Chapter 6

Role of Rho-kinase in maintaining airway smooth muscle contractile phenotype

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Abstract

This study aims to investigate the role of Rho-kinase in phenotype switching and proliferation of bovine tracheal smooth muscle. To induce different phenotypic states, bovine tracheal smooth muscle strips were cultured (8 days) in 10% FBS (FBS, less contractile phenotype) or insulin (1 μM, hypercontractile phenotype) and compared to strips cultured in serum-free medium. In contraction experiments, the Rho-kinase inhibitor (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y-27632) (1 μM) decreased sensitivity to methacholine and KCl and lowered maximal responsiveness to KCl in all strips irrespective of the phenotype present. To investigate effects of Rho-kinase bovine tracheal smooth muscle phenotypic regulation, strips were pretreated with Y-27632 (1 μM) for 8 days. This resulted in a decreased maximal contractility to both methacholine and KCl, quantitatively comparable to the decrease in contractility induced by platelet-derived growth factor (PDGF, 10 ng/ml). The combination of Y-27632 and PDGF responded additively. Y-27632 did not affect basal or PDGF-induced bovine tracheal smooth muscle cell proliferation, determined both as increases in [3H]thymidine incorporation and cell number. Inhibitors of the p42/p44 mitogen activated protein kinase (MAPK)-pathway, the p38 MAPK-pathway and the phosphatidylinositol (PI) 3-kinase pathway all inhibited PDGF-induced proliferation and phenotype changes. These results show that the functional contribution of Rho-kinase to bovine tracheal smooth muscle contraction is not dependent on phenotypic state. In addition, Rho-kinase is not involved in phenotypic modulation or proliferation induced by PDGF, whereas p42/p44-, p38-MAPK and PI 3-kinase are. Rho-kinase is, however, a major regulator involved in the basal maintenance of contractility in bovine tracheal smooth muscle.

Introduction

Modulation of airway smooth muscle cell phenotype affects contractile, synthetic and/or proliferative characteristics [1]. Reduction of smooth muscle specific protein expression (e.g. smooth muscle-α-actin, smooth muscle-myosin heavy chain and smooth muscle-myosin light chain kinase) can be induced in response to serum-rich media [2]. Serum withdrawal leads to reconstitution of the contractile phenotype indicating the reversible nature of phenotype switching [3]. A recent study from our laboratory shows the occurrence of phenotype switching in organ-cultured intact bovine tracheal smooth muscle [4], showing that serum and growth factors are capable of shifting bovine tracheal smooth muscle phenotype toward a less contractile phenotype, which is linearly related to their mitogenic response. Insulin on the other hand has been shown to induce hypercontractility in smooth muscle cells [5,6].

Since inflammatory cells as well as plasma are potential sources of growth factors, phenotype switching may occur as a result of recurrent periods of allergen exposure in asthmatic airways. Repeated allergen challenge indeed has been shown to increase airway smooth muscle mass, together with reductions in smooth muscle specific protein
expression and contractility in a Brown-Norway rat model of allergic asthma [7]. As such, phenotype switching has been postulated to contribute to remodeling of the airway smooth muscle layer in asthma and therefore to the chronic increase in severity of the disease [8].

It can be envisaged that the Rho/Rho-kinase pathway is able to oppose airway smooth muscle phenotypic modulation induced by growth factors as this pathway is reported to control smooth muscle specific gene expression by mediating the nuclear localization of serum-response factor (SRF) [9,10]. The organisation state of the contractile apparatus could be directly linked to transcriptional regulation through Rho-kinase dependent regulation of actin polymerization [11]. In addition, high Rho protein expression has been observed in the contractile phenotype of aortic smooth muscle cells [12]. Taken together, this suggests that the Rho/Rho-kinase pathway is involved in maintaining the contractile smooth muscle phenotype, with a relatively more profound contribution to contraction in the more contractile state of the smooth muscle.

Paradoxically, the Rho/Rho-kinase pathway has been shown to be involved in thrombin-induced rat aortic smooth muscle cell proliferation and in serum-induced rat hepatic stellate cell growth [13,14]. Mechanistically this may be explained by Rho-kinase dependent activation of p42/p44 mitogen-activated protein kinase (MAPK) [13]. Since stimulation of proliferation and modulation to the less contractile phenotype coincide and since p42/p44 MAPK is associated with phenotypic modulation in bovine tracheal smooth muscle [4], the Rho/Rho-kinase pathway might thus be related to a shift to the less contractile phenotype. Therefore, to clarify this role of Rho-kinase in phenotype switching, we used both organ cultured bovine tracheal smooth muscle strips and cultured bovine tracheal smooth muscle cells in which contractility and proliferation were measured, respectively.

