British Journal of Pharmacology (1999) 128, 925–933
http://www.stockton-press.co.uk/bjp

A major role for the Rho-associated coiled coil forming protein kinase in G-protein-mediated Ca^{2+} sensitization through inhibition of myosin phosphatase in rabbit trachea

*,[1]Kunihiko Iizuka, [1]Akihiro Yoshii, [2]Koichi Samizo, [1]Hideo Tsukagoshi, [1]Tamotsu Ishizuka, [1]Kunio Dobashi, [3]Tsugio Nakazawa & [1]Masatomo Mori

[1]First Department of Internal Medicine, Gunma University Faculty of Medicine, School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma, 371-8511, Japan; [2]Department of Pharmacology, Gunma University Faculty of Medicine, School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma, 371-8511, Japan and [3]Gunma University Faculty of Health Sciences, School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma, 371-8511, Japan

1 G protein-mediated Ca^{2+} sensitization of airway smooth muscle contraction was investigated with respect to the relative importance of Rho-associated coiled coil forming protein kinase (ROCK) and protein kinase C (PKC). We examined the effects of Y-27632, a ROCK inhibitor, and GF 109203X, a PKC inhibitor, on guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS)-induced contraction in α-toxin- or β-escin-permeabilized rabbit trachea.

2 Although pre-treatment with Y-27632 dose-dependently inhibited GTPγS (10 μM)-induced Ca^{2+} sensitization of α-toxin-permeabilized trachea, a Y-27632-insensitive component (approximately 16% of the maximum contraction) was retained during the early phase of the GTPγS response in the presence of Y-27632 (100 μM).

3 GF 109203X (5 μM) abolished 1 μM 4β-phorbol 12, 13-dibutyrate (PDBu)-induced, but only partially inhibited the GTPγS-induced Ca^{2+} sensitization. A combination of Y-27632 (100 μM) and GF 109203X (5 μM) totally abolished the GTPγS response.

4 GTPγS caused only a small contraction in the absence of Ca^{2+}. Wortmannin (30 μM), a myosin light chain kinase (MLCK) inhibitor, completely inhibited Ca^{2+}-induced contraction. ATP-triggered contraction of the strip which had been treated with calyculin A (1 μM), a phosphatase inhibitor, in rigor solutions was markedly slowed by worthmannin (30 μM), but not by Y-27632 (100 μM), in the presence of GTPγS and Ca^{2+}.

5 GTPγS, but not PDBu, contracted the β-escin-permeabilized trachea in the absence of Ca^{2+}, but the presence of Ca^{2+}-independent MLCK.

6 We conclude that ROCK plays a primary role in G-protein-mediated Ca^{2+} sensitization, which requires MLCK activity, with minor contribution of PKC to the early phase of contraction, and PDBu utilizes conventional PKC(s) in airway smooth muscle.

Keywords: Airway smooth muscle; Ca^{2+} sensitization; MLCK; PKC; ROCK; Y-27632

Abbreviations: EGTA, [ethylenebis (oxyethylenenitrilo)]-tetraacetic acid; GF 109203X, 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); IMLCK, Ca^{2+}-independent myosin light chain kinase; MCL_{20}, 20 kDa mysoin light chain; MLCK, myosin light chain kinase; PDBu, 4β-phorbol 12, 13-dibutyrate; PKC, protein kinase C; ROCK, Rho-associated coiled coil forming protein kinase; SM, smooth muscle; SMPP-1M, smooth muscle protein phosphatase 1 associated with myosin; $t_{1/2}$, the half-time of contraction; Y-27632, (+)-(R)-trans-4-(1-Aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide dihydrochloride, monohydrate

Introduction

Intracellular Ca^{2+} is the primary regulator of smooth muscle (SM) contraction, and under physiological conditions, phosphorylation of Ser^{19} of 20 kDa light chain of myosin (MLC_{20}) by myosin light chain kinase (MLCK) is necessary and sufficient for initiation of SM contraction (Itoh *et al*., 1989; Somlyo & Himpens, 1989; Somlyo & Somlyo, 1994). However, the force of SM contraction can change in response to agonists at a given concentration of Ca^{2+}. An increase in muscle tension and/or phosphorylation of MLC_{20} at a constant Ca^{2+} concentration is referred to as Ca^{2+} sensitization (Somlyo & Himpens, 1989; Somlyo & Somlyo, 1994). This has been well recognized as the secondary mechanism of force maintenance of SM stimulated by contractile agonists. Other investigators (Bremerich *et al*., 1997a,b; Gerthoffer, 1996) and ourselves (Iizuka *et al*., 1997) have demonstrated that receptor-dependent, G protein-mediated Ca^{2+} sensitization occurs in airway SM. Several lines of evidence indicate that a small G protein, RhoAp21 (Fujihara *et al*., 1997; Gong *et al*., 1996; 1997a,b; Hirata *et al*., 1992) and protein kinase C (PKC) (Fujihara *et al*., 1997; Gailly *et al*., 1997; Jensen *et al*., 1996; Iizuka *et al*., 1997; Ikebe & Brozovich, 1996; Masuo *et al*., 1994; Parsons *et al*., 1996; Rasmussen *et al*., 1987; Somlyo & Somlyo, 1994) may contribute to agonist-induced Ca^{2+} sensitization.

Recently, several proteins have been identified as RhoAp21 effectors, including Rho-associated coiled coil forming protein kinases (ROCK I and its isoform ROCK II) (Narumiya *et al*.,

*Author for correspondence; E-mail: iizukak@sb.gunma-u.ac.jp

1997). ROCKs play a key role in focal adhesion and stress fibre formation in fibroblasts, and in Ca^{2+} sensitization of SM (Amano *et al*., 1996; 1997; Ishizaki *et al*., 1996; 1997; Kimura *et al*., 1996; Kureishi *et al*., 1997, Leung *et al*., 1995; Matsui *et al*., 1996; Nakagawa *et al*., 1996). Y-27632, (+)-(R)-trans-4-(1-Aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide dihydrochloride, monohydrate, inhibited ROCK I and ROCK II, both *in vitro* and *in vivo* (Uehata *et al*., 1997). In airway SM we observed a similar inhibitory action of Y-27632; thus, subsequent addition of Y-27632 reversed carbachol- and endothelin-1-induced Ca^{2+} sensitization in α-toxin-permeabilized rabbit tracheal and human bronchial SM (Yoshii *et al*., 1999). These results indicate that activation of Rho/ROCK is a major downstream pathway of receptor-dependent G protein-mediated Ca^{2+} sensitization. However, in canine tracheal SM, Rho/ROCK-mediated signalling and PKC-mediated signalling may be distinct, because the effects of saturating concentrations of GTPγS (guanosine 5′-*O*-(3-thiotriphosphate)) and PDBu (4β-phorbol 12, 13-dibutyrate) were additive (Iizuka *et al*., 1997). Thus, the relative importance of these protein kinases remains to be elucidated in airway SM.

