Ca$^{2+}$ sensitization of smooth muscle contractility induced by ruthenium red

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1Department of Pharmacology and Therapeutics and 2Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya 467-8603, Japan; and 3Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Calgary, Calgary, Alberta T2N 4N1, Canada

Yamada, Aki, Susumu Ohya, Masaru Hirano, Minoru Watanabe, Michael P. Walsh, and Yuji Imaizumi. Ca$^{2+}$ sensitization of smooth muscle contractility induced by ruthenium red. Am. J. Physiol. 276 (Cell Physiol. 45): C566–C575, 1999.—The effects of ruthenium red (RuR) on contractility were examined in skinned fibers of guinea pig smooth muscles, where sarcoplasmic reticulum function was destroyed by treatment with A-23187. Contractions of skinned fibers of the urinary bladder were enhanced by RuR in a concentration-dependent manner (EC$\text{_{50}}$ = 60 µM at pCa 6.0). The magnitude of contraction at pCa 6.0 was increased to 320% of control by 100 µM RuR. Qualitatively, the same results were obtained in skinned fibers prepared from the ileal longitudinal smooth muscle layer and mesenteric artery. The maximal contraction induced by pCa 4.5 was not affected significantly by RuR. The enhanced contraction by RuR was not reversed by the addition of guanosine 5'-O-(2-thiodiphosphate) or a peptide inhibitor of protein kinase C [PKC (19–31)]. The application of microcystin, a potent protein phosphatase inhibitor, induced a tonic contraction of skinned smooth muscle at low Ca$^{2+}$ concentration ([Ca$^{2+}$]$\text{_{i}}$; pCa > 8.0). RuR had a dual effect on the microcystin-induced contraction-to-enhancement ratio at low concentrations and suppression at high concentrations. The relaxation following the decrease in [Ca$^{2+}$]$\text{_{i}}$ from pCa 5.0 to 8.0 was significantly slowed down by an addition of RuR. Phosphorylation of the myosin light chain at pCa 6.3 was significantly increased by RuR in skinned fibers of the guinea pig ileum. These results indicate that RuR markedly increases the Ca$^{2+}$ sensitivity of the contractile system, at least in part via inhibition of myosin light chain phosphatase.

The Ryanodine Receptor Ca$^{2+}$-release channel (RyR) of the sarcoplasmic reticulum (SR) plays an obligatory role in excitation-contraction coupling in skeletal and cardiac muscles (6). Three isoforms (RyR-1, RyR-2, and RyR-3) have been identified (for a review, see Ref. 30). RyR-1 and RyR-2 are predominantly expressed in skeletal and cardiac muscles, respectively, whereas RyR-3 is widely expressed in other cell types, including neurons and smooth muscles (23). It has been reported that all these isoforms are expressed in vascular smooth muscles, but the physiological functions of RyRs in smooth muscles have not been fully elucidated (31).

Dantrolene, a specific inhibitor of RyR-1, suppresses the twitch contraction in skeletal muscle and is an effective treatment for malignant hyperthermia but has little or no effect on smooth or cardiac muscles (24, 31). Ruthenium red (RuR), [(NH$_3$)$_3$Ru-O-Ru(NH$_3$)$_4$-O-Ru(NH$_3$)$_3$]Cl$_6$, blocks the SR Ca$^{2+}$-release channel (34) and has been widely used as an inhibitor of Ca$^{2+}$-induced Ca$^{2+}$ release (25). The ability of RuR to impair Ca$^{2+}$ release in neuronal cells has also been demonstrated (32). Because the inhibition of RyR-3 by RuR at relatively low concentrations (4, 9, 14) is similar to that of RyR-1 and RyR-2, the importance of RuR as a pharmacological tool to investigate the cellular functions of Ca$^{2+}$ release through RyRs in the SR and endoplasmic reticulum of smooth muscles and neurons, respectively, has been increasing (22, 30).