Methods

Tissue preparation and organ culture procedure
Bovine tracheae were obtained from local slaughterhouses and rapidly transported to the laboratory in Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.60, MgSO4 1.18, CaCl2 2.50, NaH2PO4 1.28, NaHCO3 25.00 and glucose 5.50, pregassed with 5% CO2 and 95% O2; pH 7.4. After dissection of the smooth muscle layer and careful removal of mucosa and connective tissue, tracheal smooth muscle strips were prepared while incubated in gassed KH-buffer at room temperature. Care was taken to cut tissue strips with macroscopically identical length (1 cm) and width (2 mm). Tissue strips were washed once in sterile Dulbecco’s modification of Eagle’s medium (DMEM), supplemented with NaHCO3 (7 mM), HEPES (10 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (1.5 μg/ml). Organ culture was performed as described previously (Gosens et al., 2002). In brief, tissue strips were transferred into suspension culture flasks and a volume of 7.5 ml
medium was added per tissue strip. Strips were maintained in culture in an incubator shaker (37 °C, 55 rpm) for 8 days, refreshing the medium on day 4. When applied, insulin, PDGF and/or kinase inhibitors (given 30 min prior to growth factors) were added in a small volume (7.5 µl per tissue strip). Culture flasks containing kinase inhibitors were protected from light during the whole experiment.

Isometric tension measurements.
Tissue strips, collected from suspension culture flasks, were washed with several volumes of KH-buffer pregassed with 5% CO2 and 95% O2, pH 7.4 at 37 °C. Subsequently, strips were mounted for isometric recording (Grass force-displacement transducer FT03) in 20 ml water-jacked organ baths, containing KH-buffer at 37 °C, continuously gassed with 5% CO2 and 95% O2, pH 7.4. During a 90 min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 3 g. Subsequently, muscle strips were precontracted with 20 mM and 30 mM isotonic KCl solutions. Following two wash-outs, maximal relaxation was established by the addition of 0.1 µM (-)-isoprenaline. In > 95% of experiments no basal myogenic tone was detected. Tension was now re-adjusted to 3 g, immediately followed by two changes of fresh KH-buffer. After another equilibration period of 30 min cumulative concentration response curves were constructed to stepwise increasing concentrations of isotonic KCl (5.6 – 50 mM) or methacholine (1 nM – 100 µM). Occasionally, Y-27632 (1 µM) was added 30 min prior to the construction of methacholine concentration response curves. When maximal KCl or methacholine-induced tension was obtained, the strips were washed several times and maximal relaxation was established using (-)-isoprenaline (10 µM).

Isolation of bovine tracheal smooth muscle cells
After the removal of mucosa and connective tissue, tracheal smooth muscle was chopped using a McIlwain tissue chopper, three times at a setting of 300 µm and three times at a setting of 100 µm. Tissue particles were washed two times with the medium mentioned above, supplemented with 0.5 % foetal bovine serum (FBS). Enzymatic digestion was performed in the same medium, supplemented with collagenase P (0.75 mg/ml), papain (1 mg/ml) and soybean trypsin inhibitor (1 mg/ml). During digestion, the suspension was incubated in an incubator shaker (Innova 4000) at 37 °C, 55 rpm for 20 min, followed by a 10 min period of shaking at 70 rpm. After filtration of the obtained suspension over 50 µm gauze, cells were washed three times in medium supplemented with 10% FBS.

[^3H]thymidine-incorporation
Bovine tracheal smooth muscle cells were plated in 24 well cluster plates at a density of 50,000 cells per well directly after isolation and were allowed to attach overnight in 10% FBS containing medium. Cells were washed twice with sterile phosphate buffered saline (PBS, composition (mM) NaCl, 140.0; KCl, 2.6; KH2PO4, 1.4; Na2HPO4.2H2O, 8.1; pH 7.4) and made quiescent by incubation in FBS-free medium, supplemented with apo-transferrin (5 µg/ml), ascorbate (100 µM) and insulin (1 µM) for 72h. Cells were then
washed with PBS and stimulated with mitogens in FBS- and insulin-free medium for 28h, the last 24h in the presence of [3H]thymidine (0.25 μCi/ml). After incubation the cells were washed twice with PBS at room temperature and once with ice-cold 5% trichloroacetic acid. Cells were treated with this trichloro-acetic acid-solution on ice for 30 min and subsequently the acid-insoluble fraction was dissolved in 1 ml NaOH (1M). Incorporated [3H]thymidine was quantified by liquid-scintillation counting using a Beckman LS1701 β-counter.