To clarify the relative roles of ROCK and PKC in G protein-mediated Ca^{2+} sensitization of airway SM, we investigated the effects of pre-treatment with a ROCK inhibitor, Y-27632, and a PKC inhibitor, 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GF 109203X), on GTPγS- and/or PDBu-induced isometric contractions at constant free Ca^{2+} concentration in α-toxin-, β-escin, or Triton X-100-permeabilized rabbit tracheal SM. We wanted to determine: (1) whether pre-treatment with Y-27632 can block GTPγS-induced Ca^{2+} sensitization completely. Because post-treatment with Y-27632 induced full relaxation of GTPγS-induced Ca^{2+} sensitization (Fu *et al*., 1998; Uehata *et al*., 1997). Appropriate pre-treatment with Y-27632 should abolish the GTPγS response, if the Rho/ROCK-mediated mechanism is necessary and sufficient for G protein-triggered Ca^{2+} sensitization; (2) if a Y-27632-insensitive component is present, whether this component is inhibited by GF 109203X; (3) whether ROCK directly phosphorylates MLC_{20} *in situ* during GTPγS-induced Ca^{2+} sensitization. Because ROCK II directly phosphorylated the same residue of MLC_{20} (Ser^{19}) *in vitro* as did MLCK (Amano *et al*., 1996). This conflicts with the idea that the main mechanism of Ca^{2+} sensitization is through inhibition of smooth muscle protein phosphatase 1 associated with myosin (SMPP-1M) activity (Somlyo & Somlyo, 1994). We designed a protocol to separate the dual ROCK-mediated pathways by treatment of the trachea with calyculin A, a potent SMPP-1M inhibitor, in ATP-free (rigor) solutions. After inhibition of SMPP-1M was accomplished by calyculin A, we compared the half-time of ATP triggered-contraction ($t_{1/2}$) in tracheal strips treated with wortmannin, Y-27632 or both in the presence of Ca^{2+} and GTPγS. Wortmannin inhibits MLCK but not ROCK II in Triton X-100-permeabilized SM (Kureishi *et al*., 1997). By contrast, Y-27632 inhibits ROCK but not MLCK both *in vitro* (Uehata *et al*., 1997) and *in situ* (Yoshii *et al*., 1999); and (5) finally, whether GTPγS-induced contraction requires MLCK activity, and whether a qualitative difference is seen between PDBu-induced and GTPγS-induced Ca^{2+} sensitization. We introduced Ca^{2+}-independent MLCK (IMLCK) to β-escin-permeabilized rabbit tracheal SM (Iizuka *et al*., 1994). In this situation the MLCK/SMPP-1M activity ratio would be expected to increase Ca^{2+}-independently, and we anticipated that the Ca^{2+} dependency of the effects of GTPγS and PDBu would be clarified. Preliminary results of this study has been presented at the Annual Biophysical Society Meeting (Iizuka *et al*., 1999).

Methods

Tissue preparation and isometric force measurement

The material preparation and force measurement have been reported elsewhere (Iizuka *et al*., 1994; 1997; 1998). In brief, the trachea was removed from Japanese albino rabbits under halothane anaesthesia in accordance with the recommendations of the Animal Care and Experimentation Committee, Gunma University, Showa Campus. Small strips of tracheal SM (width 400 μm; thickness 40–50 μm; length 3.0 mm) were dissected, and set up in a bubble chamber system to measure isometric force development (Iizuka *et al*., 1998; Yoshii *et al*., 1999). After permeabilization, all experiments were performed at 24 °C in the presence of 2 μM ibuprofen.

Solutions and permeabilization with α-toxin, β-escin or Triton X-100

The normal relaxing solution (G1) contained (in mM): potassium methanesulphonate 74.1, Mg^{2+} 2, ATP (Mg^{2+} salt), 4.5 [ethylenebis (oxyethylenenitrilo)] - tetraacetic acid (EGTA), creatine phosphate 10, Pipes- KOH 30 (pH 7.1 at 24°C, ionic strength 0.2). The same solution containing 10 mM rather than 1 mM EGTA and various amounts of calcium methanesulphonate was used to achieve the desired concentration of free Ca^{2+}.

According to Zimmermann *et al*. (1995), we prepared an EGTA (10 mM)-buffered ATP-free, Ca^{2+}-free solution (G10 rigor) and an ATP-free, high Ca^{2+} solution (pCa 4.5; rigor). These rigor solutions contained 50 μM P^1, P^5-di (adenosine-5′) pentaphosphate, an inhibitor of myokinase activity. The desired concentration of free Ca^{2+} was obtained by mixing the G10 rigor and pCa 4.5 rigor solutions.

The methods of permeabilization with α-toxin, β-escin, and Triton X-100 have been described previously (Iizuka *et al*., 1998; Yoshii *et al*., 1999). The concentrations of α-toxin, β-escin, and Triton X-100 were 16.4 μg ml^{-1}, 60 μM, and 0.1% (v v^{-1}), and the incubation periods with α-toxin, β-escin, and Triton X-100 were 30, 15 and 10 min at 30°C, respectively. Cold-preincubation at 4°C for 45 min was preceded in the β-escin or Triton X-100 permeabilization to obtain homogeneously treated strips (Iizuka *et al*., 1998; Yoshii *et al*., 1999). We added a calcium ionophore A23187 (10 μM) to the strips during α-toxin and β-escin permeabilization functionally to remove the sarcoplasmic reticulum. We verified that the treatment with A23187 was sufficient to clamp the Ca^{2+} concentrations (Iizuka *et al*., 1998). Unless noted otherwise, calmodulin (CaM) at 0.1 and 1 μM was present in the experiments with β-escin- and Triton X-100-permeabilized strips, respectively (Iizuka *et al*., 1994; 1997; 1998).

Purification and application of 61 kDa Ca^{2+}-independent active MLCK

Smooth muscle MLCK was prepared from frozen chicken gizzard according to Hayakawa *et al*. (1994). Constitutively active 61 kDa IMLCK was obtained by tryptic cleavage of MLCK according to Ikebe *et al*. (1987) with minor modification. MLCK was proteolyzed at 25°C with N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin, and proteolytic fragments were purified using DEAE-Tyopearl 650S (Tosoh, Tokyo, Japan) followed by Superose 12 (Pharmacia, Uppsara, Sweden). Protein concentrations were determined with Bradford (Bio-Rad) procedures, using bovine serum albumin as a standard. Before use, the buffer was

exchanged for relaxing solution containing 10 mM EGTA (G10) to keep the concentration of Ca^{2+} below pCa 8.0. After adjustment of the protein concentration, 61 kDa Ca^{2+}-CaM-independent active MLCK fragment (IMLCK) was added to tracheal SM permeabilized with β-escin (Iizuka *et al*., 1994).

Effects of Y-27632 and GF 109203X on GTPγS- or PDBu-induced Ca^{2+} sensitization in α-toxin-permeabilized rabbit trachea

After obtaining a reproducible maximum response to pCa 5.0, we treated the strips without or with Y-27632 (1–100 μM) or GF 109203X (5 μM) in G1 for 30 min. Control strips were treated with the vehicle (water for Y-27632 and 0.5% dimethyl sulphoxide (DMSO) for GF 109203X). The reagents were present during Ca^{2+} sensitization. When submaximal contraction induced by pCa 6.5 was stable, 10 μM GTPγS or 1 μM PDBu was added to the strips. The concentrations of GTPγS and PDBu used were supramaximal (Iizuka *et al*., 1997; Yoshii *et al*., 1999). A similar protocol was employed in the IMLCK experiments using β-escin-permeabilized strips.

Estimation of MLCK and ROCK activities toward MLC_{20} during Ca^{2+} sensitization

First, we simply added 10 μM GTPγS to α-toxin-permeabilized strips in the relaxing solution (G1), followed by quickly transferring the strips to the bubble chamber containing 10 μM GTPγS and Ca^{2+} (pCa 6.5). Next, we compared MLCK and ROCK activities towards MLC_{20} during GTPγS-induced Ca^{2+} sensitization. The putative regulatory mechanism of MLC_{20} phosphorylation is shown in Figure 1. Once Ca^{2+} plus calyculin A-induced contraction reached a peak, Y-27632 had no effect on force (Uehata *et al*., 1997). Thus, Y-27632 cannot affect SMPP-1M activity which has been completely inhibited by calyculin A. Phosphatase inhibitor (e.g. microcystin-LR and calyculin A)-induced contraction is dependent on the activity of MLCK in the presence of Ca^{2+} (Lee *et al*., 1997). Note that the rate of force rise, but not the final amplitude, reflects the kinase activity toward MLC_{20} (Lee *et al*., 1997; Masuo *et al*., 1994). When SMPP-1M has been already inactivated, ROCK, MLCK, and other kinases each can phosphorylate MLC_{20} to the maximum level. If ROCK directly phosphorylates MLC_{20} like MLCK *in situ*, therefore, inhibition of ROCK by Y-27632 would affect the rate of contraction induced by calyculin A. The α-toxin-permeabilized strips were incubated in a Ca^{2+}-free, ATP-free solution (G10 rigor) for 10 min to wash out resultant ATP from the strips, then transferred to a pCa 6.5 rigor solution containing calyculin A (1 μM), and incubated for 60 min. GTPγS (10 μM) was present 7 min before and during contraction. Even in the presence of Ca^{2+}, GTPγS, and calyculin A, phosphorylation of MLC_{20} by MLCK and/or by ROCK, and phosphorylation of SMPP-1M by ROCK should not occur under the experimental conditions, because ATP was absent. Contraction (phosphorylation) was initiated by addition of ATP (4.5 mM, Mg^{2+} salt). On the other hand, wortmannin, Y-27632, and calyculin A selectively inhibited MLCK, ROCK, and SMPP-1M in the rigor solutions. We carried out the ATP-triggered experiments in the presence of wortmannin (30 μM), Y-27632 (100 μM), or both, 60 min before and during contractions, and measured $t_{1/2}$. Appropriate amounts of vehicle (0.3% DMSO for wortmannin and 1% water for Y-27632) were added to the control strips.

