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

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$_{50}$ = 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$^{2+}$]: 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$^{2+}$] 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.

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. 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$^{2+}$]] (15, 27, 28). The present study was undertaken, therefore, to further investigate the effects of RuR on smooth muscle contractility and led 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 spikelike 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 K 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 K solution were measured, the strips were incubated in relaxing solution containing 2 mM EGTA (R2G) for 15 min. The skinning of the smooth muscle preparations was achieved by incubation with 60 µM β-scn in a solution of pCa 6.3 at room temperature. After the skining, 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 skinnering.

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) over night with blocking reagent, then with anti-myosin light chain (20 kDa; MLC20) antibody in 0.5% blocking solution. After further washing, immunoreaction was visualized with FUJIFILM immuno-enzymatic analyzer LAS-1000. Each band was scanned, and the peaks of integrated density were quantitated with FUJI-FILM bioimaging analyzer Mac BAS, version 2.5.

Table 1. Solutions used for skinned-fiber experiments

<table>
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<tr>
<th>Solution</th>
<th>ATP</th>
<th>Mg(Ms)₂</th>
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<td>5.27</td>
<td>0</td>
<td>84.73</td>
<td>2.0</td>
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<tr>
<td>R0.1G</td>
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<td>5.20</td>
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<td>90.48</td>
<td>0.1</td>
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<tr>
<td>pCa 7.0</td>
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<td>1.00</td>
<td>73.73</td>
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<td>4.45</td>
<td>66.96</td>
<td>5.0</td>
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<td>pCa 5.0</td>
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<td>5.04</td>
<td>65.60</td>
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The composition of the standard HEPES buffered solution was (in mM) 137.0 NaCl, 5.9 KCl, 1.2 CaCl₂, 14.0 glucose, and 10.0 HEPES. The pH values of all HEPES-buffered solutions were adjusted to 7.4 with NaOH. A high-K⁺ solution was prepared by replacing NaCl with equimolar KCl. A nominally Ca²⁺-free solution was prepared by replacing Ca²⁺ with 0.5 mM EGTA. The solutions used for skinned-fiber experiments are listed in Table 1 and were prepared as described previously (33). The apparent stability constant of Ca₄-EGTA at 23°C and pH 7.0 was 10⁶.36 M⁻¹, and the method for calculating pCa (computer program SP 6802) was that of Horiuchi (12). The ionic strength of the solution was maintained at 0.2 M by adjusting the concentration of potassium methanesulfonate. The pH was adjusted to 7.0 with KOH at 23°C. The composition of the buffer for MLC extraction was 6 M urea, 20 mM Tris, 22 mM glycine, 10 mM dithiothreitol, 0.04% bromphenol blue, 10 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonfluoride, and 0.6 M KI.

Statistics. Data are expressed as means ± SE. Statistical significance was examined by using the paired Student’s t-test.

Drugs. The following compounds were obtained (sources are in parentheses): RuR (Sigma, lot 126H1196; Wako Pure Chemical Industries, lot LEN0492), ATP disodium salt (Oriental), creatine phosphate disodium salt (Wako Pure Chem. Indust.), EGTA and PIPES (Dojindo), protein kinase C inhibitor peptide [PKC (19–31); Sekagaku], microcystin-LR (Research Biochemicals International), and ML-9 (Sigma). The ML-9 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 K⁺. 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-scn 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.

Increase in Ca²⁺ sensitivity caused by RuR. Figure 2A shows the effects of 60 µM RuR on contractions induced by a cumulative increase in [Ca²⁺] in skinned fibers of the guinea pig urinary bladder. In the presence of RuR, the contractions induced by pCa 6.0 and 5.5 solutions were larger than those in the control. Figure 2B shows the relationship between pCa and relative tension in the absence of RuR and in the presence of 60 µM RuR. The [Ca²⁺] giving half-maximal contraction was decreased from 2.14 ± 0.26 (n = 5) to 1.50 ± 0.19 µM (n = 5) by the application of 60 µM RuR (P < 0.05). The relative magnitudes of the maximal responses to pCa 4.5 in the absence of RuR were 26.9 ± 14.1% of that
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 Ca²⁺ 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 Ca²⁺ sensitivity of the mesenteric artery was...
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 Ca\(^{2+}\) sensitivity of the contractile system without affecting the maximal response.