**MTT assay**

Bovine tracheal smooth muscle cells were treated similarly as described above. Subsequently, cells were stimulated with mitogens for 7 days, after which cell number was estimated using the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan. Briefly, cells were washed twice with PBS and 200 μl medium containing 0.5 mg/ml MTT was added to each well. After five hours, 200 μl solubilisation solution (composition: 10% sodium dodecylsulphate in 0.01 N HCl) was added and the cells were solubilised overnight at 37 °C. The amount of formazan in the obtained solution was estimated by measuring optical density at a test wavelength of 550 and a reference wavelength of 650 nm.

**Data analysis**

All data represent means ± s.e.m. from n separate experiments. Concentration response curves of contractile responses were analyzed by measuring myogenic tension only. No corrections were made for basal tone. Maximal tension (Emax) and EC50 were calculated from the concentration response curves. Curves were fitted using the logistic 4-parameter model (Sigmaplot 8.0, SPSS Inc.). The statistical significance of differences between data was determined by the Student’s t-test for paired observations or one-way analysis of variance, where appropriate. Differences were considered to be statistically significant when P < 0.05.

**Materials**

Dulbecco’s modification of Eagle’s Medium (DMEM) and methacholine hydrochloride were obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). FBS, NaHCO3 solution (7.5%), HEPES solution (1 M), sodium pyruvate solution (100 mM), non-essential amino acid mixture, gentamycin solution (10 mg/ml), penicillin/streptomycin solution (5000 U/ml ; 5000 μg/ml) and amphotericin B solution (250 μg/ml) (Fungizone) were obtained from Gibco BRL Life Technologies (Paisley, U.K.). Platelet-derived growth factor AB (PDGF-AB, human recombinant), insulin (from bovine pancreas), MTT, sodium-dodecyl sulphate, apo-transferrin (human), soybean trypsin inhibitor and (-)-isoprenaline hydrochloride were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 4-[5-(4-Fluorophenyl)-2-[4-(methylsulphonyl)phenyl]-1H-imidazol-4-yl]pyridine (SB-203580), (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y-27632), 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD-98059) and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY-294002) were obtained from Tocris.
Cookson Ltd. (Bristol, UK.). L(+)-ascorbic acid was from Merck (Darmstadt, Germany). [methyl-3H]thymidine (specific activity 25 Ci/mmol) was obtained from Amersham (Buckinghamshire, U.K.) Papain and Collagenase P were from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade.

Results

Effects of Y-27632 on contraction in different phenotypic states
To obtain different phenotypic states, bovine tracheal smooth muscle strips were cultured in serum-free medium (vehicle treatment), serum-free medium containing insulin (1 μM, hypercontractile state) or in serum-rich medium containing 10% FBS (less contractile state). Both methacholine- and KCl-induced contractions were susceptible to this phenotypic shift: Emax increased upon insulin pretreatment to 128 ± 11% and 134 ± 18% of vehicle pretreated preparations and decreased upon 10% FBS treatment to 73 ± 10% and 54 ± 9% of vehicle pretreated preparations, for methacholine and KCl, respectively. The role of Rho-kinase in methacholine-induced contraction in these different phenotypic states was evaluated by treatment with Y-27632 (1 μM) for 30 min. In 8 days vehicle pretreated strips, Y-27632 induced a slight decrease (ΔpD₂ = 0.33 ± 0.05, P<0.01) in sensitivity to methacholine with no effect on maximal contraction (Fig. 1a). Comparable shifts in sensitivity were obtained in insulin pretreated (ΔpD₂ = 0.33 ± 0.07, P<0.05; Fig. 1b) and FBS pretreated bovine tracheal smooth muscle strips (ΔpD₂ = 0.37 ± 0.08, P<0.001).