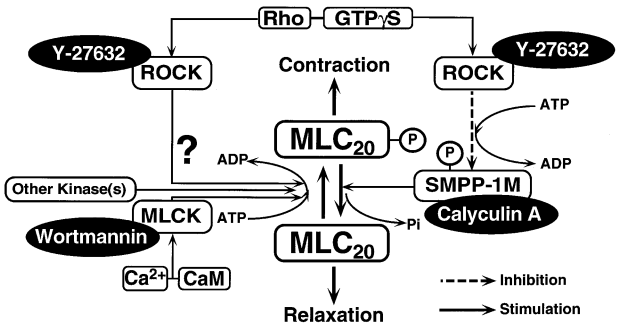


Figure 1 Putative mechanism of phosphorylation of mysoin light chain in smooth muscle. In ATP free (rigor) solutions Y-27632, wortmannin, and calyculin A inhibit ROCK, MLCK, and SMPP-1M, respectively, although ROCK, MLCK, and other kinase(s) cannot phosphorylate the substrates even when Ca^{2+} and GTPγS are present because ATP is absent. Once SMPP-1M has been inhibited by calyculin A, Rho/ROCK-mediated signalling cannot affect SMPP-1M activity. ATP application triggers phosphorylation of MLC_{20}, resulting in contraction. The rate of force rise, but not the final amplitude, reflects ROCK, MLCK and other kinase(s) activities towards MLC_{20}. CaM, calmodulin; MLC_{20}, 20 kDa myosin light chain; MLCK, myosin light chain kinase; ROCK, Rho-associated coiled coil forming protein kinase; SMPP-1M, smooth muscle protein phosphatase 1 associated with myosin.

Reagents

Staphylococcus aureus α-toxin was obtained from RBI (Natick, MA, U.S.A.); CaM and P^1, P^5-di (adenosine-5′) pentaphosphate were from Sigma (St. Louis, MO, U.S.A.). Y-27632 was the gift from Yoshitomi Pharmaceutical Industries, Ltd. (Osaka, Japan). Y-27632 was dissolved in distilled water as a stock solution (10 mM), and stored at -20°C until use. GTPγS was from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Calphostin C, calyculin A, GF 109203X, PDBu, staurosporine, and thapsigargin were purchased from Calbiochem (La Jolla, CA, U.S.A.). Wortmannin was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of reagent grade.

Statistical analysis

Data were normalized to the pCa 5.0 response prior to reagent treatment in each strip, and are shown as means ± s.e.mean of the indicated numbers of experiments. Data were compared by the Mann-Whitney *U*-test, or Student's *t*-test with the Bonferroni correction for multiple comparisons. A P value of <0.05 was considered to be statistically significant.

Results

Effect of Y-27632 on GTPγS-induced Ca^{2+} sensitization in α-toxin-permeabilized trachea

As shown in Figure 2, GTPγS (10 μM) caused rapid contractions from 1.80 ± 0.8% before GTPγS application to 96.1 ± 2.3% ($n=5$) at a constant free Ca^{2+} level of pCa 6.5. The GTPγS response was completely reversed by Y-27632 (100 μM) within 16.2 ± 3.9 min ($n=5$) (post-treatment with Y-27632). In pre-treatment experiments (Figure 3), GTPγS (10 μM) increased force from 4.5 ± 1.5% before GTPγS application to 99.9 ± 7.1% ($n=6$) 7 min after the GTPγS application. Pre-treatment with Y-27632 dose-dependently inhibited this GTPγS-induced Ca^{2+} sensitization; the peak

amplitudes with Y-27632 at 1, 10, and 100 μM were 74.7±4.9% (at 5 min, $P<0.05$, $n=4$), 38.6±3.8% (at 4 min, $P<0.05$, $n=4$), and 15.7±3.2% (at 6 min, $P<0.05$, $n=7$), respectively. During the early phase of the GTPγS response, a small but significant contraction was still observed, even in the presence of the highest concentration of Y-27632 at 100 μM (Y-27632-insensitive component). Because GTPγS-induced Ca^{2+} sensitization was completely reversed by Y-27632 within 20 min (post-treatment), the preincubation period (30 min) was sufficient. This was supported by the finding that the Y-27632-insensitive component was still observed when the preincubation time was prolonged to 60 min (data not shown). To exclude another possibility that the Y-27632-insensitive component was due to Ca^{2+} release from sarcoplasmic reticulum which escaped the A23187 treatment, we observed GTPγS-induced Ca^{2+} sensitization in the presence of thapsigargin (30 μM) and Y-27632 (100 μM). Under these conditions again approximately 20% of the control contraction was still retained (data not shown). Thus, we verified that the sarcoplasmic reticulum was functionally removed from the preparation, and that the Ca^{2+} concentration was clamped exactly at pCa 6.5.

Complete prevention of PDBu- but not GTPγS-induced Ca^{2+} sensitization by GF 109203X

As shown in Figure 4a, PDBu (1 μM) gradually increased contractile force from the steady state level at pCa 6.5 (3.74±1.3%) to 74.5±8.9% ($n=4$), and the developed force increased further on addition of GTPγS (10 μM) as previously reported. Pre-treatment with GF 109203X (5 μM) completely prevented the response to PDBu (2.04±1.1%, $n=6$), but not to GTPγS (Figure 4b). GF 109203X at less than 5 μM partially inhibited, but did not entirely abolish the PDBu response (data not shown). We also tested other PKC inhibitors, staurosporine and calphostin C. Staurosporine at 10 nM inhibited both PDBu and the submaximal Ca^{2+}-induced contractions. Calphostin C at 30 μM had no effect on the PDBu response, even though calphostin C was photo-activated by light 20 min before and during the PDBu response. To exclude the possibility that GF 109203X inhibits MLCK, we tested the effect of GF 109203X (5 μM) on Ca^{2+} (pCa 6.2)-induced contraction in Triton X-100-permeabilized trachea. Figure 5 shows that GF 109203X did not change the force. In contrast, wortmannin (30 μM) relaxed the trachea. Thus, GF 109203X at 5 μM was the best method selectively to inhibit the PDBu response under the experimental conditions.

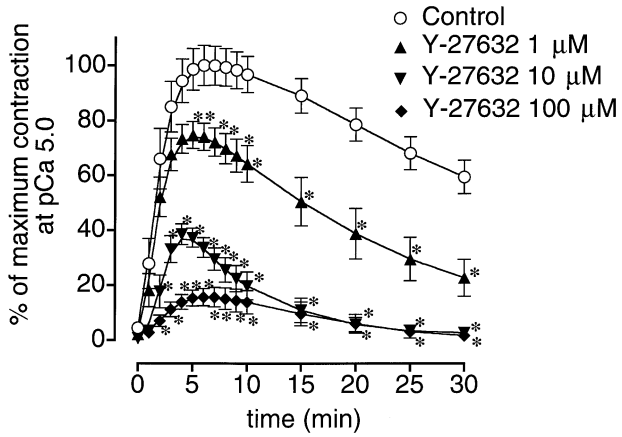
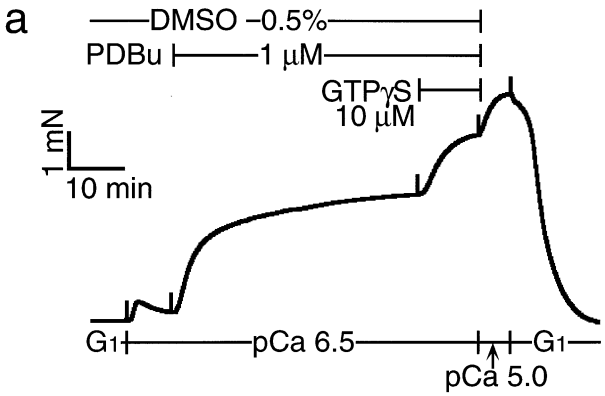


Figure 3 Time course of GTPγS-induced Ca^{2+} sensitization in the presence or absence of Y-27632. After stable pCa 5.0 response was obtained, the α-toxin-permeabilized tracheal strips were pre-incubated for 30 min without or with Y-27632 at indicated concentrations. When the submaximal contraction at pCa 6.5 became stable, GTPγS (10 μM) was added to the strips, and the serial changes in tension were observed for 30 min. Y-27632 was present during GTPγS-induced Ca^{2+} sensitization. Developed force was normalized to the initial pCa 5.0 response. *$P<0.05$ vs control. ($n=4-7$).



