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Inhibition of Rho-Associated Kinase Reduces MLC_{20} Phosphorylation and Contractility of Intact Myometrium and Attenuates Agonist-Induced Ca^{2+} Sensitization of Force of Permeabilized Rat Myometrium

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ABSTRACT. The role of rhoA/rho-associated kinase (ROK) signaling pathways in agonist-induced contraction of the rat myometrium was investigated. We measured the $[Ca^{2+}]_i$-force relationship, phosphorylation of myosin regulatory light chains (MLC_{20}) in intact tissue and the Ca^{2+}-sensitization of force in permeabilized myometrial cells of rat. In measurements of the relationship between $[Ca^{2+}]_i$ and tension in intact tissue, Y-27632, a ROK inhibitor, significantly attenuated the carbachol-induced contraction without changing $[Ca^{2+}]_i$. Phosphorylation of MLC_{20} was increased by carbachol and this increased phosphorylation was blocked by treatment of tissue with Y-27632. In tension measurements of single hyperpermeable cells, carbachol evoked sustained contraction at constant pCa 6.7 and these agonist-induced contractions were decreased by treatment with Y-27632. These results suggest that activation of a ROK-mediated signalin g pathway(s) plays an important role in agonist-induced alterations in MLC_{20} phosphorylation and force of rat myometrium.
KEY WORDS: MLC_{20} phosphorylation, rat myometrium, rho-associated kinase (ROK), rhoA, Y-27632.

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Agonist-stimulation of uterine smooth muscle primarily involves an elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) via increased Ca^{2+} influx through plasma membrane Ca^{2+} channels and/or Ca^{2+} release from intracellular IP3-sensitive (inositol 1,4,5-trisphosphate) Ca^{2+} stores [1]. The elevation in $[Ca^{2+}]_i$ results in activation of the Ca^{2+}/calmodulin-dependent enzyme myosin light chain kinase and subsequent phosphorylation of the regulatory myosin light chains (MLC_{20}) [2,3]. This increased phosphorylation of MLC_{20} enhances actomyosin ATPase activity and uterine force production. Smooth muscle relaxation is preceded by dephosphorylation of MLC_{20} by myosin light chain phosphatase (MLCP) [4]. In addition, it has been shown that an increase in the Ca^{2+}-sensitivity of the smooth muscle myofilaments contributes to the contraction induced by Ca^{2+}-mobilizing agonists [1, 5, 6]. Recent studies in other smooth muscles have indicated that both rhoA and rhoA-associated kinase (ROK) are involved in this mechanism [7]. ROK is able to regulate the phosphorylation of MLC_{20} by the inactivation of myosin light chain phosphatase (MLCP) through the phosphorylation of myosin binding subunit [8].

The spontaneous contractions of the myometrium are augmented by a variety of uterotonic agents playing an important role at parturition. These agonists have been reported to increase myometrial contraction, not only by increasing $[Ca^{2+}]_i$, but also by increasing the Ca^{2+} sensitivity of myometrial force production through a receptor-coupled, G-protein-mediated mechanism [5, 9] that has been suggested, in part at least, to be mediated via activation of ROK [6, 10]. Interestingly, in myometria from both rat and humans the mRNA expressions of rhoA and ROK in the pregnant myometrium were increased in comparison to those in the non-pregnant myometrium [11, 12]. Our preliminary data has suggested that agonist-induced Ca^{2+}-sensitization of force of single permeabilized uterine cells can be attenuated by inhibition of ROK [10]. We have also previously demonstrated in single isolated myometrial cells that rhoA and ROKα translocate from the cytosol to the membrane after carbachol stimulation [13] supportive of the hypothesis that membranous relocalization of rhoA/ROK is necessary for agonist-induced Ca^{2+}-sensitization to occur [7, 10, 13]. Although the above reports are suggestive of an involvement of rhoA and/or ROK in the Ca^{2+} sensitization phenomenon, the extent of the putative role of rhoA and ROK in the contractile regulation of pregnant rat myometrium has not been fully elucidated. Therefore, in this study we have further examined the role of ROK-mediated pathways in regulating myometrial contractile function. This has involved comparing the effects of ROK inhibition with Y-27632 on (i) the relationships between $[Ca^{2+}]_i$ and force and MLC_{20} phosphorylation and force in intact myometrial tissue and (ii) the agonist-induced Ca^{2+} sensitivity of contraction in single permeabilized cells.