Figure 1. Methacholine-induced contraction of organ-cultured BTSM strips, pretreated for 8 days with (a) serum-free medium; (b) insulin (1 μM) or (c) 10% FBS. Cumulative concentration-response curves to methacholine were constructed both in the absence (open symbols) and presence (closed symbols) of 1 μM Y-27632. Data represent means ± s.e.m. of 4 experiments each performed in duplicate.
Rho-kinase and ASM phenotype

Figure 2. KCl-induced contraction of organ-cultured BTSM strips, pretreated for 8 days with (a) serum-free medium; (b) insulin (1 μM) or (c) 10% FBS. Cumulative concentration-response curves to KCl were constructed both in the absence (open symbols) and presence (closed symbols) of 1 μM Y-27632. Data represent means ± s.e.m. of 4 experiments each performed in duplicate.

Furthermore, maximal contraction was not affected by Y-27632 in either state. Interestingly, KCl-induced contraction was more sensitive to Y-27632 when compared to methacholine (Fig. 2). Treatment with Y-27632 induced a rightward shift in the KCl concentration-response curve of vehicle-treated preparations (ΔEC50 = 7.6 ± 1.4 mM, P<0.05). In addition, maximal contraction was significantly decreased (Emax = 69 ± 11 % of control, P<0.05). After 8d pretreatment of bovine tracheal smooth muscle strips with insulin or FBS, the relative effects of Y-27632 on KCl-induced contraction were not altered (ΔEC50 = 7.2 ± 0.7 mM and 7.6 ± 0.8 mM and Emax = 75 ± 6 % and 66 ± 12 % for insulin and FBS treated, respectively, Fig. 2).

Effects of Y-27632 on bovine tracheal smooth muscle contractile phenotype

The role of Rho-kinase in long-term regulation of bovine tracheal smooth muscle contractility was determined by culturing bovine tracheal smooth muscle strips for 8 days in serum-free medium containing Y-27632 (1 μM). Y-27632 pretreated strips responded with a decreased maximal contraction to 78 ± 7% and 86 ± 4% for methacholine and KCl, respectively (Fig. 3). No shift in sensitivity was observed for methacholine or for KCl after 8 days pretreatment with Y-27632. The effects of Y-27632 were both quantitatively and qualitatively similar to the effects induced by 8 days treatment with 10 ng/ml PDGF, known to shift bovine tracheal smooth muscle phenotype to a less contractile state (Fig. 3). However, 8 days pretreatment with the combination of PDGF and Y-27632 further decreased maximal contraction in an additive fashion to 54 ± 9% and 59 ± 11% for methacholine and KCl, respectively.
Figure 3. (a) Methacholine- and (b) KCl-induced contractions of organ-cultured BTSM strips, pretreated for 8 days with serum-free medium containing Y-27632 (1 μM), PDGF (10 ng/ml) or both. Data represent means ± s.e.m. of 5 experiments each performed in duplicate.

In contrast to inhibition of Rho-kinase, combined pretreatment (8 days) with inhibitors of either p42/p44 MAPK (PD98059, 30 μM), phosphatidyl inositol (PI) 3-kinase (LY294002, 10 μM) or p38 MAPK (SB203580, 10 μM) prevented PDGF-induced depression of methacholine-induced contraction (Fig. 4). Similar results were obtained using KCl as a contractile stimulus (data not shown). Note that maximal methacholine-induced contraction was not affected by acute treatment (30 min) with any of the kinase inhibitors mentioned. Rather, they induced a slight rightward shift in the concentration response curve to methacholine (Table 1).

**Table 1.** Acute effects of the kinase inhibitors used on methacholine-induced contraction of bovine tracheal smooth muscle strips.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>E&lt;sub&gt;max&lt;/sub&gt; (%)</th>
<th>pD&lt;sub&gt;2&lt;/sub&gt; (-log M)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>6.92 ± 0.10</td>
</tr>
<tr>
<td>LY294002 10 μM</td>
<td>101 ± 8</td>
<td>6.45 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PD98059 30 μM</td>
<td>101 ± 9</td>
<td>6.62 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SB203580 10 μM</td>
<td>106 ± 12</td>
<td>6.59 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Y27632 1 μM</td>
<td>103 ± 10</td>
<td>6.49 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data represent means ± s.e.m. of 4 experiments each performed in duplicate.