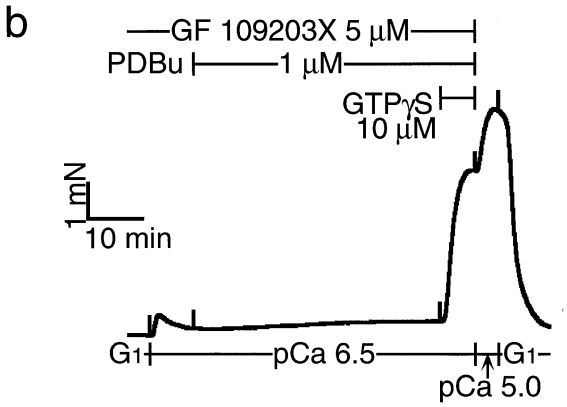


Figure 4 Complete inhibition of PDBu- but not GTPγS-induced Ca^{2+} sensitization by GF 109203X. After stable pCa 5.0 response was obtained, the α-toxin-permeabilized tracheal strips were pre-incubated for 30 min with either GF 109203X (5 μM, b) or 0.5% dimethyl sulphoxide (DMSO, a). When the submaximal contraction at pCa 6.5 became stable, PDBu (1 μM) was added to the strips, followed by application of GTPγS (10 μM) as indicated. The traces are representative of four experiments.

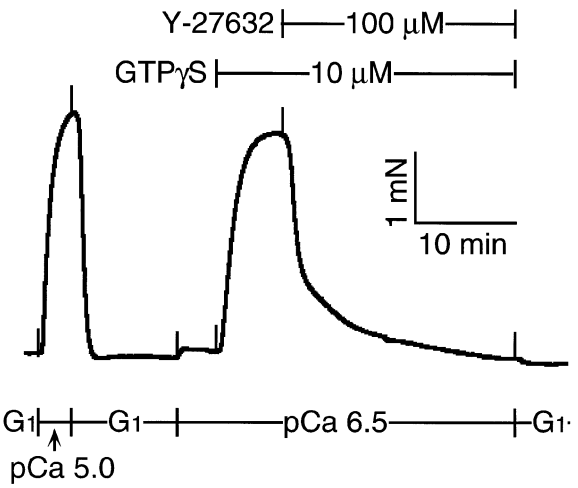


Figure 2 Effect of subsequent addition of Y-27632 on GTPγS-induced Ca^{2+} sensitization. When the GTPγS-induced Ca^{2+} sensitization reached a peak, a high concentration of Y-27632 was applied to the strip. The traces are representative of five experiments.

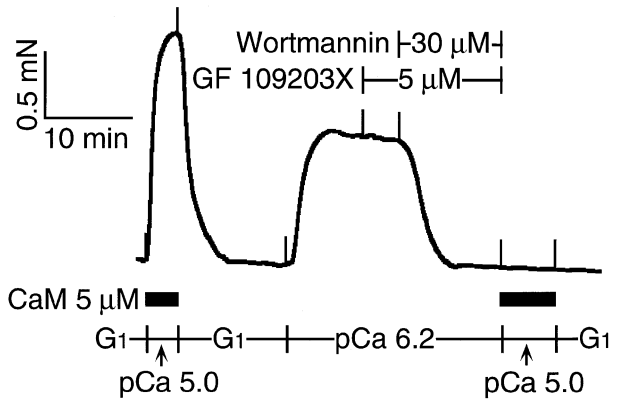


Figure 5 Lack of effect of GF 109203X on Ca^{2+}-induced contraction of Triton X-100-permeabilized trachea. The trachea was contracted by a solution of pCa 5.0 containing calmodulin (CaM, 5 μM), and relaxed in a relaxing solution (G1). When the pCa 6.2-induced contraction became stable, GF 109203X followed by wortmannin were added to the strip. The trace was representative of four experiments. All solutions except for pCa 5.0 contained 1 μM CaM.

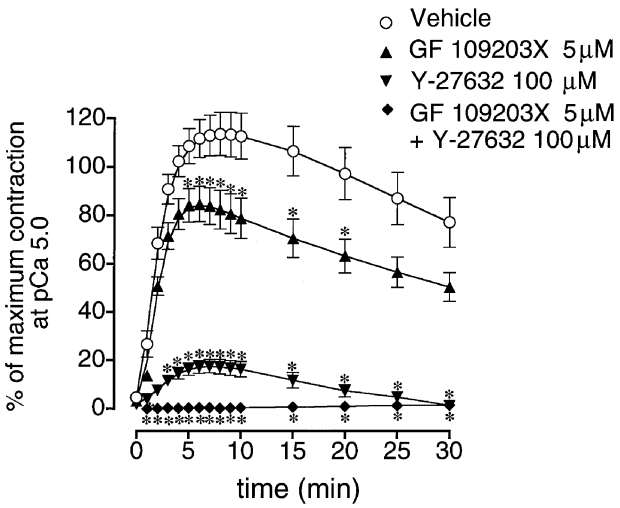


Figure 6 GTPγS-induced Ca^{2+} sensitization in the presence of Y-27632, GF 109203X, or both. After stable pCa 5.0 response was obtained, the α-toxin-permeabilized tracheal strips were preincubated for 30 min without or with either GF 109203X, Y-27632, or both. When the submaximal contraction at pCa 6.5 became stable, GTPγS (10 μM) was added to the strips, and the changes in tension were observed for 30 min. The reagents were present during GTPγS-induced Ca^{2+} sensitization, and control experiments were carried out in the presence of vehicle 0.5% dimethyl sulphoxide (DMSO), water, or both. Developed force was normalized to the initial pCa 5.0 response. $^*P<0.05$ vs control. ($n=4-11$).

The inhibitory effects of Y-27632, GF 109203X, and their combination

To compare the relative contributions of ROCK and PKC to G protein-mediated Ca^{2+} sensitization, the permeabilized strips were treated with Y-27632 (100 μM), GF 109203X (5 μM), or Y-27632 plus GF 109203X prior to being stimulated by 10 μM GTPγS. As shown Figure 6, the peak amplitude of the control strips was $113.7\pm8.9\%$ at 8 min ($n=10$), and that was reduced by Y-27632 (100 μM) to $17.6\pm2.5\%$ at 7 min ($n=11$). GF 109203X showed a lesser, but significant inhibition; the peak contraction evoked by GTPγS was $84.7\pm7.2\%$ at 6 min ($n=4$). On the other hand, Y-27632 with GF 109203X caused total suppression of the GTPγS-induced Ca^{2+} sensitization.