Several additional effects of RuR have been reported (3, 21). An important finding with respect to the use of RuR as a pharmacological tool in smooth muscle is its inhibition of the binding of Ca$^{2+}$ to calmodulin (26), which may cause inhibition of contraction. We report here that RuR markedly enhances the contraction induced by pCa 6.5–5.0 in skinned smooth muscle fibers of the guinea pig ileum. Our results are not consistent with the results of Sasaki et al. (26) that would predict that RuR causes a decrease in the Ca$^{2+}$ sensitivity of contraction. Ca$^{2+}$ sensitization of smooth muscle contraction is involved in enhanced responses to some agonists without an increase in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$\text{_{i}}$) (15, 27, 28). The present study was undertaken, therefore, to further investigate the effects of RuR on smooth muscle contractility and to the characterization of RuR-induced Ca$^{2+}$ sensitization in skinned smooth muscles.

METHODS

Tension measurement. Male Hartley guinea pigs, weighing 300–350 g, were stunned by a blow on the head and immediately exsanguinated. The urinary bladder, the terminal portion of the ileum, and the fourth branches of the mesenteric artery were isolated. Muscle strips (250 µm in width, 100 µm in depth, and 1.5–2 mm in length) were dissected from the urinary bladder and ileum. Ring preparations (300 µm in width) were dissected from the mesenteric artery. For tension measurement, 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 (33). To prevent the change in surface tension and ionic strength of the solution caused by evaporation, the muscle was transferred from pool to pool at an
interval of ~10 min. The transfer occasionally resulted in a spike-like artifact in a trace. 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 ~60 min before the start of experiments. The preparation was then repeatedly exposed to 142.9 mM KCl solution at intervals of ~30 min until the contraction was reproducible. Experiments were carried out at room temperature (21–24°C).

Skinned-fiber preparation. After the steady contractions induced by the 142.9 mM KCl solution were measured, the strips were incubated in relaxing solution containing 2 mM EGTA (R2G) for 15 min. The skinnings of the smooth muscle preparations were achieved by incubation with 60 µM β-escin in a solution of pCa 6.3 at room temperature. After the skinnings, the solution was changed to R2G. The pCa in R2G solution was <8.0, assuming that the Ca²⁺ contamination of the solution was <50 µM. The contractile response to caffeine was tested in relaxation solution containing 0.1 mM EGTA (R0.1G). When the Ca²⁺ sensitivity of the contractile response was studied in detail, it was found that the function of intracellular Ca²⁺ storage sites was removed by treatment with 10 µM A-23187 for 20 min in R2G solution after skinnings.

Measurement of myosin light-chain phosphorylation. Tissue was frozen in acetone-5% (wt/vol) TCA on dry ice for 30 min, transferred to 100% acetone, incubated for 30 min at room temperature, washed repeatedly with acetone, dried, and incubated in an extraction buffer (20 µl/mg tissue dry wt) for 2 h on a shaker before centrifugation at 6,400 rpm for 10 min at room temperature. The supernatant was subjected to urea-glycerol gel electrophoresis. Myosin from chicken gizzards was used as the control. Electrophoretic transfer of proteins to nitrocellulose sheets was carried out in 25 mM Tris-192 mM glycine-20% methanol, pH 8.3, at 200 mA for 4 h. Anti-myosin light-chain antibody [PKC-(19–31); Seikagaku], microcystin-LR (Research Biochemicals International), and ML-9 (Sigma). The ML20 antibody was prepared in Dr. M. P. Walsh's laboratory. Myosin from chicken gizzards was obtained from Sigma. The purity of RuR from Sigma was >40%, and the purity of that from Wako was >95%. There was no marked difference between the potentiating effects on contraction in skinned fibers of RuR from these two sources. All the data shown in RESULTS were obtained with RuR from Wako.