<sup>a</sup> P<0.05 compared to control.
Effects of Y-27632 on bovine tracheal smooth muscle DNA-synthesis and proliferation

Since stimulation of proliferation and phenotypic modulation are tightly correlated in bovine tracheal smooth muscle [4], [3H]thymidine incorporation measurements were also performed in response to the above mentioned inhibitors, both in the absence and presence of 10 ng/ml PDGF. Y-27632 (1 μM) did not affect basal [3H]thymidine incorporation (96 ± 2%). The response induced by 10 ng/ml PDGF was not affected significantly by Y-27632 as well (Fig. 5).

**Figure 4.** Maximal methacholine-induced contraction (E_{max}) of organ cultured BTSM strips after 8 days pretreatment with Y-27632 (Y, 1 μM), PD-98059 (PD, 30 μM), LY-294002 (LY, 10 μM) or SB-203580 (SB, 10 μM). Vehicle-treated strips served as controls (C). BTSM strips were organ cultured both in the absence (open bars) and presence (hatched bars) of PDGF (10 ng/ml). Data represent means ± s.e.m. of 5-6 experiments each performed in duplicate. *P<0.05; **P<0.01 compared to control, †P<0.05 compared to Y-27632.

**Figure 5.** [3H]-thymidine incorporation of unpassaged BTSM cells, made quiescent in serum-free medium for a period of 3 days. Basal responses (open bars) and those in response to PDGF (10 ng/ml, hatched bars) were measured in the presence of vehicle (C), Y-27632 (Y, 1 μM), PD-98059 (PD, 30 μM), LY-294002 (LY, 10 μM) or SB-203580 (SB, 10 μM). Data represent means ± s.e.m. of 6 experiments each performed in triplicate. ***P<0.001 compared to basal; ‡P<0.01 compared to PDGF response.
In contrast, PD98059, LY294002 and SB203580 all strongly inhibited basal incorporation to 64 ± 4%, 50 ± 4% and 20 ± 1% of basal, respectively. PDGF-induced DNA-synthesis was strongly reduced by these inhibitors as well (Fig. 5).

Cell proliferation as measured by cell number again was not affected by Y-27632. As expected, PD-98059, SB-203580 and LY-294002 reduced PDGF-induced increases in cell number (Fig. 6). Interestingly, basal cell number was reduced only after treatment with LY-294002 and SB-203580 to 70 ± 7% and 70 ± 3% of serum-free treated cell number, respectively.

**Discussion**

Rho-kinase has been shown to be involved in airway smooth muscle contraction through a number of mechanisms. It is known to affect the phosphorylation state of myosin, either by direct phosphorylation [15] or by inhibition of myosin light chain phosphatase [16,17]. In addition, a role for Rho-kinase in non-capacitative calcium entry has been observed in guinea pig airway smooth muscle [18]. Therefore, Rho-kinase may be involved in both calcium-dependent and independent regulation of contraction. In the present study, we show that Y-27632 at a Rho-kinase selective concentration [19] inhibits contraction induced by both methacholine and KCl. The relatively large inhibition of KCl-induced contraction was unexpected as Rho-kinase has been described so far to be activated only through receptor-dependent mechanisms [20]. This suggests that potassium depolarization-induced contraction, mediated by opening L-type Ca\(^{2+}\)-channels, relies more on Rho-kinase activation than muscarinic agonist-induced contraction which acts mainly through inositol 1,4,5-triphosphate-induced Ca\(^{2+}\)-mobilization [21].

Figure 6. MTT assay of unpassaged BTSM cells, made quiescent in serum-free medium for a period of 3 days. Basal responses (open bars) and those in response to PDGF (10 ng/ml, hatched bars) were measured in the presence of vehicle (C), Y-27632 (Y, 1 μM), PD-98059 (PD, 30 μM), LY-294002 (LY, 10 μM) or SB-203580 (SB, 10 μM). Data represent means ± s.e.m. of 6 experiments each performed in triplicate. **P<0.01, ***P<0.001 compared to basal; † P<0.05, ‡ P<0.01 compared to control PDGF response.
We further investigated if Rho-kinase might contribute to phenotype switching or long-term maintenance of contractility. Indeed, prolonged (8 days) pretreatment of bovine tracheal smooth muscle strips with Y-27632 induced a significant reduction in contractility. This reduction is comparable to that induced by high concentrations of growth factor (PDGF 10 ng/ml or IGF-1 30 ng/ml, [4]), indicating that Rho-kinase may be a major signaling pathway in the maintenance of contractility. As observed after pretreatment with growth factors, $E_{\text{max}}$ to KCl and methacholine were influenced similarly, both quantitatively and qualitatively. These two contractile agonists use totally distinct mechanisms to achieve elevated $[\text{Ca}^{2+}]_i$. Therefore changes at the contractile machinery are likely to explain these effects. The observed depression with no effect on sensitivity for methacholine after pretreatment with Y-27632 rules out the possibility that the observed effects are the consequence of remaining Y-27632 in the tissue during measurements of contraction, since acute treatment with Y-27632 induces a rightward shift with no effect on $E_{\text{max}}$ at all (Table 1). The similarities between KCl and methacholine after 8 days pretreatment with Y-27632 provide additional evidence, since acute effects of Y-27632 are more eminent for KCl when compared to methacholine (cf. Figures 1-2).

