Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 483 (2004) 71–78
www.elsevier.com/locate/ejphar

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

Reinoud Gosens*, Dedmer Schaafsma, Herman Meurs, Johan Zaagsma, S. Adriaan Nelemans

Department of Molecular Pharmacology, University Centre for Pharmacy, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands

Received 7 August 2003; received in revised form 15 October 2003; accepted 21 October 2003

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% foetal bovine serum (foetal bovine serum, 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 the 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 [^{3}H]thymidine incorporation and cell number. Inhibitors of the p42/p44 mitogen-activated protein kinase (MAPK) pathway, the p38 MAPK pathway and the phosphatidyl inositol (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 MAPK, 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.

Keywords: Y-27632; Smooth muscle; Airway; MAP (mitogen-activated protein) kinase; Phosphatidyl inositol 3-kinase; PDGF (platelet-derived growth factor)

1. Introduction

Modulation of airway smooth muscle cell phenotype affects contractile, synthetic and/or proliferative characteristics (Halayko and Solway, 2001). 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 (Halayko et al., 1996). Serum withdrawal leads to reconstitution of the contractile phenotype indicating the reversible nature of phenotype switching (Halayko et al., 1999). A recent study from our laboratory shows the occurrence of phenotype switching in organ-cultured intact bovine tracheal smooth muscle (Gosens et al., 2002), 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 (Hayashi et al., 1998; Gosens et al., 2003).

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 (Moir et al., 2003). As such, phenotype switching has been postulated to contribute to remodelling of the airway smooth muscle layer in asthma and therefore to the chronic increase in severity of the disease (Hirst et al., 2000).

* Corresponding author. Tel.: +31-50-363-3321; fax: +31-50-363-6908.
E-mail address: R.Gosens@farm.rug.nl (R. Gosens).

0014-2999/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2003.10.027

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 localisation of serum-response factor (SRF) (Camoretti-Mercado et al., 2000; Liu et al., 2003). The organisation state of the contractile apparatus could be directly linked to transcriptional regulation through Rho-kinase dependent regulation of actin polymerisation (Mack et al., 2001). In addition, high Rho protein expression has been observed in the contractile phenotype of aortic smooth muscle cells (Worth et al., 2001). 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 (Iwamoto et al., 2000; Seasholtz et al., 1999). Mechanistically, this may be explained by Rho-kinase dependent activation of p42/p44 mitogen-activated protein kinase (MAPK) (Iwamoto et al., 2000). 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 (Gosens et al., 2002), 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.

2. Methods

2.1. 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, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00 and glucose 5.50, pregassed with 5% CO_2 and 95% O_2; 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 $NaHCO_3$ (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, platelet-derived growth factor (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.

2.2. Isometric tension measurements

Tissue strips, collected from suspension culture flasks, were washed with several volumes of KH-buffer pregassed with 5% CO_2 and 95% O_2, 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% CO_2 and 95% O_2, 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 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).

2.3. 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. 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 a 50-μm gauze, cells were washed three times in medium supplemented with 10% foetal bovine serum.

2.4. [^{3}H]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% foetal bovine serum containing medium. Cells were washed twice with sterile phosphate-buffered saline (PBS, composition (mM) NaCl, 140.0; KCl, 2.6; KH_2PO_4, 1.4; $Na_2HPO_4{\cdot}2H_2O$, 8.1; pH 7.4) and made quiescent by incubation in foetal bovine serum-free medium, supplemented with apo-transferrin (5 μg/ml), ascorbate (100 μM) and insulin (1 μM) for 72 h. Cells were then washed with PBS and stimulated with mitogens in foetal bovine serum- and insulin-free medium for 28 h, the last 24 h in the presence of [^{3}H]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 trichloroacetic acid solution on ice for 30 min, and, subsequently, the acid-insoluble fraction was dissolved in 1 ml NaOH (1 M). Incorporated [^{3}H]thymidine was quantified by liquid-scintillation counting using a Beckman LS1701 β-counter.

2.5. 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 5 h, 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.

2.6. 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 (E_{max}) and EC_{50} were calculated from the concentration–response curves. Curves were fitted using the logistic four-parameter model (Sigmaplot 8.0, SPSS). 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 $P < 0.05$.