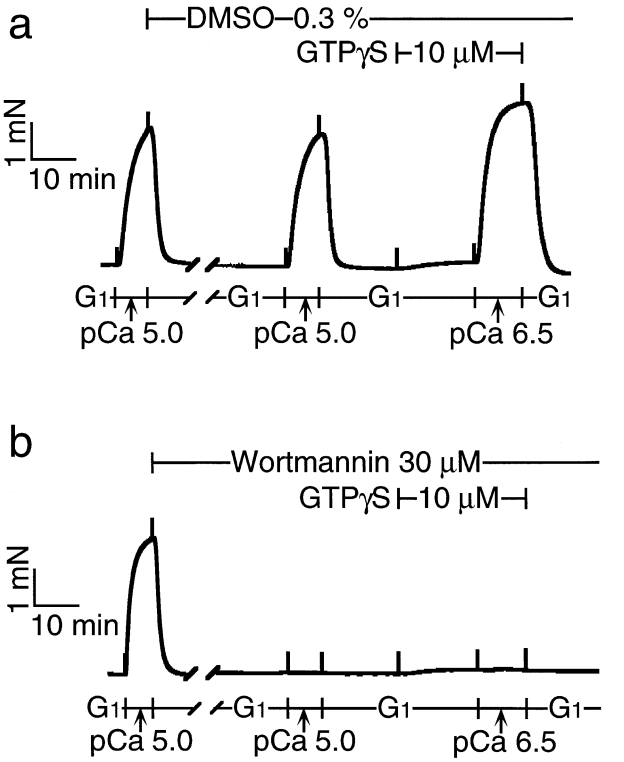


Figure 7 Complete inhibition of high Ca^{2+} alone and low Ca^{2+} plus GTPγS-induced contractions by wortmannin. After obtaining the pCa 5.0 response, the strips were treated with wortmannin (30 μM) (b) or the vehicle (0.3% dimethyl sulphoxide, DMSO) (a) for 30 min in a relaxing solution (G1), followed by exposure of high Ca^{2+} (pCa 5.0) and its washing. Then, GTPγS (10 μM) was added to the strips in G1, and quick transferring the strips to the pCa 6.5 solution containing GTPγS. The traces are representative of six experiments.

Comparison of MLCK and ROCK activities toward MLC_{20} in situ

In α-toxin-permeabilized trachea wortmannin (30 μM) completely inhibited the pCa 5.0 response, and after the wortmannin-treatment GTPγS did not induce any contraction either in the presence or absence of Ca^{2+} (Figure 7b). By contrast, when wortmannin was absent, a reproducible pCa 5.0 response was observed. GTPγS caused a marginal contraction in G1 ($3.92\pm1.1\%$, $n=6$), and addition of Ca^{2+} (pCa 6.5) evoked a large contraction (Figure 7a). To test the possibility that phosphatidylinositol 3-kinase may contribute to GTPγS-induced Ca^{2+} sensitization, we treated the strip with a low concentration of wortmannin (30 nM) for 30 min, and observed GTPγS-induced contraction in the pCa 6.5 solution containing wortmannin. There was no significant difference in the GTPγS response between the wortmannin-treated strips ($90.6\pm3.2\%$, $n=4$) and the control strips ($97.2\pm2.5\%$, $n=4$). As shown in Figure 8, ATP elicited rapid contractions of the strips which had been treated with GTPγS and calyculin A in the pCa 6.5 rigor solution. The final force developments were not different among the four groups; $105.7\pm7.2\%$, $113.9\pm11.3\%$, $97.4\pm4.7\%$, $105.1\pm7.5\%$ in the control, Y-27632-, wortmannin-, and Y-27632 plus wortmannin-treated strips, respectively ($n=4-5$). However, the contractile response was much slower when wortmannin was present (Figure 8b). Values of $t_{1/2}$ in the control, Y-27632-,

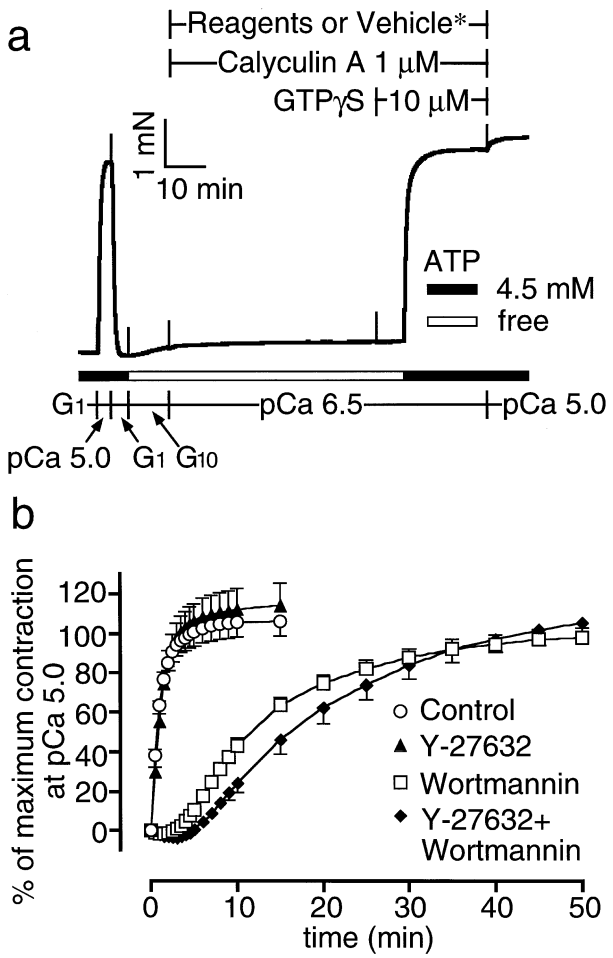


Figure 8 The effects of wortmannin and Y-27632 on ATP-triggered contraction of calyculin A-treated trachea. After the pCa 5.0 response was obtained, the α-toxin-permeabilized tracheal strips were incubated in an ATP free Ca^{2+} free solution (G10) for 10 min to remove ATP from the strip, and then incubated in a pCa 6.5 rigor solution containing calyculin A (1 μM) for 60 min. GTPγS (10 μM) was present as indicated. Contraction was initiated by addition of ATP (a). Time matched experiments were carried out in the presence of wortmannin (30 μM), Y-27632 (100 μM), or both 60 min before and during contraction as indicated*. Data are summarized in (b) ($n=4-5$).

wortmannin-, and Y-27632 plus wortmannin-treated strips were 0.74 ± 0.1, 1.00 ± 0.2, 11.5 ± 0.8, and 18.1 ± 3.4 min ($n=4-5$), respectively. Thus, wortmannin significantly slowed the ATP-triggered contractions either in the presence or absence of Y-27632 under the experimental conditions ($P<0.01$, control vs wortmannin and wortmannin plus Y-27632-treated groups).

Introduction of IMLCK to β-escin-permeabilized SM

As shown in Figure 9b, IMLCK (2 μM) contracted the β-escin-permeabilized SM in the absence of Ca^{2+}. Once the developed force had stabilized, addition of PDBu (1 μM) failed to cause further contraction. However, a GTPγS (10 μM) response was clearly observed. The lack of response to CaM (5 μM) demonstrated that these events occurred in the absence of Ca^{2+}. In control experiments (Figure 9a), PDBu and GTPγS caused Ca^{2+} sensitization of the strip contracted by pCa 6.0 in an additive manner, and CaM contracted the strips further. A summary of the data is shown in Figure 9c. Values of the PDBu and the CaM responses were significantly different between the pCa 6.0- and the IMLCK-treated groups. In a separate set of experiments, we added Y-27632 (30 μM) to strips at the peak of contraction induced by GTPγS (10 μM) in the presence of IMLCK. Y-27632 reversed the GTPγS response to the prior IMLCK-contracted level, and calyculin A (300 nM) contracted the strips further (Figure 9d).

Discussion

Ca^{2+} sensitization of SM contraction is a well established phenomenon, and inhibition of SMPP-1M that dephosphorylates MLC_{20} has been proposed as the main mechanism of Ca^{2+} sensitization (Kitazawa *et al*., 1991a,b; Masuo *et al*., 1994; Somlyo & Somlyo, 1994). Rho/ROCK- and PKC-mediated pathways are most commonly implicated in the Ca^{2+} sensitization, but phospholipase A_2 (Gailly *et al*., 1997; Parsons *et al*., 1996) and various tyrosine kinases (Steusloff *et al*., 1995) have also been suggested to be involved. In the present study we employed Y-27632 and GF 109203X to clarify the roles of ROCK and PKC in G protein-mediated Ca^{2+} sensitization.