Effect of GDP\(_b\)S on RuR-induced enhancement of contraction. It is widely accepted that smooth muscle contraction by agonist stimulation is mainly due to an increase in [Ca\(^{2+}\)] and Ca\(^{2+}\)-sensitization of contractile elements (29), although additional Ca\(^{2+}\)-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 Ca\(^{2+}\)-sensitivity of MLC\(_{20}\) phosphorylation and contraction of smooth muscle (7, 18). This GTP-mediated Ca\(^{2+}\)-sensitization is inhibited by nonhydrolyzable GDP analogue guanosine 5'-O-(2-thiodiphosphate) (GDP\(_b\)S). The contraction of skinned guinea pig ileum strips enhanced by 100 µM ACh was reduced by the addition of 10 and 300 µM GDP\(_b\)S (Fig. 6Aa). On the other hand, the enhancement of contraction by 100 µM RuR (n = 5; not shown) or 300 µM

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**Ruthenium Red and Ca\(^{2+}\) Sensitivity in Smooth Muscles**

Fig. 3. Effects of RuR on Ca\(^{2+}\) sensitivity and maximal response. A: effects of 100 µM RuR on a submaximal contraction at pCa 6.0 (a) and a maximal contraction at pCa 4.5 (b) in skinned urinary bladder smooth muscles. B: summarized results obtained from experiments like those shown in A. The amplitude of tonic contraction in absence of RuR was taken as 100% in each trial. The contraction in pCa 6.0 solution was increased to 286.6 ± 22.6% of control (n = 4; \(* P < 0.01\)) and that in pCa 4.5 solution was not affected (99.9 ± 0.1% of control, n = 4) by addition of 100 µM RuR.

Fig. 4. Concentration dependence of RuR-induced enhancement of contraction of skinned urinary bladder smooth muscle. A: original traces of contraction during cumulative addition of RuR in pCa 6.0 solution. Experiments were performed in a paired fashion. A set of 2 skinned fibers was prepared, and solutions were simultaneously changed in presence (a) or absence (b) of RuR. B: summarized results of concentration-response relationship of RuR-induced enhancement. No. of samples (n) was 5 except for experiments with 10 and 600 µM RuR, for which n = 3. Amplitude of tonic contraction in absence of RuR was taken as 100% in each trial. Response in presence of RuR was corrected for time-matched control in each pair. \(* P < 0.05\), \(** P < 0.01\) vs. control.

Fig. 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 Ca\(^{2+}\) sensitivity of the contractile system without affecting the maximal response.
was markedly enhanced by cumulative addition of 10–600 µM RuR in skinned mesenteric artery. Submaximal contraction at pCa 6.3 (14.1% of maximum) was abolished by PKC-(19—31), a peptide activator of PKC, strongly enhanced the contraction at pCa 6.0 (53.1 ± 5.4% of maximum). No. of samples (n) was 6 except for experiment with 600 µM RuR, for which n = 3. B: 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). Dotted lines (B and C), relationship for urinary bladder at pCa 6.0 (see Fig. 4).* P < 0.05 vs. control.

(n = 4) RuR was not affected by the addition of GDPβS (Fig. 6A). Figure 6B shows the summarized results. The magnitude of the enhancement of contraction by 100 µM ACh was smaller than that by 300 µM RuR (to 209.1 ± 26.7% vs. 387.8 ± 70.3% of control, respectively; n = 4). The ACh-induced enhancement of contraction was significantly reduced by 300 µM GDPβS, whereas the enhancement by 300 µM RuR was unaffected.

Effect of PKC inhibitor on RuR-induced enhancement of contraction. The activation of PKC induces contraction in intact and permeabilized smooth muscle preparations via an increase in Ca2+ sensitivity and/or a Ca2+-independent pathway (5, 29, 36). The application of 3 µM phorbol 12,13-dibutyrate (PDBu), a potent activator of PKC, strongly enhanced the contraction at pCa 6.3 in skinned smooth muscle strips of the ileal longitudinal layer (Fig. 7Aa). The PDBu-induced enhancement was abolished by PKC-(19—31), a peptide inhibitor of PKC. To investigate the involvement of PKC in Ca2+ sensitization caused by RuR, the effect of PKC-(19—31) was studied (Fig. 7Ab). The enhanced contraction was not affected significantly by PKC-(19—31). 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).

Effects of microcystin-LR on RuR-induced contraction. Contraction of smooth muscle is triggered by Ca2+ binding to calmodulin, which activates MLC kinase (MLCK). Activated MLCK phosphorylates Ser-19 of MLC20, resulting in activation of the myosin MgATPase by actin (8). The principal mechanism of relaxation involves dephosphorylation of MLC20 by MLC phosphatase. Therefore, 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...
tion in the presence of 300 µM RuR was examined. 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 ± 3.3%). The concentration of microcystin required for half-maximal effect were 84 and 44 nM (n = 6, P < 0.05) in the absence of RuR and in the presence of 300 µM RuR, respectively. The minimum concentration of microcystin required to induce a detectable contraction was also decreased. The maximum contraction obtained at 1 µM microcystin was significantly decreased from 42.4 ± 4.6 to 27.4 ± 4.2% (n = 6, P < 0.05). RuR also decreased the maximum contraction obtained at 1 µM microcystin.