MATERIALS AND METHODS

Tissue preparation: All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee. Female Sprague-Dawley

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rats in late pregnancy (on day 19–21 of gestation; term = 22 days) were killed by cervical dislocation. The myometrium was then dissected in a longitudinal direction, and then connective tissue was removed under normal Tyrode solution [composition (in mM): NaCl (154), KCl (5.4), $CaCl_2$ (2), $MgSO_4$ (1.2), HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (10), glucose (12), pH 7.4) aerated with 100% oxygen.

Simultaneous measurement of [Ca^{2+}]i and tension: $[Ca^{2+}]_i$ was measured according to the method described by Ozaki *et al.* [14], using the fluorescent Ca^{2+} indicator, fura-2. Myometrial strips were exposed to acetoxymethyl ester of fura-2 (fura-2/AM, 5 μM) and 0.02% cremophor EL in normal Tyrode solution for 3–4 hr at room temperature. At the end of the loading period, the muscle strip was washed with normal Tyrode solution for 30 min to remove extracellular fura-2/AM and was held horizontally in a temperature-controlled 5 m*l* organ chamber, one end of the muscle strip being connected to a force-displacement transducer to monitor the muscle contraction. The normal Tyrode solution was maintained at 37°C and was continuously aerated with 100% O_2. After equilibration, muscle strips were illuminated altemately (48 Hz) at two excitation wavelengths (340 and 380 nm). The intensity of 500 nm fluorescence (F_{340} and F_{380}) was measured by using a fluorimeter (CAF-100; Jasco, Tokyo). The ratio of F_{340} to F_{380} [$R(F_{340}/F_{380})$] was taken as a measure of $[Ca^{2+}]_i$. The absolute Ca^{2+} concentration was not calculated in these experiments due to the well-documented difficulties of calibration of intact smooth muscle tissues with the use of ionophores [3].

Single cell isolation: Single cells were isolated according to previously published methods [13]. Thirty to thirty-five strips (3–4 mm long, 1 mm wide) of longitudinal uterine smooth muscle were dissected free of underlying circular smooth muscle and endometrial layers in Hanks solution [composition (in mM): KH_2PO_4 (0.44), Na_2PO_4 (0.42), $NaHCO_3$ (4.17), HEPES (10), NaCl (137), KCl (5.4), dextrose (5.5), $MgCl_2$ (4.9), $CaCl_2$ (1)]. The muscle strips were washed three times in Ca^{2+}-free Hanks and further dissected into 1-mm^2 pieces. The tissues were then incubated for two 30-min washed (34°C) in 7 m*l* of Ca^{2+}-free, Mg^{2+}-free Hansk supplemented with 1.9 mg/m*l* collagenase tyep 2 (228 U/mg; Worthington), 1 mg/m*l* grade II elastase (3.65 U/mg; Boehringer-Manheim), 1.5 mg/m*l* trypsin inhibitor type II-S (Sigma), and 0.2% bovine serum albumin fraction V (Gibco-BRL). The isolation medium was then passed over a 500 μm-diameter woven mesh (Spectrum) and the digested strips were washed with 15 m*l* of cold (4°C) Mg^{2+}-containing Hanks. The filter effluent, containing isolated single smooth muscle cells, was plated over five coverslips and left at 4°C for 60 min. Cells were neither centrifuged nor triturated. $CaCl_2$ was gradually readmitted to the cells at 4°C to a final concentration of 1 mM $CaCl_2$ over 1 h. Cells were then allowed to warm to room temperature (20–22°C) in Hanks solution for 30 min before experimental maneuvers. Strict cell selection criteria were employed whereby only flat smooth cells of relaxed appearance, with no evidence of membrane ruffling or blebbing, and of length > 150 μm and width > 8 μm were assessed. For all experiments, isolated cells were first tested to confirm that they shortened in response to phenylephrine.

Single cell force measurements: Isolated single cells plated onto glass coverslips were prepared for permeabilization and force recording according to previously published methods [13, 15]. Briefly, coverslips were placed on the stage of an inverted microscope (Nikon) and the cells equilibrated in relaxing (pCa 9.0) solution (ionic composition: Ca^{2+}, 1 nM-10 nM; Mg^{2+}, 1 mM; K^+, 135 mM; MgATP, 3 mM; EGTA 15 mM; MOPS, 15 mM; phosphocreatine, 15 mM; creatine kinase, 20 U/m*l*; ionic strength, 0.2 M; pH 7.0; propionate the major anion) followed by permeabilization with 30 μg/m*l* saponin in pCa 9.0 solution. The solution was then changed to pCa 6.7. The Ca^{2+} concentrations of each solution were calculated as previously detailed [16]. The tips of two microtools of diameter < 5 μm (Glass 1BRL, W/FIL 1.0 mm, 1B100F-4, 12773-08H, World Precision Instruments) were placed on the cell surface at an angle of approximately 45° and the cell was left undisturbed for 5 min. One microtool was attached to a force transducer (Cambridge Model 400A). Force recordings were performed at room temperature (20–22°C). Control recordings illustrated that transducer drift was negligible over the course of recordings.