Since the effects of pretreatment with Y-27632 are similar to the effects induced by PDGF, we hypothesised that these effects could be mediated through a common mechanism. However, combined pretreatment with Y-27632 and PDGF induced additive depression of both KCl and methacholine-induced contraction, suggesting distinct mechanisms of action. In addition, PDGF is mitogenic, whereas Y-27632 is not. The strong relationship ($r = 0.97$) between growth factor-induced effects on contractility and proliferation [4] therefore excludes the same mechanism to be involved.

In contrast to Rho-kinase, p42/p44 MAPK (PD-98059), p38 MAPK (SB-203580) and PI 3-kinase (LY-294002) all appeared to be involved in phenotypic modulation induced by PDGF, which results in depression of contraction both for KCl and methacholine. As for Y-27632, the effects of PD-98059, SB-203580 and LY-294002 cannot be explained by remaining kinase inhibitor in the tissue during the contraction experiment, as neither inhibitor showed acute effects on maximal methacholine-induced contraction (Table 1). In addition to phenotypic modulation, p42/p44 MAPK, p38 MAPK and PI 3-kinase were involved in PDGF-induced DNA-synthesis and proliferation in bovine tracheal smooth muscle cells as well, whereas no such role for Rho-kinase was found. It is noteworthy that pretreatment with SB-203580 and LY-294002 slightly lowered both basal contractility and basal proliferation, whereas PD-98059 did not. These effects may be explained by a role for p38 MAPK and PI 3-kinase, but not for p42/p44 MAPK in preserving cell number. Effects on cell number will, however, not explain the pretreatment effects of Y-27632 on contractility in view of the lack of effect on basal cell number. Studies using other smooth muscle cell types have revealed similar effects on phenotypic modulation for p42/p44 MAPK [22,23] and p38 MAPK [23]. The role for PI 3-kinase remains controversial, as it has both been associated with maintenance of the contractile [5] and modulation to the less contractile phenotype [24]. Isoform-specific
effects of PI 3-kinase or of downstream targets may explain these opposite effects, as observed for Akt1 and Akt2 in insulin induced differentiation in C2C12 cells [25].

The results presented in this study show that Rho-kinase is involved in maintaining contractility, but has no effect on the induction of the less contractile phenotype by growth factors, which corresponds to the observation that Rho-kinase is not involved in proliferation or DNA synthesis. The effects of Y-27632 on contractility may be explained by Rho-kinase dependent effects on the localisation of the transcription factor SRF or on actin remodeling, both of which regulate smooth muscle specific gene expression [11]. Lack of effect of Rho-kinase inhibition on mitogenesis as observed by us for bovine tracheal smooth muscle, was also shown for human saphenous vein smooth muscle cell proliferation induced by PDGF-AB [26]. In contrast, Rho-kinase dependent proliferation of rat aortic smooth muscle cells induced by thrombin [14] and of hepatic stellate cells induced by serum [13] were reported. These differences are likely the result of cell type- or stimulus-specific effects.

In conclusion, the functional contribution of Rho-kinase to contraction is not dependent on the phenotypic state of intact smooth muscle. In addition, Rho-kinase is not involved in phenotypic modulation or proliferation induced by PDGF. However, Rho-kinase is a major regulator involved in the basal maintenance of contractility in bovine tracheal smooth muscle. Apart from beneficial acute effects on contraction, long-term effects of Rho-kinase inhibition may also be beneficial for treatment of airway diseases, as this will induce a less contractile airway smooth muscle state. Since the effect is quantitatively comparable to that induced by growth factors, without the induction of a proliferative phenotype, treatment with Rho-kinase inhibitors do not necessarily contribute to disadvantageous airway remodeling.

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References