Comparison of pre- and post-treatment with Y-27632

Y-27632 is a high selective ROCK inhibitor at least in cell free system (Uehata *et al*., 1997). Although post-addition of Y-27632 completely reversed GTPγS-induced Ca^{2+} sensitization of α-toxin-permeabilized trachea (Figure 2), a Y-27632-insensitive component was observed in pre-treatment experiments (Figure 3). Similar results were reported by Fu and colleagues (1988) in vascular SM using Y-27632 at 30 μM. We employed a higher concentration of Y-27632 (100 μM), because we required more definitive inhibition of ROCK to demonstrate the Y-27632-insensitive component. As a result, we verified the presence of a Y-27632-insensitive component. These findings suggest that a Rho/ROCK-independent mechanism(s) is (are) involved in the initial phase of the GTPγS response. We found that the insensitive component disappeared in the presence of GF 109203X, while GF 109203X alone showed only a partial inhibition of the GTPγS response (Figure 4). Peak amplitude of the Y-27632-insensitive component appeared around 6–8 min after the GTPγS application, and this component was no longer seen 20 min after the GTPγS application. Spontaneous reduction of the component is the reason why post-treatment with Y-27632 completely reversed the GTPγS response.

We considered the possibility that disappearance of the Y-27632-insensitive component was due to inhibition of MLCK by GF 109203X. Gailly *et al*. (1997) reported that while GF 109203X (600 nM) caused complete inhibition of the PDBu response without affecting the GTPγS response, the submaximal Ca^{2+}-induced contraction was slowed by GF 109203X. The reason why a higher concentration of GF 109203X was required to obtain complete inhibition of the PDBu response in this study was presumably due to the permeabilization method, α-toxin. In β-escin-permeabilized SM (Gailly *et al*., 1997), large molecules such as lactate dehydrogenase could leak out, and soluble PKCs would also be released from the strips (Iizuka *et al*., 1994). If this is the case, a low concentration of GF 109203X would be sufficient for complete inhibition of the PDBu response. By contrast, the majority of the PKC system would be intact in the α-toxin-permeabilized trachea in this study. In the presence of GF 109203X (5 μM), we observed not only pCa

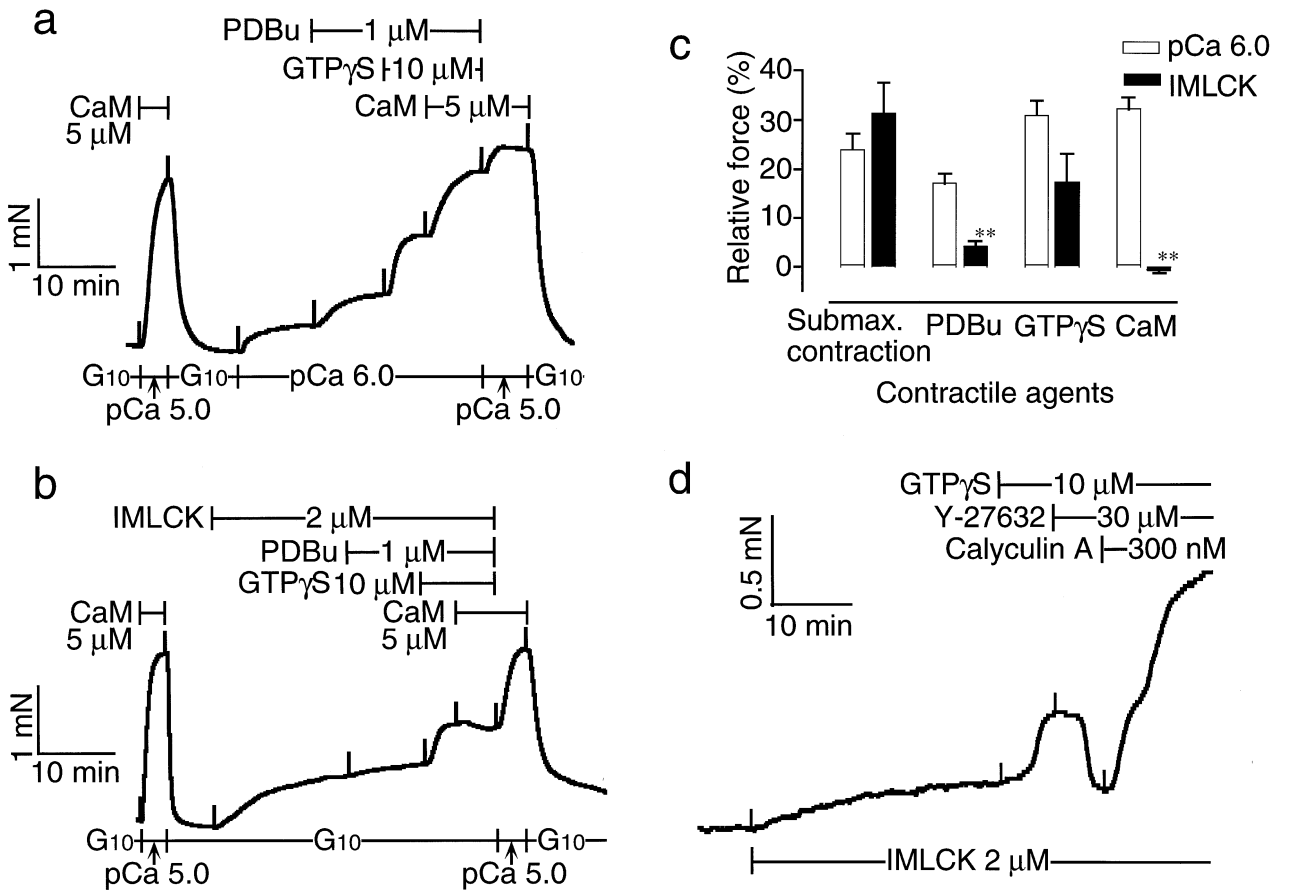


Figure 9 Requirement of MLCK activity but not Ca^{2+} itself for GTPγS-induced contraction in β-escin-permeabilized trachea. After stable pCa 5.0 plus 5 μM calmodulin (CaM) response was obtained, the β-escin-permeabilized strips were contracted either by Ca^{2+}-independent MLCK (IMLCK, 2 μM) in G10 (buffered by 10 mM EGTA) (b) or by pCa 6.0 (a). PDBu (1 μM), GTPγS (10 μM), and CaM (5 μM) were added sequentially to each strip as indicated. Unless noted otherwise all solutions contained CaM (0.1 μM). Data are summarized in (c). Submax. contraction means either pCa 6.0- or IMLCK-induced force prior to addition of Ca^{2+} sensitizing agents. The responses to pCa 6.0, IMLCK, PDBu, GTPγS, and CaM were normalized to the initial maximum contraction of each strip. $^{**}P<0.01$, (Mann-Whitney *U*-test, $n=4$). In separate set of experiments, Y-27632 was added to the peak of contraction induced by GTPγS in the presence of IMLCK, followed by application of calyculin A. The traces are representative of four experiments (d).

6.5-induced contractions but also a large GTPγS-induced Ca^{2+} sensitization (Figure 4b), which required MLCK activity to generate force *in situ* (Figure 9). Further, wortmannin (30 μM), but not GF 109203X (5 μM), relaxed the Ca^{2+} induced contraction of Triton X-100-permeabilized trachea (Figure 5). Thus, it is likely that sufficient MLCK activity for the GTPγS response was retained in the presence of GF 109203X, and that inhibition of the Y-27632-insensitive component was not due to any non-specific effect of GF 109203X. Therefore, we conclude that Rho/ROCK signalling is a major, but not the only, mechanism of GTPγS-induced Ca^{2+} sensitization, and that the GF 109203X-sensitive PKCs play a minor role during the initial phase of GTPγS-induced Ca^{2+} sensitization.

Ca^{2+} requirement for PDBu-induced Ca^{2+} sensitization of β-escin-permeabilized SM

IMLCK (2 μM) contracted the β-escin-permeabilized trachea in the absence of Ca^{2+} (Figure 9). PDBu failed to evoke an additional contraction in the absence of Ca^{2+}, indicating that conventional PKCs (phorbol ester and Ca^{2+} sensitive) are involved in the PDBu response. This is supported by the results from selective inhibition of conventional and atypical PKCs by synthesized peptides, and from down-regulation of PKC by prolonged incubation with phorbol ester (Gailly *et al*., 1997; Jensen *et al*., 1996). In contrast, GTPγS caused contractions in the presence of IMLCK as previously reported in rabbit portal vein (Iizuka *et al*., 1994) and in bovine trachea (Kubota *et al*., 1992). Thus, the IMLCK experiments demonstrated qualitative differences in Ca^{2+} requirement between GTPγS- and PDBu-induced Ca^{2+} sensitization.