2.7. Materials

DMEM and methacholine hydrochloride were obtained from ICN Biomedicals (Costa Mesa, CA, USA). Foetal bovine serum, $NaHCO_3$ 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, UK). 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 (St. Louis, MO, USA). 4-[5-(4-Fluorophenyl)-2-[4-(methylsulphonyl)phenyl]-1*H*-imidazol-4-yl]pyridine (SB-203580), (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexane carboxamide (Y-27632), 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD-98059) and 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY-294002) were obtained from Tocris Cookson (Bristol, UK). L(+)ascorbic acid was from Merck (Darmstadt, Germany). [*methyl*-^{3}H]thymidine (specific activity 25 Ci/mmol) was

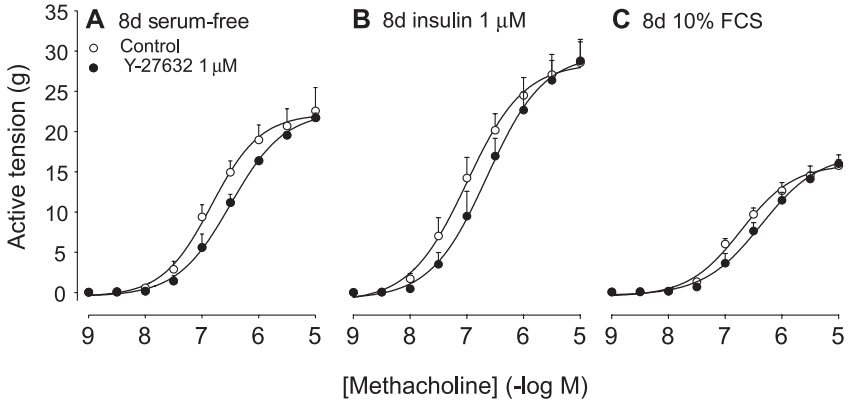

Fig. 1. Methacholine-induced contraction of organ-cultured bovine tracheal smooth muscle strips, pretreated for 8 days with (A) serum-free medium; (B) insulin (1 μM) or (C) 10% FCS. Cumulative concentration–response curves to methacholine were constructed both in the absence (open symbols) and presence (closed symbols) of 1 μM Y-27632. Data represent means ± S.E.M. of four experiments each performed in duplicate.

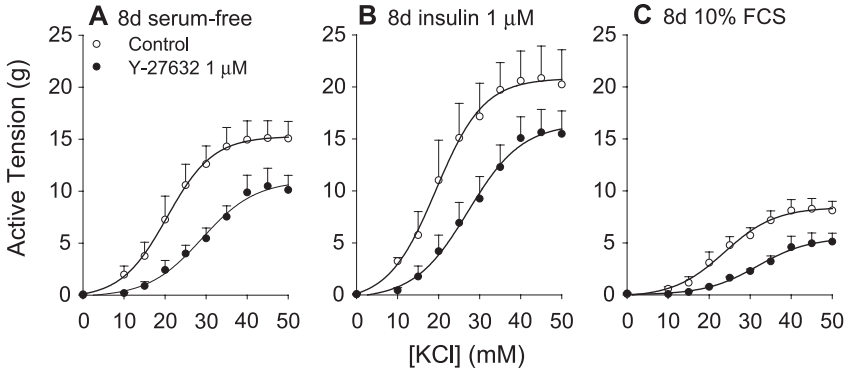

Fig. 2. KCl-induced contraction of organ-cultured bovine tracheal smooth muscle strips, pretreated for 8 days with (A) serum-free medium; (B) insulin (1 μM) or (C) 10% FCS. Cumulative concentration–response curves to KCl were constructed both in the absence (open symbols) and presence (closed symbols) of 1 μM Y-27632. Data represent means ± S.E.M. of four experiments each performed in duplicate.

obtained from Amersham (Buckinghamshire, UK). Papain and collagenase P were from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade.

3. Results

3.1. 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% foetal bovine serum (less contractile state). Both methacholine- and KCl-induced contractions were susceptible to this phenotypic shift: E_{max} increased upon insulin pretreatment to 128 ± 11% and 134 ± 18% of vehicle pretreated preparations and decreased upon 10% foetal bovine serum 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-day vehicle-pretreated strips, Y-27632 induced a slight decrease ($\Delta pD_2 = 0.33 \pm 0.05$, $P < 0.01$) in sensitivity to methacholine with no effect on maximal contraction (Fig. 1A). Comparable shifts in sensitivity were obtained in insulin pretreated ($\Delta pD_2 = 0.33 \pm 0.07$, $P < 0.05$; Fig. 1B) and foetal bovine serum pretreated bovine tracheal smooth muscle strips ($\Delta pD_2 = 0.37 \pm 0.08$, $P < 0.001$). 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 ($\Delta EC_{50} = 7.6 \pm 1.4$ mM, $P < 0.05$). In addition, maximal contraction was significantly decreased ($E_{max} = 69 \pm 11\%$ of control, $P < 0.05$). After 8 days of pretreatment of bovine tracheal smooth muscle strips with insulin or foetal bovine serum, the relative effects of Y-27632 on KCl-induced contraction were not altered ($\Delta EC_{50} = 7.2 \pm 0.7$ and 7.6 ±