RESULTS

Opposite effects of RuR on intact and skinned smooth muscle fibers. Figure 1 shows the effects of 100 µM RuR on contractions induced by a high-K⁺ solution in an intact strip (A) and by a pCa 6.0 solution in a skinned strip (B) of the guinea pig urinary bladder. In intact muscle, a phasic contraction and a subsequent tonic contraction were induced by 142.9 mM KCl. The second trial with 142.9 mM K⁺ solution was performed in the presence of 100 µM RuR added 5 min before the high-K⁺ solution. Both phasic and tonic contractions were markedly reduced by the application of 100 µM RuR. The third trial with the high-K⁺ solution after washout of RuR showed that the inhibition by RuR was mostly removed. In a preparation permeabilized with β-escin and treated with A-23187, the tonic contraction elicited by the pCa 6.0 solution was markedly increased by the addition of 100 µM RuR (Fig. 1B). This effect was also reversed on washout.

Table 1. Solutions used for skinned-fiber experiments

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Chemicals Industries, lot LEN0492), ATP disodium salt (Oriental, creatine phosphate disodium salt (Wako Pure Chem., Indust.), EGTA and PIPES (Daijindo), protein kinase C inhibitor peptide [PKC-(19–31); Seikagaku], microcystin-LR (Research Biochemicals International), and ML-9 (Sigma). The ML20 antibody was prepared in Dr. M. P. Walsh's laboratory. Myosin from chicken gizzards was obtained from Sigma. The purity of RuR from Sigma was >40%, and the purity of that from Wako was >95%. There was no marked difference between the potentiating effects on contraction in skinned fibers of RuR from these two sources. All the data shown in RESULTS were obtained with RuR from Wako.
induced by 142.9 mM K\(^+\) solution before skinning and 22.9 ± 9.6% of that in the presence of 60 µM RuR (P > 0.05). Therefore, the Ca\(^{2+}\) sensitivity of skinned fibers was increased by 60 µM RuR, whereas the maximal response was not affected significantly. To confirm this, 100 µM RuR was added when muscles were contracted in pCa 6.0 or 4.5 solution (Fig. 3A). The contraction in pCa 6.0 solution was increased to 286.6 ± 22.6% (n = 4) of control (P < 0.01) and that in pCa 4.5 solution was not affected significantly (99.9 ± 0.1% of control, n = 4, P > 0.05) by the addition of 100 µM RuR (Fig. 3B).

Concentration-dependent effects of RuR. The contraction of skinned fibers of the guinea pig urinary bladder in pCa 6.0 solution was increased in a concentration-dependent manner by the cumulative addition of 30–300 µM RuR (Fig. 4A). The effects of 30–100 µM RuR were removed by washout, but those of 300 or 600 µM RuR were not removed completely, even after a 30-min washout in R2G. Experiments were always performed in a paired fashion in the presence (Fig. 4Aa) and absence (Fig. 4Ab) of RuR, because the effect of RuR developed slowly. In the time-matched control, the relative amplitude of contraction in pCa 6.0 just before the solution change back to R2G was 139.5 ± 22.6% of that measured 10 min after the transfer to pCa 6.0 solution (n = 4; P < 0.05). Figure 4B shows the relationship between RuR concentration and tension in the pCa 6.0 solution. The relative magnitude of contraction in pCa 6.0 solution in the presence of RuR was corrected for the time-matched control in each pair of preparations. The contraction in pCa 6.0 solution was enhanced by RuR in a concentration-dependent manner in the range of 10–600 µM. Maximal force was 324.9 ± 55.8% (n = 6, P < 0.01) of control, with half-maximal effect at 60 µM RuR.