Unimportance of direct phosphorylation of MLC_{20} by ROCK

ROCK II (Rho kinase) directly phosphorylated Ser^{19} of MLC_{20}, and the effect of ROCK II was more potent than that of MLCK in a cell-free system (Amano *et al*., 1996). However, the relation between ROCK and MLCK seemed to be different *in situ*.

First, GTPγS application to the strips in the absence of Ca^{2+} (G1), caused only a marginal contraction (approximately 4%). As shown in Figure 1, if the kinase works *in situ*, GTPγS should evoke an apparent contraction even in the absence of Ca^{2+}. Because the results *in vitro* revealed that neither Rho/ROCK-induced inhibition of SMPP-1M activity nor direct phosphorylation of MLC_{20} by ROCK is dependent on Ca^{2+} (Amano *et al*., 1996). If this dual mechanism is present *in situ*, therefore, GTPγS should increase MLC_{20} phosphorylation levels in the absence of Ca^{2+}.

Second, after MLCK was inhibited by wortmannin, GTPγS no longer evoked any contraction either in the presence or absence of Ca^{2+}, suggesting that MLCK activity is required for GTPγS-induced contraction.

Third, we attempted to separate the dual ROCK-mediated signalling using ATP-free rigor solutions and calyculin A. The ATP-triggered contraction reached a peak within 1 min, and the amplitude achieved a comparable level to that of the final pCa 5.0 response, indicating that treatment with calyculin A at 1 μM for 60 min abolished SMPP-1M activity of the strips. Under these experimental conditions, we compared $t_{1/2}$ in the presence of Y-27632, wortmannin, or both. As a result, the effects of wortmannin and Y-27632 were quite different. Inhibition of MLCK by wortmannin induced a prominent increase in $t_{1/2}$. In contrast, inhibition of ROCK(s) by Y-27632 only showed a tendency to cause an increase in $t_{1/2}$. The much lesser effect of Y-27632 is in agreement with the results of a marginal contraction induced by GTPγS in G1 (Figure 7a). These findings indicate that MLCK plays a more important role in GTPγS-induced Ca^{2+} sensitization than does ROCK(s) as a kinase toward MLC_{20}. We did not add GF 109203X to this protocol. Because, it was reported that PDBu did not affect kinase activity but did inhibit SMPP-1M activity in rabbit femoral artery SM (Masuo *et al*., 1994). In addition, we did not want to increase the concentration of DMSO more than 1% (GF 109203X, wortmannin, and calyculin A used DMSO as their vehicle). Interestingly, in the strips treated with wortmannin and Y-27632 together, slow contractions were still observed. Recently, a new Ca^{2+}-independent MLC kinase distinct from MLCK and ROCK has been reported (Weber *et al*., 1999). The kinase is insensitive to wortmannin and to HA 1077, another ROCK inhibitor. Thus, our results suggest that the new kinase may be present in airway SM, although the physiological importance of the kinase remains to be determined.

Finally, in the presence of IMLCK, but in the absence of Ca^{2+}, GTPγS caused contractions. The IMLCK experiments clearly shows that GTPγS-induced Ca^{2+} sensitization requires MLCK activity, but not Ca^{2+} itself. Hence, the results from the above different approaches indicate that ROCK does not effectively phosphorylate MLC_{20} *in situ* at least in rabbit trachea, and that a combination of MLCK activation by submaximal Ca^{2+} and SMPP-1M inhibition by ROCK is the main mechanism of G-protein-mediated Ca^{2+} sensitization. However, the extent of the direct phosphorylation of MLC_{20} by ROCK may be variable and be dependent on the SM type, because GTPγS contracted rabbit femoral artery in Ca^{2+}-free relaxing solution (Kitazawa & Somlyo, 1991).

Conclusion

We conclude that Rho/ROCK-mediated inhibition of SMPP-1M, but not direct phosphorylation of MLC_{20} by ROCK, is the major mechanism of GTPγS-induced Ca^{2+} sensitization of airway SM contraction, while PKCs also partially contribute to initial phase of the GTPγS response.

We thank Drs S. Kobayashi and K. Kohama (Gunma University) for their advice, and to Yoshitomi Pharmaceutical Industries, Ltd. for generous gifts of Y-27632. We are grateful to Drs A.P. Somlyo and A.V. Somlyo (University of Virginia) for their important suggestions. The work was partly supported by the Ministry of Education, Science and Culture of Japan (09670463).

References

AMANO, M., CHIHARA, K., KIMURA, K., FUKATA, Y., NAKAMURA, N., MATSUURA, Y. & KAIBUCHI, K. (1997). Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science,* **275,** 1308–1311.

AMANO, M., ITO, M., KIMURA, K., FUKATA, Y., CHIHARA, K., NAKANO, T., MATSUURA, Y. & KAIBUCHI, K. (1996). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J. Biol. Chem.,* **271,** 20246–20249.

BREMERICH, D.H., HIRASAKI, A., JONES, K.A. & WARNER, D.O. (1997a). Halothane attenuation of calcium sensitivity in airway smooth muscle. Mechanisms of action during muscarinic receptor stimulation. *Anesthesiology,* **87,** 94–101.

BREMERICH, D.H., WARNER, D.O., LORENZ, R.R., SHUMWAY, R. & JONES, K.A. (1997b). Role of protein kinase C in calcium sensitization during muscarinic stimulation in airway smooth muscle. *Am. J. Physiol.,* **273,** L775–L781.

FU, X., GONG, M.C., JIA, T., SOMLYO, A.V. & SOMLYO, A.P. (1998). The effects of the rho-kinase inhibitor Y-27632 on arachidonic acid-, GTPγS-, and phorbol ester-induced Ca^{2+}-sensitization of smooth muscle. *FEBS lett.,* **440,** 183–187.

FUJIHARA, H., WALKER, L.A., GONG, M.C., LEMICHEZ, E., BOQUET, P., SOMLYO, A.V. & SOMLYO, A.P. (1997). Inhibition of rhoA translocation and calcium sensitization by in vivo ADP-ribosylation with the chimeric toxin DC3B. *Mol. Biol. Cell.,* **8,** 2437–2447.

GAILLY, P., GONG, M.C., SOMLYO, A.V. & SOMLYO, A.P. (1997). Possible role of atypical protein kinase C activated by arachidonic acid in Ca^{2+} sensitization of rabbit smooth muscle. *J. Physiol.,* **500,** 95–109.

GERTHOFFER, W.T. (1996). Agonist synergism in airway smooth muscle contraction. *J. Pharmacol. Exp. Ther.,* **278,** 800–807.

GONG, M.C., FUJIHARA, H., SOMLYO, A.V. & SOMLYO, A.P. (1997a). Translocation of rhoA associated with Ca^{2+} sensitization of smooth muscle. *J. Biol. Chem.,* **272,** 10704–10709.

GONG, M.C., FUJIHARA, H., WALKER, L.A., SOMLYO, A.V. & SOMLYO, A.P. (1997b). Down-regulation of G-protein-mediated Ca^{2+} sensitization in smooth muscle. *Mol. Biol. Cell,* **8,** 279–286.

GONG, M.C., IIZUKA, K., NIXON, G., BROWNE, J.P., HALL, A., ECCLESTON, J.F., SUGAI, M., KOBAYASHI, S., SOMLYO, A.V. & SOMLYO, A.P. (1996). Role of guanine nucleotide-binding proteins – ras-family or trimeric proteins or both – in Ca^{2+} sensitization of smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 1340–1345.

HAYAKAWA, K., OKAGAKI, T., HIGASHI-FUJIME, S. & KOHAMA, K. (1994). Bundling of actin filaments by myosin light chain kinase from smooth muscle. *Biochem. Biophys. Res. Commun.,* **199,** 786–791.

HIRATA, K., KIKUCHI, A., SASAKI, T., KURODA, S., KAIBUCHI, K., MATSUURA, Y., SEKI, H., SAIDA, K. & TAKAI, Y. (1992). Involvement of rho p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. *J. Biol. Chem.,* **267,** 8719–8722.

IIZUKA, K., DOBASHI, K., YOSHII, A., HORIE, T., SUZUKI, H., NAKAZAWA, T. & MORI, M. (1997). Receptor-dependent G protein-mediated Ca^{2+} sensitization in canine airway smooth muscle. *Cell Calcium,* **22,** 21–30.