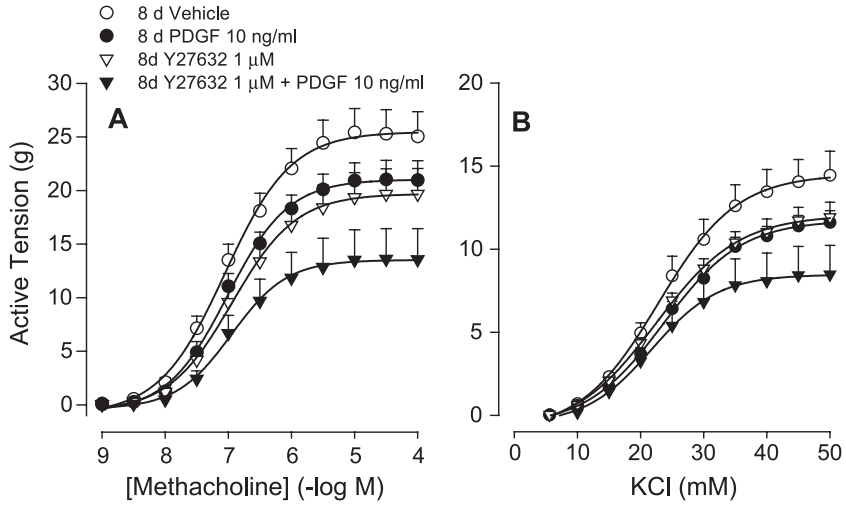

Fig. 3. (A) Methacholine- and (B) KCl-induced contractions of organ-cultured bovine tracheal smooth muscle strips, pretreated for 8 days with serum-free medium containing Y-27632 (1 μM), PDGF (10 ng/ml) or both. Data represent means ± S.E.M. of five experiments each performed in duplicate.

0.8 mM and $E_{max} = 75 \pm 6\%$ and $66 \pm 12\%$ for insulin and foetal bovine serum treated, respectively, Fig. 2).

3.2. 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 $78 \pm 7\%$ and $86 \pm 4\%$ for methacholine and KCl, respectively (Fig. 3). No shift in sensitivity was observed for methacholine or for KCl after 8 days of pretreatment with Y-27632. The effects of Y-27632 were both quantitatively and qualitatively similar to the effects induced by 8 days of treatment with 10 ng/ml PDGF, known to shift bovine tracheal smooth muscle phenotype to a less contractile state (Fig. 3). However, 8 days of pretreatment with the combination of PDGF and Y-27632 further decreased maximal contraction in an additive fashion to $54 \pm 9\%$ and $59 \pm 11\%$ for methacholine and KCl, respectively.

In contrast to inhibition of Rho-kinase, combined pretreatment (8 days) with inhibitors of either p42/p44 MAPK (PD-98059, 30 μM), phosphatidyl inositol (PI) 3-kinase (LY-294002, 10 μM) or p38 MAPK (SB-203580, 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

	E_{max} (%)	pD_2 ($-\log M$)
Control	100	6.92 ± 0.10
LY-294002, 10 μM	101 ± 8	6.45 ± 0.13[a]
PD-98059, 30 μM	101 ± 9	6.62 ± 0.11[a]
SB-203580, 10 μM	106 ± 12	6.59 ± 0.12[a]
Y27632, 1 μM	103 ± 10	6.49 ± 0.15[a]

Data represent means ± S.E.M. of four experiments each performed in duplicate.
[a] $P < 0.05$ compared to control.