Translocation of rhoA: Carbachol-treated and non-treated strips were quick-frozen and homogenized in ice-cold homogenization buffer [10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 2 mM EDTA, 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 1 mM 4-(2-aminoethy)benzenesulfonyl fluoride, 20 μg/m*l* leupeptin, 20 KIU aprotinin] and centrifuged at 100,000 × g for 1 hr at 4°C (Optima XL-100K ultracentrifuge; Beckman Instruments), and the supernatant was collected as the cytosolic fraction. Pellets were resuspended, and membrane proteins were extracted by incubation for 10 min at 4°C in homogenization buffer containing 0.1% Triton X-100. The extract was centrifuged at 100,000× g for 1 hr at 4°C. The supernatant was collected and is referred to as the membrane fraction. Immunoreactive bands for and rhoA (mouse monoclonal primary antibody, 1:500 dilution; Santa Cruz Biotechnology) in cytosolic and membrane fraction were processed as common methods for western blots.

MLC_{20} phosphorylation measurements: Phosphorylation of MLC_{20} was measured using glycerol-urea minigels. The strips were mounted for isometric recording as described above. The contraction induced by carbachol (10 μM) was then examined, and the strips were rapidly removed from the experimental apparatus at the time of maximal contraction and immediately frozen by immersion for 1 hr in an acetone-dry ice slurry containing 10% TCA and 10 mM DTT. Frozen strips were gradually warmed up to room temperature, then ground with glass pestles, and washed with acetone containing 5 mM DTT to remove TCA, and then were stored at –80°C before use. The samples suspended in 20 μ*l* of urea sample buffer [8 M urea, 20 mM Tris base, 23 mM glycine (pH 8.6), 10 mM DTT, 10% glycerol, and

0.04% bromphenol blue], applied to glycerol-urea minigels (10% acrylamide/0.8% bisacrylamide, 40% glycerol, 20 mM Tris base, and 23 mM glycine), and subjected to electrophoresis at 400 V constant voltage until the dye front reached the bottom of the gel. Electrophoretic transfer of proteins from the gels on to nitrocellulose membranes was carried out. The membrane was blocked in 5% dried milk in PBS-Tween buffer for 30 min and then was incubated overnight at 4°C with a specific MLC_{20} monoclonal antibody as a primary antibody (1:1,000; Sigma). The blot was then incubated with an anti-mouse IgG (goat) antibody conjugated with horseradish peroxidase (1:1,000; Calbiochem) as a secondary antibody and was visualized with ECL. The MLC_{20} bands were quantitated densitometrically using Fuji Photo Film Image, and the MLC_{20} phosphorylation levels were expressed as the area of phosphorylated MLC_{20} divided by the total area of MLC_{20} times 100%.

To evaluate the effects of the ROK inhibitor Y-27632 on phosphorylation of MLC, the uterine strips were preincubated with Y-27632 (1 μM) for 10 min and then stimulated without removal of Y-27632. The strips were rapidly removed from the experimental apparatus and the immediately frozen at the same time as the corresponding strips without Y-27632 were frozen, and treated as described above.

Statistics: All values given in text are mean± SE. Differences between means tested using Student's *t* test. Significant differences were taken at the $p<0.05$ level.

RESULTS

In order to determine whether the effector molecule of rhoA activation, ROK, is involved in agonist-induced Ca^{2+}-sensitization of pregnant rat myometrium, we tested effect of Y-27632, a selective inhibitor of ROK [17], on the carbachol-mediated contractions. As shown in Fig. 1, 10μM carbachol evoked two types of contractions, tonic (Fig. 1A) and phasic (Fig. 1B) type. One μM Y-27632 was applied when the carbachol-induced increase in $[Ca^{2+}]_i$ ratio and tonic or phasic tension was stable. As can be observed in Fig. 1, Y-27632 attenuated carbachol-induced contraction without changing $[Ca^{2+}]_i$. In the tonic response, the magnitude of carbachol-induced $[Ca^{2+}]_i$ and tension changes in the presence of Y-27632 were 96.6 ± 4.3% and 43.9 ± 16.3% ($p<0.05$, n=7), respectively, of the $[Ca^{2+}]_i$ and tension alterations produced by carbachol in the absence of Y-27632 (Fig. 2A). In the phasic response, the magnitude of carbachol-induced $[Ca^{2+}]_i$ and tension changes in the presence of Y-27632 were 91.2 ± 6.2% and 57.2 ± 16.3% ($p<0.05$, n=7), respectively (Fig. 2B).