Effect of RuR on skinned fibers of ileum and mesenteric artery. Figure 5A shows the relationship between pCa and the relative amplitude of contraction in skinned fibers of the urinary bladder, ileal longitudinal smooth muscle layer, and mesenteric artery of the guinea pig. The Ca\(^{2+}\) sensitivity of the mesenteric artery was enhanced by RuR in a concentration-dependent manner in the range of 10–600 µM. Maximal force was 324.9 ± 55.8% (n = 6, P < 0.01) of control, with half-maximal effect at 60 µM RuR.
higher than those of the urinary bladder and ileum. In pCa 6.0 solution, contractions of skinned fibers of the mesenteric artery, ileum, and urinary bladder were 88.4 ± 2.0 (n = 5), 53.1 ± 5.4 (n = 5, P < 0.01 vs. the mesenteric artery and urinary bladder), and 18.2 ± 3.2% (n = 6, P < 0.01 vs. the mesenteric artery) of the maximal contraction. Figure 5, B and C, shows the relationship between RuR concentration and the relative magnitude of contraction for the ileum (B) and mesenteric artery (C) when RuR was added cumulatively in pCa 6.0 and 6.3 solutions, respectively. The dotted lines indicate the relationship for the urinary bladder at pCa 6.0 (see Fig. 4B). Although 300 µM RuR markedly enhanced the contraction in the ileum (222.2 ± 46.3% of control; n = 6, P < 0.05), this enhancement was smaller than that for the urinary bladder (324.9 ± 55.8% of control; n = 5, P < 0.05). Moreover, in the mesenteric artery, the contraction in pCa 6.0 solution was 88% of the maximum contraction at pCa 4.5 and the enhancement by 300 µM RuR was not significant (113.3 ± 5.8% of that before application of RuR; n = 3). The contraction at pCa 6.3 in this preparation was, however, markedly enhanced by 300 µM RuR (Fig. 5C) (461.8 ± 117.5% of control; n = 6, P < 0.05). These results again suggest that RuR increases the Ca²⁺ sensitivity of the contractile system without affecting the maximal response.

Effect of GDPβS on RuR-induced enhancement of contraction. It is widely accepted that smooth muscle contraction by agonist stimulation is mainly due to an increase in [Ca²⁺]i and Ca²⁺ sensitization of contractile elements (29), although additional Ca²⁺-independent mechanisms are involved (1, 15). It has been reported that guanine nucleotide-binding proteins (G proteins) are involved in the agonist-induced increase in the Ca²⁺ sensitivity of MLC²₀ phosphorylation and contraction of smooth muscle (7, 18). This GTP-mediated Ca²⁺ sensitization is inhibited by nonhydrolyzable GDP analogue guanosine 5’-O-(2-thiodiphosphate) (GDPβS). The contraction of skinned guinea pig ileum strips enhanced by 100 µM ACh was reduced by the addition of 10 and 300 µM GDPβS (Fig. 6Aa). On the other hand, the enhancement of contraction by 100 µM RuR was also reduced (100 µM RuR + 10 and 300 µM GDPβS) (Fig. 6Ab). The enhancement of contraction by 300 µM RuR was, however, not significantly different from that of 300 µM RuR alone (Fig. 6Ac). These results suggest that GDPβS inhibits the RuR-induced increase in Ca²⁺ sensitivity of the contractile system.
Ruthenium Red (RuR) markedly enhanced the contraction of skinned smooth muscle of urinary bladder, ileum, and mesenteric artery. Submaximal contraction at pCa 6.3 (14.1 ± 2.6% of maximum) was significantly enhanced by cumulative addition of 10–600 µM RuR. The enhanced contraction was abolished by PKC-(19—31), a peptide inhibitor of PKC. To investigate the involvement of PKC in the Ca2+ sensitivity of skinned smooth muscle, the effects of PKC-(19—31) on PDBu- and RuR-induced contractions were studied (Fig. 7Aa). The enhanced contraction by 300 µM RuR was unaffected. Figure 7B shows the summarized results of the effects of PKC-(19—31) on PDBu- and RuR-induced contractions. The application of 3–30 µM PKC-(19—31) decreased the PDBu-induced enhancement of contraction in a concentration-dependent manner (n = 5, P < 0.05 vs. control at 10 µM and P < 0.01 vs. control at 30 µM) but did not affect the RuR-induced enhancement of contraction (n = 4, P > 0.05 vs. control).