3.3. 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 (Gosens et al., 2002), [^{3}H]thymidine incorporation measurements were also performed in response to the abovementioned inhibitors, both in the absence and presence of 10 ng/ml PDGF. Y-27632 (1 μM) did not affect basal [^{3}H]thymidine incorporation ($96 \pm 2\%$). The response induced by 10 ng/ml PDGF was not affected significantly by Y-27632 as well (Fig. 5). In contrast, PD-98059, LY-294002 and SB-203580 all strongly inhibited basal incorporation to $64 \pm 4\%$, $50 \pm 4\%$ and $20 \pm 1\%$ 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

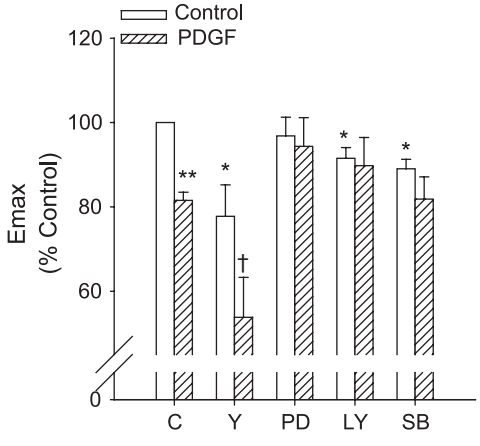

Fig. 4. Maximal methacholine-induced contraction (E_{max}) of organ-cultured bovine tracheal smooth muscle strips after 8 days of pretreatment with Y-27632 (Y, 1 μM), PD-98059 (PD, 30 μM), LY-294002 (LY, 10 μM) or SB-203580 (SB, 10 μM). Vehicle-treated strips served as controls (C). Bovine tracheal smooth muscle 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 five to six experiments each performed in duplicate. *$P < 0.05$; **$P < 0.01$ compared to control, †compared to Y-27632.

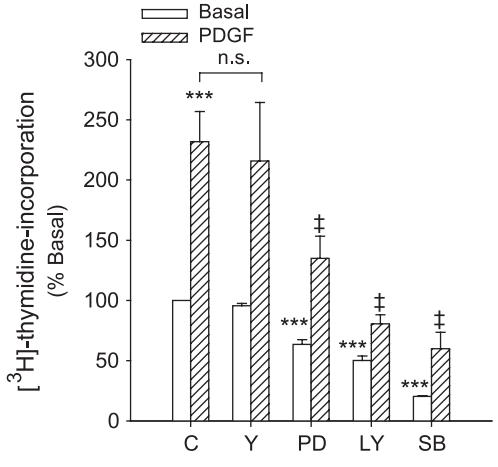

Fig. 5. [^{3}H]Thymidine incorporation of unpassaged bovine tracheal smooth muscle cells, made quiescent in serum-free medium for a period of 3 days. Basal responses (open bars) and those in response to PDGF (10 ng/ml, hatched bars) were measured in the presence of vehicle (C), Y-27632 (Y, 1 μM), PD-98059 (PD, 30 μM), LY-294002 (LY, 10 μM) or SB-203580 (SB, 10 μM). Data represent means ± S.E.M. of six experiments each performed in triplicate. ***$P < 0.001$ compared to basal; ‡$P < 0.01$ compared to control PDGF response.

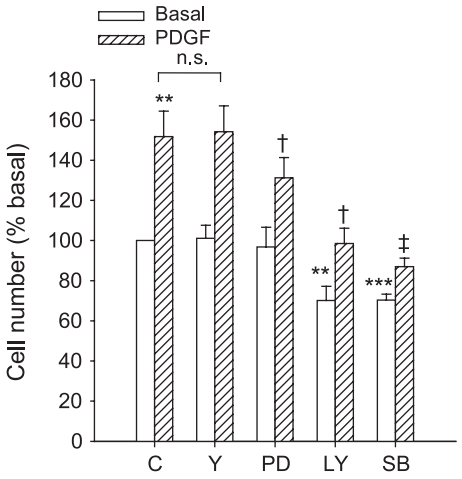

Fig. 6. MTT assay of unpassaged bovine tracheal smooth muscle cells, made quiescent in serum-free medium for a period of 3 days. Basal responses (open bars) and those in response to PDGF (10 ng/ml, hatched bars) were measured in the presence of vehicle (C), Y-27632 (Y, 1 μM), PD-98059 (PD, 30 μM), LY-294002 (LY, 10 μM) or SB-203580 (SB, 10 μM). Data represent means ± S.E.M. of six experiments each performed in triplicate. $^{**}P<0.01$; $^{***}P<0.001$ compared to basal; $^{\dagger}P<0.05$; $^{\ddagger}P<0.01$ compared to control PDGF response.

with LY-294002 and SB-230580 to 70 ± 7% and 70 ± 3% of serum-free treated cell number, respectively.