To determine if protein kinase C may play a role in carbachol-induced contraction of intact pregnant rat myometrium, we also tested the effects of protein kinase C inhibitor, calphostin C on the carbachol-induced increase in the fura-2 $[Ca^{2+}]_i$ ratio and tension. As shown in Fig. 3A, 1 μM calphostin C had no effect on the carbachol-induced increases in $[Ca^{2+}]_i$ and tension. The magnitude of carbachol-induced $[Ca^{2+}]_i$ and tension changes in the presence of calphostin C were 97.2 ± 2.1% and 80 ± 6.1% (n=5), respec-

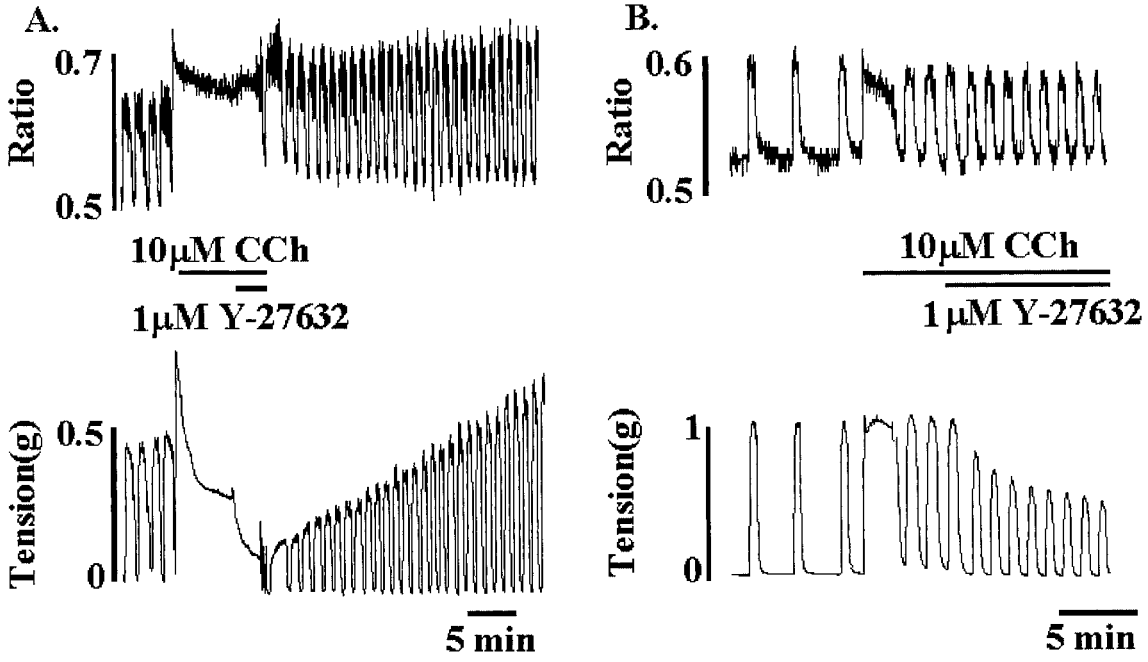

Fig. 1. Typical record of effect of Y-27632 on the carbachol-induced increase in the Fura-2 Ca^{2+} Ratio [$R(F_{340}/F_{380})$] and tension. 1 μM Y-27632 was added when carbachol (10 μM CCh)-induced increase in ratio and tension was stable.

