{2+} sensitization of permeabilized smooth muscle: EC_{50} values for the potentiation at pCa 6.0 in permeabilized muscle strips prepared from urinary bladder and ileal longitudinal layer were approx. 60 and 110 μ M respectively, and that at pCa 6.3 in mesenteric artery was approx. 50 μ M [28]. This difference between IC_{50} and EC_{50} values is due, at least in part, to the inhibitory effect of RuR on Ca^{2+} binding to CaM (K_i 9 μ M [27]). Exogenously added CaM markedly enhanced the potentiating effect of RuR on the contraction at pCa 6.3, whereas the enhancement by CaM of microcystin-induced potentiation was significantly smaller. Ca^{2+} sensitization induced by RuR is therefore partly attenuated by the inhibition of Ca^{2+} binding to CaM by RuR.

The results obtained with ATP[S] and ML-9 support the hypothesis that MP inhibition by RuR is the major mechanism underlying RuR-induced sensitization of permeabilized muscle strips to Ca^{2+} . It was clearly shown that the enhancement of contraction at pCa 6.3 by RuR was not observed when the permeabilized muscle strip had already been sensitized by MP thiophosphorylation. Of importance is the observation that the thiophosphorylation of MP was inhibited by the simultaneous presence of RuR and ATP[S]. This suggests that the binding of RuR to MP masks the site of (thio)phosphorylation. Alternatively, RuR might inhibit MP kinase in addition to MP itself. It has been reported that MP activity is down-regulated by phosphorylation via signal transduction pathways including the PKC-catalysed phosphorylation of CPI-17, a 17 kDa protein substrate of PKC that becomes a potent inhibitor of MP after phosphorylation [7], and the Rho-associated kinase-catalysed phosphorylation of MYPT [11]. In our previous study, RuR-induced Ca^{2+} sensitization was not inhibited by a PKC inhibitor peptide [28], suggesting that PKC did not contribute to the sensitization. It is therefore possible that RuR inhibits MYPT kinases such as Rho-associated kinase [40] or MP-associated MYPT kinase [41] via an unknown pathway and thereby protects MP from thiophosphorylation. Further experiments will be required to address this issue.

Smooth-muscle MP is composed of three subunits: the δ isoform of the catalytic subunit (37 kDa) of type 1 phosphatase (PP1c δ), MYPT (130 kDa), which targets the phosphatase to myosin, and a 20 kDa subunit of unknown function [42–44]. Three principal ways of inhibiting this phosphatase activity have been described: phosphorylation of MYPT lowers the V_{max} [14]; dissociation of PP1c δ from the phosphatase holoenzyme disrupts targeting [4]; and binding of an inhibitor to PP1c δ attenuates phosphatase activity [45]. RuR might act via the third mechanism because it was found to inhibit the activity of purified MP in the absence of $MgATP^{2-}$. The major mechanism underlying the Ca^{2+} sensitization by RuR might therefore be the direct inhibition of PP1c δ function; further direct evidence will be required to

support this conclusion. In addition, as has been speculated above, RuR conceivably prevents the phosphorylation of MP by kinases such as Rho-associated kinase.

Both microcystin and RuR induced Ca^{2+} -independent contraction in R2G solution ($\text{pCa} > 8$) with EC_{50} values of 59 nM and 843 μM respectively. These Ca^{2+} -independent contractions might be due to LC_{20} phosphorylation by a kinase distinct from MLCK [39,46]. The ratio of the IC_{50} of RuR for the inhibition of purified MP activity to the EC_{50} of RuR for the activation of Ca^{2+} -independent contraction was 37, whereas that for microcystin was approx. 300. This difference between IC_{50} for purified phosphatase and EC_{50} for the contraction of permeabilized muscle by microcystin has previously been reported [47] and might be common among inhibitors of smooth-muscle MP.

The addition of 300 μM to microcystin-induced Ca^{2+} -independent contraction in R2G solution exhibited a dual effect: the potentiation of contraction induced by low concentrations of microcystin (less than 10 nM) and the inhibition of contraction induced by high concentrations of microcystin (more than 300 nM). The synergistic effects of 30 nM microcystin and 300 μM RuR were consistent with the assumption that both agents inhibit MP. The activity of purified MP was totally blocked by 300 μM RuR, regardless of the further addition of 0.01–1 nM microcystin, suggesting that RuR might not compete directly with microcystin to inhibit MP. The mechanism for the marked decrease in 1 μM microcystin-induced contraction by RuR was not elucidated in this study.

It can be concluded that, in addition to several effects of RuR reported so far, RuR directly binds to and inhibits smooth-muscle MP. It is obvious that the inhibition of MP by RuR can occur in Ca^{2+} -free conditions and without interaction with other factors including Ca^{2+} -binding proteins. Although the phosphorylation of MP is not involved in the RuR-induced inhibition of purified MP, we cannot rule out the possibility that MP kinase could also be down-regulated by RuR in permeabilized smooth muscle. However, the inhibition of MP is likely to be the major mechanism underlying Ca^{2+} sensitization of permeabilized smooth-muscle strips by RuR. The inhibitory effect of RuR on MLCK by preventing Ca^{2+} binding to CaM seemed to be functionally masked because an increase in LC_{20} phosphorylation was observed.

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