IIZUKA, K., IKEBE, M., SOMLYO, A.V. & SOMLYO, A.P. (1994). Introduction of high molecular weight (IgG) proteins into receptor coupled, permeabilized smooth muscle. *Cell Calcium,* **16,** 431–445.

IIZUKA, K., YOSHII, A., DOBASHI, K., HORIE, T., MORI, M. & NAKAZAWA, T. (1998). *Ins*P_3, but not novel Ca^{2+} releasers, contributes to agonist-initiated contraction in rabbit airway smooth muscle. *J. Physiol.,* **511,** 915–933.

IIZUKA, K., YOSHII, A., DOBASHI, K., NAKAZAWA, T. & MORI, M. (1999). The roles of ROCK (rho-associated coiled coil forming kinase) and protein kinase C (PKC) in calcium sensitization of airway smooth muscle. *Biophys. J.,* **76,** A288.

IKEBE, M. & BROZOVICH, F.V. (1996). Protein kinase C increases force and slows relaxation in smooth muscle: evidence for regulation of the myosin light chain phosphatase. *Biochem. Biophys. Res. Commun.,* **225,** 370–376.

IKEBE, M., STEPINSKA, M., KEMP, B.E., MEANS, A.R. & HARTSHORNE, D.J. (1987). Proteolysis of smooth muscle myosin light chain kinase. Formation of inactive and calmodulin-independent fragments. *J. Biol. Chem.,* **262,** 13828 – 13834.

ISHIZAKI, T., MAEKAWA, M., FUJISAWA, K., OKAWA, K., IWAMATSU, A., FUJITA, A., WATANABE, N., SAITO, Y., KAKIZUKA, A., MORII, N. & NARUMIYA, S. (1996). The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO J.,* **15,** 1885 – 1893.

ISHIZAKI, T., NAITO, M., FUJISAWA, K., MAEKAWA, M., WATANABE, N., SAITO, Y. & NARUMIYA, S. (1997). p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. *FEBS Lett.,* **404,** 118 – 1124.

ITOH, T., IKEBE, M., KARGACIN, G.J., HARTSHORNE, D.J., KEMP, B.E. & FAY, F.S. (1989). Effects of modulators of myosin light-chain kinase activity in single smooth muscle cells. *Nature,* **338,** 164 – 167.

JENSEN, P.E., GONG, M.C., SOMLYO, A.V. & SOMLYO, A.P. (1996). Separate upstream and convergent downstream pathways of G-protein- and phorbol ester-mediated Ca^{2+} sensitization of myosin light chain phosphorylation in smooth muscle. *Biochem. J.,* **318,** 469 – 475.

KIMURA, K., ITO, M., AMANO, M., CHIHARA, K., FUKATA, Y., NAKAFUKU, M., YAMAMORI, B., FENG, J., NAKANO, T., OKAWA, K., IWAMATSU, A. & KAIBUCHI, K. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science,* **273,** 245 – 248.

KITAZAWA, T., GAYLINN, B.D., DENNY, G.H. & SOMLYO, A.P. (1991a). G-protein-mediated Ca^{2+} sensitization of smooth muscle contraction through myosin light chain phosphorylation. *J. Biol. Chem.,* **266,** 1708 – 1715.

KITAZAWA, T., MASUO, M. & SOMLYO, A.P. (1991b). G protein-mediated inhibition of myosin light-chain phosphatase in vascular smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 9307 – 9310.

KITAZAWA, T. & SOMLYO, A.P. (1991). Modulation of Ca^{2+} sensitivity by agonists in smooth muscle. *Adv. Exp. Med. Biol.,* **304,** 97 – 109.

KUBOTA, Y., NOMURA, M., KAMM, K.E., MUMBY, M.C. & STULL, J.T. (1992). GTPγS-dependent regulation of smooth muscle contractile elements. *Am. J. Physiol.,* **262,** C405 – C410.

KUREISHI, Y., KOBAYASHI, S., AMANO, M., KIMURA, K., KANAIDE, H., NAKANO, T., KAIBUCHI, K. & ITO, M. (1997). Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J. Biol. Chem.,* **272,** 12257 – 12260.

LEE, M.R., LI, L. & KITAZAWA, T. (1997). Cyclic GMP causes Ca^{2+} desensitization in vascular smooth muscle by activating the myosin light chain phosphatase. *J. Biol. Chem.,* **272,** 5053 – 506.

LEUNG, T., MANSER, E., TAN, L. & LIM, L. (1995). A novel serine/threonine kinase binding the ras-related rhoA GTPase which translocates the kinase to peripheral membranes. *J. Biol. Chem.,* **270,** 29051 – 29054.

MASUO, M., REARDON, S., IKEBE, M. & KITAZAWA, T. (1994). A novel mechanism for the Ca^{2+}-sensitizing effect of protein kinase C on vascular smooth muscle: inhibition of myosin light chain phosphatase. *J. Gen. Physiol.,* **104,** 265 – 286.

MATSUI, T., AMANO, M., YAMAMOTO, T., CHIHARA, K., NAKAFUKU, M., ITO, M., NAKANO, T., OKAWA, K., IWAMATSU, A. & KAIBUCHI, K. (1996). Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein rho. *EMBO J.,* **15,** 2208 – 2216.

NAKAGAWA, O., FUJISAWA, K., ISHIZAKI, T., SAITO, Y., NAKAO, K. & NARUMIYA, S. (1996). ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett.,* **392,** 189 – 193.

NARUMIYA, S., ISHIZAKI, T. & WATANABE, N. (1997). Rho effectors and reorganization of actin cytoskeleton. *FEBS Lett.,* **410,** 68 – 72.

PARSONS, S.J., SUMNER, M.J. & GARLAND, C.J. (1996). Phospholipase A_2 and protein kinase C contribute to myofilament sensitization to 5-HT in the rabbit mesenteric artery. *J. Physiol.,* **491,** 447 – 453.

RASMUSSEN, H., TAKUWA, Y. & PARK, S. (1987). Protein kinase C in the regulation of smooth muscle contraction. *FASEB J.,* **1,** 177 – 185.

SOMLYO, A.P. & HIMPENS, B. (1989). Cell calcium and its regulation in smooth muscle. *FASEB J.,* **3,** 2266 – 2276.

SOMLYO, A.P. & SOMLYO, A.V. (1994). Signal transduction and regulation in smooth muscle. *Nature,* **372,** 231 – 236.

STEUSLOFF, A., PAUL, E., SEMENCHUK, L.A., DI SALVO, J. & PFITZER, G. (1995). Modulation of Ca^{2+} sensitivity in smooth muscle by genistein and protein tyrosine phosphorylation. *Arch. Biochem. Biophys.,* **320,** 236 – 242.

UEHATA, M., ISHIZAKI, T., SATOH, H., ONO, T., KAWAHARA, T., MORISHITA, T., TAMAKAWA, H., YAMAGAMI, K., INUI, J., MAEKAWA, M. & NARUMIYA, S. (1997). Calcium sensitization of smooth muscle mediated by a rho-associated protein kinase in hypertension. *Nature,* **389,** 990 – 994.

WEBER, L.P., VAN LIEROP, J.E. & WALSH, M.P. (1999). Ca^{2+}-independent phosphorylation of myosin in rat caudal artery and chicken gizzard myofilaments. *J. Physiol.,* **516,** 805 – 824.

YOSHII, A., IIZUKA, K., DOBASHI, K., HORIE, T., HARADA, T., NAKAZAWA, T. & MORI, M. (1999). Relaxation of contracted rabbit tracheal and human bronchial smooth muscle by Y-27632 through inhibition of Ca^{2+} sensitization. *Am. J. Respir. Cell Mol. Biol.,* **20,** 1190 – 1200.

ZIMMERMANN, B., SOMLYO, A.V., ELLIS-DAVIES, G.C.R., KAPLAN, J.H. & SOMLYO, A.P. (1995). Kinetics of prephosphorylation reactions and myosin light chain phosphorylation in smooth muscle. *J. Biol. Chem.,* **270,** 23966 – 23974.

(Received July 5, 1999
Revised July 26, 1999
Accepted July 30, 1999)