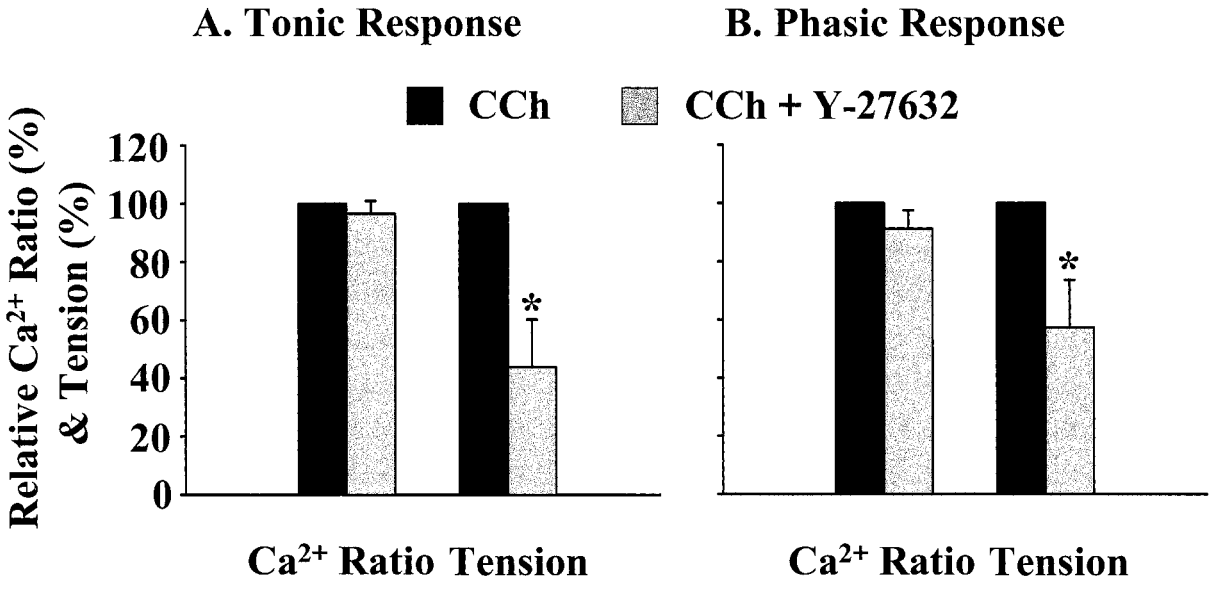

Fig. 2. Statistical analysis of the effects of Y-27632 on the carbachol-induced increase in the Fura-2 Ca^{2+} Ratio [$R(F_{340}/F_{380})$] and tension. 1 μM Y-27632 was added when carbachol (10 μM CCh)-induced increase in ratio and tension was stable. Data are expressed as relative percentage of carbachol response. Results are expressed as mean ± S.E. (n=7). *: $p<0.05$ compared with contraction induced by carbachol alone.

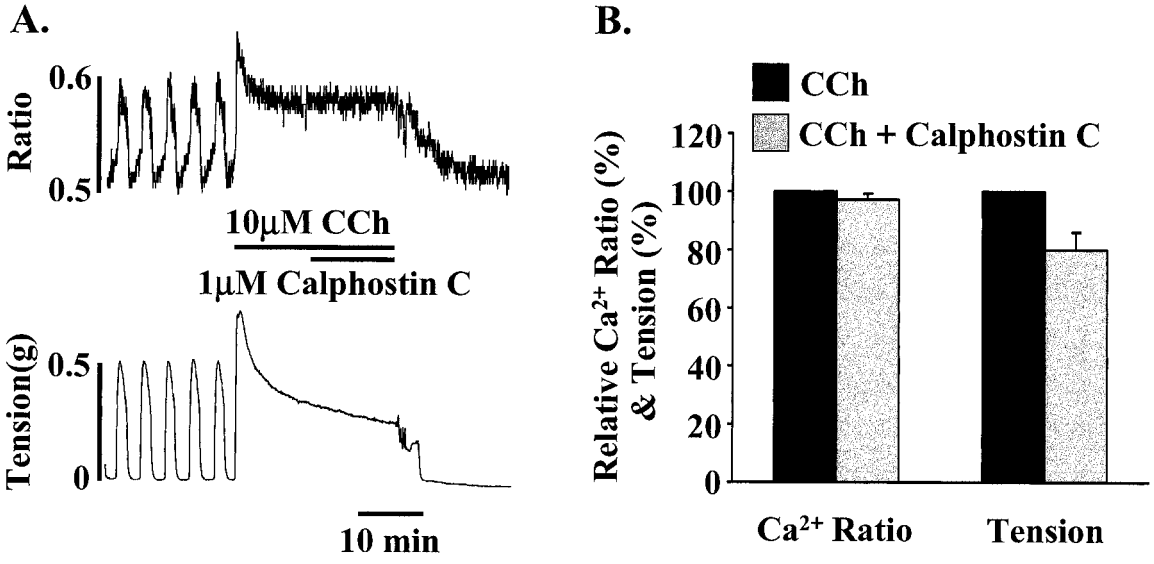

Fig. 3. A. Typical record of effect of calphostin C on the carbachol-induced increase in the Fura-2 Ca^{2+} Ratio [$R(F_{340}/F_{380})$] and tension. 1 μM calphostin C was added when carbachol (10 μM CCh)-induced increase in ratio and tension was stable. B. Statistical analysis of the effects of calphostin C on the carbachol-induced increase in the Fura-2 Ca^{2+} Ratio [$R(F_{340}/F_{380})$] and tension. Data are expressed as relative percentage of carbachol response. Results are expressed as mean ± S.E (n=5).

tively, of the $[Ca^{2+}]_i$ and tension alterations produced by carbachol in the absence of calphostin C (Fig. 3B).