4. 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 (Amano et al., 1996) or by inhibition of myosin light chain phosphatase (Kimura et al., 1996; Iizuka et al., 1999). In addition, a role for Rho-kinase in non-capacitative calcium entry has been observed in guinea pig airway smooth muscle (Ito et al., 2002). 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 (Uehata et al., 1997) 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 (Fukata et al., 2001). 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 (Meurs et al., 2001).

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, Gosens et al., 2002), indicating that Rho-kinase may be a major signalling pathway in the maintenance of contractility. As observed after pretreatment with growth factors, E_{max} to KCl and methacholine were influenced similarly, both quantitatively and qualitatively. These two contractile agonists use totally distinct mechanisms to achieve elevated $[Ca^{2+}]_i$. 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 outrules 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_{max} at all (Table 1). The similarities between KCl and methacholine after 8 days of pretreatment with Y-27632 provide additional evidence, since acute effects of Y-27632 are more eminent for KCl when compared to methacholine (cf. Figs. 1 and 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 (Gosens et al., 2002) therefore exlcudes 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 (Roy et al., 2001; Hayashi et al., 1999) and p38 MAPK (Hayashi et al., 1999). The role for PI 3-kinase remains controversial, as it has both been associated with maintenance of the contrac-

tile (Hayashi et al., 1998) and modulation to the less contractile phenotype (Reusch et al., 2001). 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 (Sumitani et al., 2002).

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 remodelling, both of which regulate smooth muscle specific gene expression (Mack et al., 2001). 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 (Liu et al., 2002). In contrast, Rho-kinase-dependent proliferation of rat aortic smooth muscle cells induced by thrombin (Seasholtz et al., 1999) and of hepatic stellate cells induced by serum (Iwamoto et al., 2000) 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 remodelling.

Acknowledgements

This work was financially supported by the Netherlands Asthma Foundation, grants NAF 99.53 (R.G.) and 01.83 (D.S.). The authors wish to thank Maartje Hiemstra for expert technical assistance.

References

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.

Camoretti-Mercado, B., Liu, H.W., Halayko, A.J., Forsythe, S.M., Kyle, J.W., Li, B., Fu, Y., McConville, J., Kogut, P., Vieira, J.E., Patel, N.M., Hershenson, M.B., Fuchs, E., Sinha, S., Miano, J.M., Parmacek, M.S., Burkhardt, J.K., Solway, J., 2000. Physiological control of smooth muscle-specific gene expression through regulated nuclear translocation of serum response factor. J. Biol. Chem. 275, 30387–30393.

Fukata, Y., Amano, M., Kaibuchi, K., 2001. Rho–Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. Trends Pharmacol. Sci. 22, 32–39.

Gosens, R., Meurs, H., Grootte Bromhaar, M.M., McKay, S., Nelemans, S.A., Zaagsma, J., 2002. Functional characterization of serum- and growth factor-induced phenotypic changes in intact bovine tracheal smooth muscle. Br. J. Pharmacol. 137, 459–466.

Gosens, R., Nelemans, S.A., Hiemstra, M., Grootte Bromhaar, M.M., Meurs, H., Zaagsma, J., 2003. Insulin induces a hypercontractile airway smooth muscle phenotype. Eur. J. Pharmacol. 481, 125–131.

Halayko, A.J., Solway, J., 2001. Molecular mechanisms of phenotypic plasticity in smooth muscle cells. J. Appl. Physiol. 90, 358–368.

Halayko, A.J., Salari, H., Ma, X., Stephens, N.L., 1996. Markers of airway smooth muscle cell phenotype. Am. J. Physiol. 270, L1040–L1051.

Halayko, A.J., Camoretti-Mercado, B., Forsythe, S.M., Vieira, J.E., Mitchell, R.W., Wylam, M.E., Hershenson, M.B., Solway, J., 1999. Divergent differentiation paths in airway smooth muscle culture: induction of functionally contractile myocytes. Am. J. Physiol. 276, L197–L206.

Hayashi, K., Saga, H., Chimori, Y., Kimura, K., Yamanaka, Y., Sobue, K., 1998. Differentiated phenotype of smooth muscle cells depends on signaling pathways through insulin-like growth factors and phosphatidylinositol 3-kinase. J. Biol. Chem. 273, 28860–28867.