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. 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. 9Ab). In the presence of RuR, the relaxation phase was delayed as indicated by the increase in the time constant (Fig. 9Ab). The relaxation phase was also delayed in the presence of 300 µM RuR (Fig. 9Ab). The relaxation phase was also delayed in the presence of 300 µM RuR (Fig. 9Ab).
contraction at pCa 5.0 slightly but significantly increased (67.4 ± 4.5% of that in the 1st trial; P < 0.05 vs. the 60.1 ± 4.4% measured in the 2nd trial in the absence of RuR; Fig. 9Aa; n = 6). The relaxation phase in the presence of 100 µM RuR was slower than that of the control (Fig. 9Ab) and was also described by a single-exponential function (t = 27.3 ± 3.7 s; P < 0.01 vs. the results of the 2nd trial in the absence of RuR; Fig. 9C).

Effect of RuR on MLC20 phosphorylation. Figure 10A shows the detection of unphosphorylated and phosphorylated MLC20 bands with the MLC20 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 MLC20 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). MLC20 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 MLC20 phosphorylation along with the enhancement of contraction. The relationship between relative tension and MLC20 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 MLC20 phosphorylation. The increase in the relative tension produced by 300 µM RuR was also coupled with the enhancement of MLC20 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 Ca\(^{2+}\) channels. The complete recovery of high-K\(^+\)-induced contraction after washout of RuR (Fig. 1) supports this interpretation. 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 Ca\(^{2+}\) sensitivity in skinned fibers (>30 µM) was higher than that required for interaction with Ca\(^{2+}\)-binding proteins such as calmodulin, Ca\(^{2+}\)-ATPase, and the ryanodine receptor, and the endogenous calmodulin receptor (Ins(1,4,5)P\(_3\)) receptor (~10 µM) or for the inhibition of Ca\(^{2+}\) current (IC\(_{50}\) ~3 µM). This would suggest that the site of action of RuR is not a Ca\(^{2+}\)-binding protein.

RuR blocks Ca\(^{2+}\)-release channels in the skeletal muscle SR membrane and thereby reduces Ca\(^{2+}\) release through the channel (19, 34). It has been reported that RuR inhibits smooth muscle Ca\(^{2+}\)-release channels reconstituted into planar lipid bilayers (9) and caffeine-induced contraction of skinned smooth muscle strips (14). Ca\(^{2+}\) release via Ins(1,4,5)P\(_3\) receptors is also inhibited by RuR, presumably because RuR competes with Ca\(^{2+}\) at the binding sites. It has also been reported that SR Ca\(^{2+}\) pump activity is reduced by RuR. The RuR-induced enhancement of contraction is, however, not due to changes in SR Ca\(^{2+}\) uptake and release, because it was observed in skinned fibers in which SR Ca\(^{2+}\) storage and release functions were destroyed by A-23187.

The enhancement of contraction by RuR was observed only at relatively low [Ca\(^{2+}\)] 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) sensitivity of the contractile system produced by RuR in cardiac muscle has been reported, but the mechanism remains unknown (37).

An increase in the Ca\(^{2+}\) sensitivity (Ca\(^{2+}\)-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 Ca\(^{2+}\)-sensitization of smooth muscle contraction has been shown (2, 7, 16, 18). On the other hand, Ca\(^{2+}\)-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 Ca\(^{2+}\)-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 MLC\(_{20}\) phosphatase (20) and also via the phosphorylation of MLC\(_{20}\) and inhibits Ca\(^{2+}\) binding to calmodulin. Electrophysiological experiments using whole cell voltage-clamp techniques, however, indicate clearly that RuR inhibits the voltage-dependent Ca\(^{2+}\) channel current in urinary bladder myocytes with an IC\(_{50}\) of ~3 µM (10). This strongly suggests that the RuR-induced reduction of high-K\(^+\)-induced contraction is mainly attributable to the blocking of Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels. The complete recovery of high-K\(^+\)-induced contraction after washout of RuR (Fig. 1) supports this interpretation. The direct

\[\text{Ca}^{2+}/\text{calmodulin} \xrightarrow{\text{PKC}} \text{MLC}_{20}\text{phosphatase} \xrightarrow{\text{RuR}} \text{MLC}_{20}\text{phosphatase}\]
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 inhibit MLCK via inhibition of Ca$^{2+}$-binding to calcmodulin (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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