As the above data indicates that ROK activation may play an important role in carbachol-induced Ca^{2+}-sensitization of pregnant rat myometrium, we performed experiments with permeabilized single uterine smooth muscle cells to examine the contribution of rhoA/ROK-mediated pathways in Ca^{2+}-sensitization of pregnant rat myometrium. At suprabasal, but sub-maximal activating Ca^{2+} (pCa 6.7) [18] carbachol evoked a gradual but sustained contraction (Fig. 4A) and this carbachol-induced contraction was significantly inhibited by treatment of single uterine smooth muscle cells with 1 μM Y-27632 (Fig. 4B). The amplitude of contraction in response to carbachol without and with Y-27632 were

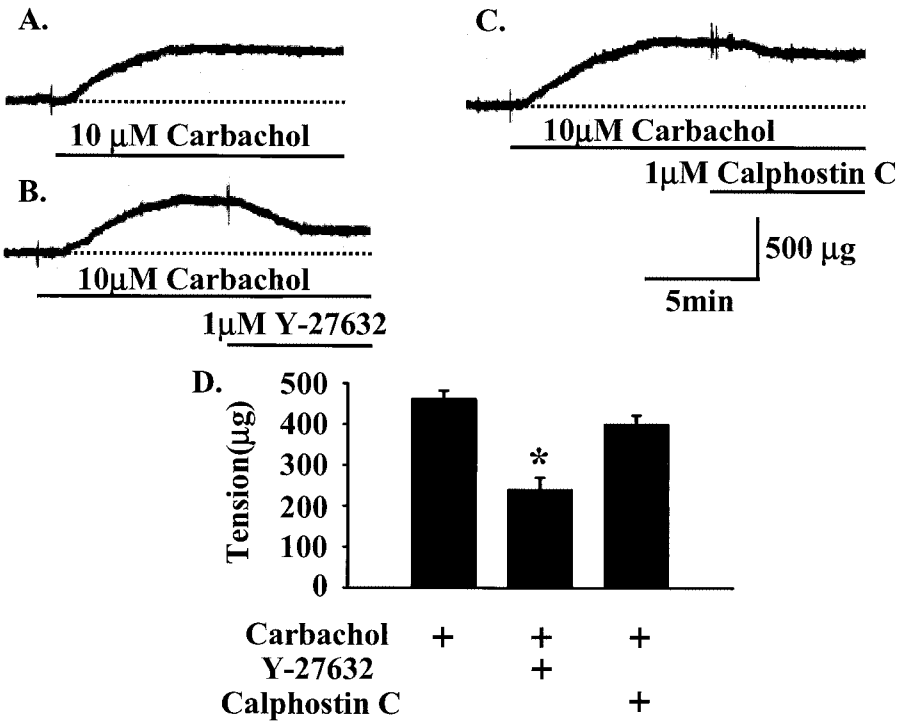

Fig. 4. Effect of Y-27632 and calphostin C on carbachol-induced contraction in single peremeabilized myometrial cell at pCa 6.7. A: Force recording in response to carbachol (10 μM). B: Effect of Y-27632 (1 μM) on carbachol-induced contraction. C: Effect of calphostin C (1 μM) on carbachol-induced contraction. D: Statistical analysis of the effects of Y-27632 and calphostin C on carbachol-induced contraction. Dashed lines indicate baseline force at pCa 6.7. Results are expressed as mean ± S.E. (n=5). *: $p<0.05$ compared with contraction induced by carbachol alone.

468.5 ± 13.8 μg and 241.9 ± 21 μg (n=5), respectively (Fig. 4D). We also tested the effect of protein kinase C inhibitor, calphostin C, on the carbachol-induced contraction in permeabilized single uterine smooth muscle cells to examine the contribution of protein kinase C in Ca^{2+}-sensitization of pregnant rat myometrium. As shown in Fig. 4C and 4D, 1 μM calphostin C inhibited carbachol-induced contraction, but not significant. The amplitude of contraction in response to carbachol without and with calphostin C were 468.5 ± 13.8 μg and 398.1 ± 14 μg (n=4), respectively (Fig. 4D).