Hayashi, K., Takahashi, M., Kimura, K., Nishida, W., Saga, H., Sobue, K., 1999. Changes in the balance of phosphoinositide 3-kinase/protein kinase B (Akt) and the mitogen-activated protein kinases (ERK/p38MAPK) determine a phenotype of visceral and vascular smooth muscle cells. J. Cell Biol. 145, 727–740.

Hirst, S.J., Twort, C.H., Lee, T.H., 2000. Differential effects of extracellular matrix proteins on human airway smooth muscle cell proliferation and phenotype. Am. J. Respir. Cell Mol. Biol. 23, 335–344.

Iizuka, K., Yoshii, A., Samizo, K., Tsukagoshi, H., Ishizuka, T., Dobashi, K., Nakazawa, T., Mori, M., 1999. A major role for the rho-associated coiled coil forming protein kinase in G-protein-mediated Ca2+ sensitization through inhibition of myosin phosphatase in rabbit trachea. Br. J. Pharmacol. 128, 925–933.

Ito, S., Kume, H., Yamaki, K., Katoh, H., Honjo, H., Kodama, I., Hayashi, H., 2002. Regulation of capacitative and noncapacitative receptor-operated Ca2+ entry by rho-kinase in tracheal smooth muscle. Am. J. Respir. Cell Mol. Biol. 26, 491–498.

Iwamoto, H., Nakamuta, M., Tada, S., Sugimoto, R., Enjoji, M., Nawata, H., 2000. A p160ROCK-specific inhibitor, Y-27632, attenuates rat hepatic stellate cell growth. J. Hepatol. 32, 762–770.

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.

Liu, B., Itoh, H., Louie, O., Kubota, K., Kent, K.C., 2002. The signaling protein Rho is necessary for vascular smooth muscle migration and survival but not for proliferation. Surgery 132, 317–325.

Liu, H.W., Halayko, A.J., Fernandes, D.J., Harmon, G.S., McCauley, J.A., Kocieniewski, P., McConville, J., Fu, Y., Forsythe, S.M., Kogut, P., Bellam, S., Dowell, M., Churchill, J., Lesso, H., Kassiri, K., Mitchell, R.W., Hershenson, M.B., Camoretti-Mercado, B., Solway, J., 2003. The RhoA/Rho kinase pathway regulates nuclear localization of serum response factor. Am. J. Respir. Cell Mol. Biol. 29, 39–47.

Mack, C.P., Somlyo, A.V., Hautmann, M., Somlyo, A.P., Owens, G.K., 2001. Smooth muscle differentiation marker gene expression is regulated by RhoA-mediated actin polymerization. J. Biol. Chem. 276, 341–347.

Meurs, H., Roffel, A.F., Elzinga, C.R.S., Zaagsma, J., 2001. Muscarinic receptor-β-adrenoceptor cross-talk in airway smooth muscle. In: Zaags-

ma, J., Meurs, H., Roffel, A.F. (Eds.), Muscarinic Receptors in Airways Diseases. Birkhäuser, Basel, Switzerland, pp. 121–158.

Moir, L.M., Leung, S.Y., Eynott, P.R., McVicker, C.G., Ward, J.P., Chung, K.F., Hirst, S.J., 2003. Repeated allergen inhalation induces phenotypic modulation of smooth muscle in bronchioles of sensitized rats. Am. J. Physiol., Lung Cell. Mol. Physiol. 284, L148–L159.

Reusch, H.P., Zimmermann, S., Schaefer, M., Paul, M., Moelling, K., 2001. Regulation of Raf by Akt controls growth and differentiation in vascular smooth muscle cells. J. Biol. Chem. 276, 33630–33637.

Roy, J., Kazi, J.M., Hedin, U., Thyberg, J., 2001. Phenotypic modulation of arterial smooth muscle cells is associated with prolonged activation of ERK 1/2. Differentiation 67, 50–58.

Seasholtz, T.M., Majumdar, M., Kaplan, D.D., Brown, J.H., 1999. Rho and Rho kinase mediate thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration. Circ. Res. 84, 1186–1193.

Sumitani, S., Goya, K., Testa, J.R., Kouhara, H., Kasayama, S., 2002. Akt1 and Akt2 differently regulate muscle creatine kinase and myogenin gene transcription in insulin-induced differentiation of C2C12 myoblasts. Endocrinology 143, 820–828.

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.

Worth, N.F., Campbell, G.R., Rolfe, B.E., 2001. A role for rho in smooth muscle phenotypic regulation. Ann. N.Y. Acad. Sci. 947, 316–322.