The inhibition of MLC20 phosphatase is a possible mechanism of Ca2+ sensitization (28). The application of microcystin-LR, a potent protein phosphatase inhibitor (11), induced a concentration-dependent tonic contraction of permeabilized guinea pig ileum strips in R2G solution (Fig. 8Aa). The relationship between the concentration of microcystin and contraction was significantly inhibited by 300 µM RuR (n = 4, 70.3% of control). No. of samples (n) was 6 except for experiment with 600 µM RuR, for which n = 3. C: concentration-response relationship of RuR-induced enhancement of contraction of skinned ileal longitudinal smooth muscle. RuR was added cumulatively to fibers submaximally contracted at pCa 6.0 (53.1 ± 5.4% of maximum). No. of samples (n) was 6 except for experiment with 600 µM RuR (n = 6). Dotted lines (B and C), relationship for urinary bladder at pCa 6.0 (see Fig. 4). *P < 0.05 vs. control.
tion in the presence of 300 µM RuR was examined (Fig. 8A). RuR exhibited a dual action on microcystin-induced contraction: 300 µM RuR significantly enhanced the effect of microcystin at low concentrations (30 and 50 nM) and significantly reduced that at high concentrations (1 and 3 µM). The contraction induced by 30 nM microcystin was significantly increased in the presence of 300 µM RuR (from 2.7 ± 1.0 to 10.2 ± 3.2% (n = 6, P < 0.05, **P < 0.01 vs. control).

Effect of RuR on the relaxation rate. The rate of relaxation following the decrease in [Ca²⁺] from pCa 5.0 to nominally free (R2G solution; pCa 8.0) was measured (Fig. 9A). To prevent MLC phosphorylation, 100 µM ML-9, an inhibitor of MLCK, was added to the solution. Under these conditions, the relaxation mainly depends on the dephosphorylation rate of phosphorylated MLC by phosphatase (28, 33). The relaxation phase was well described by a function of single exponential with a time constant (τ) of 15.8 ± 2.7 s. Although the peak amplitude of the contraction in the pCa 5.0 solution in the second trial decreased to 60.1 ± 4.4% of that of the first one (n = 6), the rate of relaxation did not change (τ = 15.7 ± 2.5 s, n = 6, P > 0.05 vs. the results of the 1st trial). The effect of 100 µM RuR on the relaxation phase was examined in the second trial (Fig. 9A). In the presence of RuR, the

![Diagram](image-url)

Fig. 7. Effects of protein kinase C (PKC) inhibitor peptide PKC-(19–31) on phorbol ester- and RuR-induced Ca²⁺ sensitization. A: In skinned smooth muscle strips of ileum, 3 µM phorbol 12,13-dibutyrate (PDBu) (a) or 100 µM RuR (b) enhanced contraction in pCa 6.3 solution. After contraction reached a steady level, 3–30 µM PKC-(19–31) was added. B: Summarized results obtained from experiments like those shown in A. Magnitude of tonic contraction in pCa 6.3 solution was taken as 100% in each preparation. Open and solid bars, effects of PKC inhibitor on PDBu-induced (n = 5) and RuR-induced (n = 4) enhancement of contraction, respectively. *P < 0.05, **P < 0.01 vs. control.