Translocation of rhoA/ROK to the cell membrane from the cytosol is thought to be essential for their activation and subsequent Ca^{2+}-sensitizing actions in a variety of smooth muscles [19, 20]. Therefore, we determined the effect of carbachol on the localization of rhoA in the cytosol and the membrane fractions of pregnant rat myometrium as a further index of activation of this signaling pathway by muscarinic stimulation. As shown in Fig. 5A, carbachol indeed resulted in the translocation of rhoA from the cytosol to the membrane fraction.

As receptor-coupled stimulation of ROK has been suggested to inhibit the activity of MLCP in other smooth muscles, and thereby increase MLC_{20} phosphorylation and force [8, 21], we measured MLC_{20} phosphorylation in carbachol-treated myometrial strips with and without Y-27632. As shown in Fig. 5B, 10 μM carbachol significantly increased MLC_{20} phosphorylation. However, the increase in MLC_{20} phosphorylation caused by carbachol was inhibited by treatment of strips with 1 μM of the ROK inhibitor Y-27632.

DISCUSSION

The present study clearly demonstrated that rhoA/ROK-mediated pathways are involved in agonist-induced activation of intact and permeabilized uterine smooth muscle from pregnant rats. Muscarinic stimulation of myometria resulted in translocation of rhoA to the plasma membrane from the cytosol, increases in $[Ca^{2+}]_i$, MLC_{20} phosphorylation and force of contraction. Thus, in agreement with earlier studies [1], agonist-induced Ca^{2+}-sensitization occurred in intact myometrial tissues. We also demonstrated that carbachol provoked gradual but sustained contractions in permeabilized single myometrial cells confirming the existence of agonist-induced Ca^{2+}-sensitization in this tissue [13].

It has been suggested that rhoA, and its downstream effector ROK, may play an important role for agonist-induced Ca^{2+}-sensitization of smooth muscle contractility.