Fig. 8. Dual action of RuR on contraction induced by microcystin-LR. A: Application of microcystin-LR induced a concentration-dependent contraction in skinned strips of guinea pig ileum in R2G solution (pCa > 8.0) (a). In presence of 300 µM RuR, minimum concentration of microcystin ([microcystin]) required to induce a contraction was decreased and maximal contraction was decreased (b). B: Concentration-response relationships of microcystin-induced contraction in absence of RuR (○) and in presence of 300 µM RuR (●). Maximal contraction induced by pCa 5.0 or 4.5 was taken as 100% in each preparation. Contractions induced by 30 and 50 nM microcystin were significantly potentiated by RuR. Those induced by 1 and 3 µM microcystin were significantly reduced by RuR. EC₅₀ of microcystin was significantly changed by presence of 300 µM RuR, from 84 to 44 nM (n = 6; *P < 0.05).
Fig. 9. Effects of RuR on relaxation after decrease in Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]). A: contractions were induced by pCa 5.0 solution twice. The relaxation phase after decrease in [Ca\(^{2+}\)] from pCa 5.0 to nominally free (R2G solution, pCa > 8.0) was recorded on a time scale 10 times faster than normal scale. R2G solution for relaxation included 100 µM ML-9 to prevent myosin light-chain (MLC) phosphorylation. To examine effects of RuR on relaxation, 100 µM RuR was applied 45 min before 2nd trial at a pCa of 5.0 and was continuously included during following relaxation (b). B: time course of relaxation in absence (○) or presence (■) of RuR in 2nd trial. Amplitude of contraction just before decrease in [Ca\(^{2+}\)] in 2nd trial was taken as 100% in each preparation. [Ca\(^{2+}\)] was decreased at time 0. Tension in presence of RuR was significantly larger than that in control until 100 s (n = 6). Data in B were well described by a single-exponential function. Values of time constant for fitted lines are 16 and 27 s in absence of RuR and in presence of 100 µM RuR, respectively. *P < 0.05, **P < 0.01 vs. control. C: relaxation phase was well described by a single-exponential function in 1st and 2nd trials and also in presence of RuR as shown in B. Time constant (τ) in presence of 100 µM RuR (solid bar) in 2nd trial was significantly larger (**P < 0.01) than that in 2nd trials in absence of RuR (open bar; n = 6).

Effect of RuR on MLC\(_{20}\) phosphorylation. Figure 10A shows the detection of unphosphorylated and phosphorylated MLC\(_{20}\) bands with the MLC\(_{20}\) antibody after separation by urea-glycerol gel electrophoresis. When skinned ileum strips were exposed to the pCa 6.3 solution, the extent of phosphorylation of MLC\(_{20}\) was 20.7 ± 1.9% (n = 6; Fig. 10B). The addition of 300 µM RuR significantly increased the level of phosphorylation to 28.1 ± 1.5% (n = 6, P < 0.05 vs. the results in the absence of RuR; Fig. 10B). MLC\(_{20}\) phosphorylation in intact strips, which were extensively contracted in 142.9 mM K\(^{+}\) solution, was 37.7 ± 3.0% (n = 8, P < 0.01 vs. the phosphorylation in pCa 6.3 solution). These results indicate that RuR significantly increased MLC\(_{20}\) phosphorylation along with the enhancement of contraction. The relationship between relative tension and MLC\(_{20}\) phosphorylation was obtained from skinned fibers in the absence of RuR and in the presence of 300 µM RuR (Fig. 10C). The increase in [Ca\(^{2+}\)] over the range pCa 6.3–4.5 increased both relative tension and MLC\(_{20}\) phosphorylation. The increase in the relative tension produced by 300 µM RuR was also coupled with the enhancement of MLC\(_{20}\) phosphorylation. It is, however, also noteworthy that the phosphorylation level at pCa 6.3 in the presence of 300 µM RuR (28%) tends to be smaller than those at pCa values of 5.5 (31.6 ± 3.0%, n = 4; P > 0.05 vs. 28%) and 4.5 (35.6 ± 3.5%, n = 4; P > 0.05 vs. 28%) in the absence of RuR, whereas the corresponding relative tensions are comparable.

DISCUSSION

The present results clearly show that RuR increases the Ca\(^{2+}\) sensitivity of the contractile system in skinned smooth muscle preparations. This observation was unexpected in light of the report that RuR suppresses Ca\(^{2+}\) binding to calmodulin (26). Although the reduction of the high-K\(^{+}\)-induced tonic contraction by RuR (Fig. 1) confirmed the previous observation (26), our interpretation of the cause of the reduction is different. Sasaki et al. (26) suggested that RuR enters the
RuR inhibits the voltage-dependent Ca²⁺ current. Electrophysiological experiments using whole cell voltage-clamp techniques, however, indicate clearly that the reduction in urinary bladder myocytes with an IC₅₀ of ~3 µM is attributable to the blocking of Ca²⁺ entry through voltage-dependent Ca²⁺ channels.