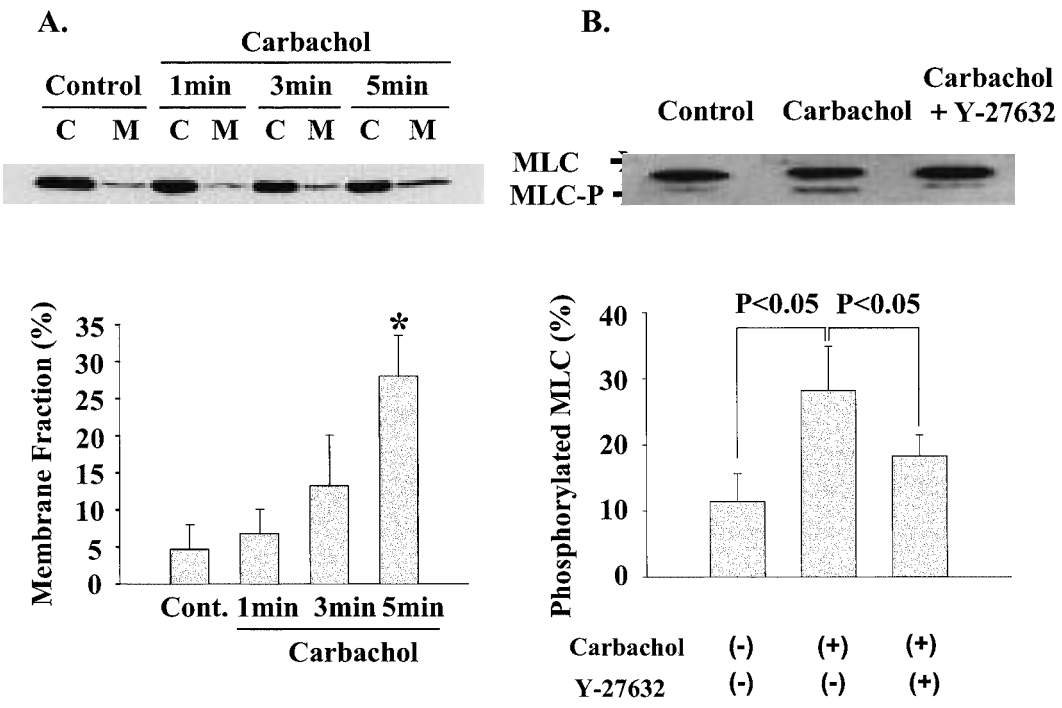

Fig. 5. A. Translocation of rhoA by carbachol stimulation. Top: Typical record for carbachol-induced translocation of rhoA. Results are representative of six experiments. C: cytosol, M: membrane. Bottom: Statistical analysis for changes in membrane fraction by carbachol. Results are expressed as mean ± S.E. (n=6). Cont. & Control:no treated carbachol strips. B. Changes in 20-KDa myosin light chain (MLC_{20}) phosphorylation with carbachol (10 μM) and effects of Y-27632 (1 μM). Top: Changes in MLC_{20} phosphorylation by carbachol (10 μM) stimulation. Results are representative of seven experiments. Bottom: Statistical analysis for changes in phosphorylation fraction. Results are expressed as mean ± S.E. (n=7).

Inactivation of rhoA in a variety of smooth muscles by ADP-ribosylation [20, 22] or monoglucosylation [22] results in inhibition of Ca^{2+}-sensitization. Also, membranous localization of rhoA is thought to be critical for its Ca^{2+}-sensitizing actions in other smooth muscles. Thus, in support of a role of rhoA-mediated events in the myometrial Ca^{2+}-sensitization phenomenon reported here, sub-cellular fractionation experiments illustrated an agonist-induced translocation of rhoA from the cytosol - where it is diffusely distributed at rest [13, 23] - to membranous pools of myometrial tissue in accordance with studies following rhoA distribution in isolated single cells by confocal immunofluorescent microscopy [13, 24].

In order to further elucidate the signaling pathway involved in carbachol-induced contractile activation of pregnant rat myometrium, we tested effect of Y-27632, a selective inhibitor of ROK [17], on the $[Ca^{2+}]_i$-force and MLC_{20}-force relationships in intact tissue. Although, in the present study, we did not compared the $[Ca^{2+}]_i$-tension relationship between carbachol and high K^+, following several experiments noticed that Ca^{2+} sensitization mechanisms may involve in the carbachol-induced contraction of rat myometrium. In experiments where we simultaneously monitored $[Ca^{2+}]_i$ and force production, Y-27632 significantly inhibited carbachol-induced contraction without a concomitant effect on elevated $[Ca^{2+}]_i$. Furthermore, in accordance with our previous preliminary results [10], Y-27632 also inhibited carbachol-induced contractions in permeabilized single cell experiments where the $[Ca^{2+}]_i$ surrounding the myofilaments is clamped by that in the bathing milieu. In other words, ROK inhibition results in a reduction in agonist-induced myometrial contraction, in both intact and permeabilized tissues, in a manner independent from the level of $[Ca^{2+}]_i$.

Recent studies have suggested that rhoA/ROK regulates MLC_{20} phosphorylation through phopshorylation, and thus inactivation, of the targeting (myosin binding) subunit of MLCP [8,25]; net MLC_{20} phosphorylation [25] is thus enhanced and smooth muscle force production elevated [26]. Y-27632 application also resulted in a significant reduction in the extent of MLC_{20} phosphorylation in uterine smooth muscle. As mentioned above, $[Ca^{2+}]_i$ remains elevated in these situations and one presumes, therefore, that myosin light chain kinase activity is little affected by this manipulation. It is thus highly likely that physiological, agonist-induced ROK-mediated attenuation of MLCP activity is unmasked by application of Y-27632.

It has suggested that protein kinase C plays an important

role in agonist-induced Ca^{2+} sensitization of other smooth muscles [15] and shows a receptor-coupled relocalization to the plasma membrane of uterine smooth muscle cells [13]. In addition to the involvement of RhoA/ROK pathway in the carbachol-induced contraction of uterine smooth muscle, we investigated the role of protein kinase C in the agonist-induced Ca^{2+} sensitization of uterine smooth muscle cells. However, calphostin C, inhibitor of protein kinase C, had no significant effect on carbachol-induced contraction in intact and permeabilized single uterine smooth muscle cells.

Taken together, these results suggest that rhoA/ROK-mediated pathways play an important role in receptor-coupled contractile activation of pregnant rat uterine smooth muscle. It has been reported that the mRNA expressions of both rhoA and ROK in myometria from pregnant rats and humans are increased in comparison to those in the non-pregnant myometria [11, 12]. It is highly possible, therefore, that activation of rhoA/ROK signaling processes contribute to the contractile actions of uterotonic agents during labour and thereby the efficacy of events surrounding successful parturition. Furthermore, these results suggest that rhoA and/or ROK may be suitable targets for the future development of therapeutic agents to be used in the clinical management of labour.

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