The direct effects of RuR on the contractile system can be clarified only by use of skinned smooth muscle fibers. The concentration of RuR required for the increase in Ca²⁺ sensitivity in skinned fibers (>30 µM) was higher than that required for interaction with Ca²⁺-binding proteins |calmodulin, Ca²⁺-ATPase, the ryanodine receptor, and the ß-myoinositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] receptor [~10 µM] or for the inhibition of Ca²⁺ current (IC₅₀ ~3 µM). This would suggest that the site of action of RuR is not a Ca²⁺-binding protein.

RuR blocks Ca²⁺-release channels in the skeletal muscle SR membrane and thereby reduces Ca²⁺ release through the channel (19, 34). It has been reported that RuR inhibits smooth muscle Ca²⁺-release channels reconstituted into planar lipid bilayers (9) and caffeine-induced contraction of skinned smooth muscle strips (14). Ca²⁺ release via Ins(1,4,5)P₃ receptors is also inhibited by RuR, presumably because RuR competes with Ca²⁺ at the binding sites. It has also been reported that SR Ca²⁺ pump activity is reduced by RuR. The RuR-induced enhancement of contraction is, however, not due to changes in SR Ca²⁺ uptake and/or release, because it was observed in skinned fibers in which SR Ca²⁺ storage and release functions were destroyed by A-23187.

The enhancement of contraction by RuR was observed only at relatively low [Ca²⁺] values (pCa 6.0 and 5.5). The maximal contraction at pCa 4.5 was not affected by RuR, strongly suggesting that RuR increases the Ca²⁺ sensitivity of the contractile system without a change in the maximal interaction between actin and myosin. It is also noteworthy that a significant enhancement of contraction by RuR in the mesenteric artery was observed at pCa 6.3 but not at pCa 6.0, because the contraction at pCa 6.0 was 88.4% of the maximum and further significant enhancement was not available. The possibility that 30–300 µM RuR changes pCa in the solutions via direct interaction with 5 mM EGTA seems to be low. It is clear, therefore, that RuR increases the Ca²⁺ sensitivity of the contractile system but does not change the maximal contractile ability in both phasic and tonic smooth muscles: the urinary bladder, ileal longitudinal layer, and mesenteric artery. A smaller but significant increase in the Ca²⁺ sensitivity of the contractile system produced by RuR in cardiac muscle has been reported, but the mechanism remains unknown (37).

An increase in the Ca²⁺ sensitivity (Ca²⁺ sensitization) of the contractile system elicited by the addition of GTP and ACh was inhibited by GDPβS, as previously reported (17). The involvement of the small GTPase, Rho, in the Ca²⁺ sensitization of smooth muscle contraction has been shown (2, 7, 16, 18). On the other hand, Ca²⁺ sensitization by RuR was not affected by GDPβS, suggesting that the activation of a small GTPase is not involved in the mechanism of RuR-induced Ca²⁺ sensitization.

The activation of PKC by phorbol ester induces or enhances the tonic contraction of intact and permeabilized smooth muscle preparations via the inhibition of MLC₂₀ phosphatase (20) and also via the phosphorylation of MLC₂₀ binding to calmodulin.

**Fig. 10. Effects of RuR on MLC₂₀ phosphorylation in pCa 6.3 solution.**

- **A:** unphosphorylated MLC₂₀ and phosphorylated MLC₂₀ (P-MLC₂₀) extracted from guinea pig ileum strips were separated by urea-glycerol-polyacrylamide gel electrophoresis and detected by immunoblotting. Lane 1: standard myosin from chicken gizzard; lane 2: pCa 6.3; lane 3: pCa 6.3 + 300 µM RuR; lane 4: pCa 4.5; lane 5: pCa 5.5; lane 6: 142.9 mM K⁺. Lanes 2-5 were obtained from skinned strips, and lane 6 was from intact strip.

- **B:** summarized results from several experiments like that shown in A. In presence of 300 µM RuR, MLC₂₀ phosphorylation in pCa 6.3 solution was significantly increased (from 20.7 ± 1.9 to 28.1 ± 1.5%, n = 6; *P < 0.05). Level of phosphorylation of MLC₂₀ in intact ileum strips in high-K⁺ solution was 37.6 ± 3.0% (n = 8; **P < 0.01 vs. skinneed fiber in pCa 6.3 solution).

- **C:** relationship between relative tension and MLC₂₀ phosphorylation. Tension in pCa 6.3 solution was taken as 1.0 in each preparation. Symbols: ○ and ☐, results obtained at pCa 6.3 in absence of RuR and in presence of 300 µM RuR, respectively; △ and □, results obtained at pCa 5.5 and 4.5, respectively, in absence of RuR.
tion of actin-binding protein calponin through a pathway independent of MLC$_{20}$ phosphorylation (13, 35, 36). Although the mechanisms of agonist-induced Ca$^{2+}$ sensitization have not been fully clarified, the inhibition of MLC$_{20}$ phosphatase by PKC is implicated as one of the major components (29). In the present study, however, the addition of 3–30 μM PKC-(19–31), a selective peptide inhibitor of PKC, did not reduce the Ca$^{2+}$ sensitization induced by 100 μM RuR, whereas it did inhibit PDBu-induced Ca$^{2+}$ sensitization. The activation of PKC, therefore, is not the major cause of RuR-induced Ca$^{2+}$ sensitization.

MLC$_{20}$ phosphorylation was significantly increased in skinned fibers treated with RuR at submaximal [Ca$^{2+}$]. This could result from the activation of MLCK or the inhibition of MLC$_{20}$ phosphatase. The latter is more likely because RuR has actually been reported to reduce MLCK activity via inhibition of Ca$^{2+}$ binding to calmodulin (26). Moreover, the concentration-response relationship of Ca$^{2+}$-independent, microcystin-induced contraction was affected significantly, suggesting that RuR may affect MLC$_{20}$ phosphatase: the addition of RuR reduced the EC$_{50}$ of microcystin for contraction from 84 to 44 nM and, interestingly, reduced the maximum response to microcystin. RuR may have a lower potency of phosphatase inhibition than microcystin and could compete with microcystin for inhibition of the phosphatase. Of potential relevance here is the observation that high concentrations of RuR (>1 mM) caused contraction in the absence of Ca$^{2+}$ and microcystin (data not shown). The relaxation following the change in [Ca$^{2+}$] from pCa 5.0 to nominally free (R2G; pCa > 8.0) was significantly slowed down by the treatment with RuR, under conditions in which MLC$_{20}$ phosphorylation was blocked by ML-9. These results strongly suggest that RuR reduces MLC$_{20}$ phosphatase activity to increase Ca$^{2+}$ sensitivity in these smooth muscles.

Although the increase in Ca$^{2+}$ sensitivity by RuR is mainly due to the increase in MLC$_{20}$ phosphorylation (Fig. 10), the extent of the increase in MLC$_{20}$ phosphorylation may not completely explain the enhancement of the relative contraction. An additional mechanism for RuR-induced enhancement of contraction may be via an increase in actomyosin ATPase activity through an actin-linked pathway that was not examined in the present study. For example, if RuR directly enhances the activity of calponin or inhibits calponin phosphatase, the result could be a decrease in the calponin-mediated inhibition of actin-activated myosin MgATPase activity (36).

In conclusion, RuR has a novel Ca$^{2+}$ sensitization effect on the contractile apparatus of smooth muscle. This is the first report of such a sensitization effect. The effect is not mediated by the activation of a G protein or PKC. Results rather indicate that RuR inhibits MLC$_{20}$ phosphatase. Although the concentration of RuR required for Ca$^{2+}$ sensitization is higher than that for inhibition of the RyR, these findings are important because of the frequent use of RuR as a pharmacological tool.

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REFERENCES

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