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Allergic sensitization enhances the contribution of Rho-kinase to airway smooth muscle contraction

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1 Repeated allergen challenge has been shown to increase the role of Rho-kinase in airway smooth muscle (ASM) contraction. We considered the possibility that active allergic sensitization by itself, that is, without subsequent allergen exposure, could be sufficient to enhance Rho-kinase-mediated ASM contraction.

2 Guinea pigs were actively IgE-sensitized to ovalbumin (OA), using $Al(OH)_3$ as adjuvant. Contractile responsiveness to G_q-coupled receptor agonists (methacholine, histamine or $PGF_{2\alpha}$) was investigated in tracheal rings. No effect of sensitization was observed on basal- and methacholine-induced myogenic tone. In contrast, potency of histamine and $PGF_{2\alpha}$ increased, that is, EC_{50} decreased, after OA-sensitization by 2.6- and 4.7-fold, respectively, without effect on maximal contraction (E_{max}).

3 Basal tone in preparations from both control and OA-sensitized animals was strongly decreased in the presence of the Rho-kinase inhibitor (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexane carboxamide (Y-27632) (1 μM). In control preparations, the E_{max} and potency of histamine were unaffected by Y-27632, but were decreased for $PGF_{2\alpha}$ (by 38.2% and 2.0-fold, respectively). However, in preparations from OA-sensitized animals, Y-27632 induced a significant reduction in E_{max} (33.5%) and potency (2.3-fold) of histamine and of $PGF_{2\alpha}$ (48.3% and 6.6-fold, respectively), normalizing the OA-sensitization-induced increase in sensitivity toward these agonists.

4 We also investigated the contribution of Rho-kinase *in vivo* by measuring airway responsiveness toward inhaled histamine in permanently instrumented, unanaesthetized control and OA-sensitized guinea pigs. Treatment with Y-27632 by inhalation (5 mM, nebulizer concentration) decreased airway responsiveness toward histamine both in control and OA-sensitized animals. However, the histamine PC_{100} ratio pre/post Y-27632 inhalation was significantly smaller in OA-sensitized animals as compared to control animals, indicating an enhanced contribution of Rho-kinase.

5 Expression of RhoA, an upstream activator of Rho-kinase, was significantly increased (2.6-fold) in lung homogenates of OA-sensitized guinea pigs compared to control animals, as determined by Western analysis.

6 In conclusion, the results show a receptor-dependent role of Rho-kinase in agonist-induced ASM contraction. The contribution of Rho-kinase to contractile airway responsiveness, both *in vivo* and *ex vivo*, is augmented after active allergic sensitization, as a consequence of increased expression of RhoA presumably. Inhibition of the RhoA/Rho-kinase pathway may be considered a useful pharmacotherapeutical target in allergy and asthma.
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Abbreviations: AHR, airway hyperresponsiveness; ASM, airway smooth muscle; CRC, cumulative concentration response curve; KH, Krebs–Henseleit; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; OA, ovalbumin; pEC_{50}, $-\log_{10}$ of the concentration causing 50% of the effect

Introduction

Smooth muscle contraction is governed to an important extent by the phosphorylation level of myosin light chain (MLC) (Pfitzer, 2001). MLC phosphorylation may be caused by an increase in intracellular Ca^{2+}-concentration ($[Ca^{2+}]_i$) *via* the formation of Ca^{2+}-calmodulin and subsequent activation of myosin light chain kinase (MLCK). However, it has been shown that $[Ca^{2+}]_i$ does not always parallel the level of MLC phosphorylation and contraction. The level and the extent of MLC phosphorylation are determined by the ratio of MLCK (MLC phosphorylation) to myosin light chain phosphatase (MLCP) (MLC dephosphorylation) activities (Somlyo & Somlyo, 2003). Hence, inhibition of MLCP, for example, after agonist-induced activation of the RhoA/Rho-kinase pathway (Fukata *et al*., 2001; Wettschureck & Offermanns, 2002), can effectively enhance MLC phosphorylation at a fixed $[Ca^{2+}]_i$,

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thereby contributing to an augmented level of contraction. Indeed, Rho-kinase has been reported to contribute to smooth muscle contraction (Gong *et al*., 1996; Uehata *et al*., 1997; Ito *et al*., 2001) by such an increased sensitivity of the contractile apparatus toward Ca^{2+}, also referred to as Ca^{2+}-sensitization (Fukata *et al*., 2001).

Contractility of airway smooth muscle (ASM) preparations obtained from patients suffering from asthma has been reported increased in some (De Jongste *et al*., 1987; Bramley *et al*., 1994; Ma *et al*., 2002), but not all patients (Taylor *et al*., 1985; Cerrina *et al*., 1986; 1989). It has also been demonstrated that passive allergic sensitization *in vitro* as well as active (ragweed) sensitization *in vivo* may increase contractile responsiveness of human and canine ASM preparations, respectively (Black *et al*., 1989; Jiang *et al*., 1992; Schmidt *et al*., 2000).

Acetylcholine-induced, Rho-mediated Ca^{2+}-sensitization of bronchial smooth muscle has been found to be increased in repeatedly allergen-challenged rats (Chiba *et al*., 1999b), and since the specific Rho-kinase inhibitor (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexane carboxamide (Y-27632) did suppress airway hyperresponsiveness (AHR) in mice repeatedly challenged with ovalbumin (OA) after sensitization in the absence and presence of respiratory syncytial virus infection (Hashimoto *et al*., 2002), changes in contraction mediated by the Rho/Rho-kinase pathway could contribute to the development of AHR under these conditions.

In the present study, we considered the possibility that active allergic sensitization by itself, that is, without subsequent allergen exposure, may already induce an enhanced contribution of Rho-kinase to contractile responsiveness of guinea pig airways, both *in vivo* and *ex vivo*.

Methods

Animals

Outbred specified pathogen-free male Dunkin Hartley guinea pigs (Harlan, Heathfield, U.K.) were used. The animals were divided into two groups, that is, control animals and OA-sensitized animals. These animals were actively IgE-sensitized to OA as described previously (Van Amsterdam *et al*., 1989). In short, 0.5 ml of an allergen solution containing 100 μg ml^{-1} OA and 100 mg ml^{-1} $Al(OH)_3$ in saline was injected intraperitoneally, while another 0.5 ml was divided over seven intracutaneous injection sites in the proximity of lymph nodes in the paws, lumbar regions and the neck. Sensitization with OA induces both IgG_1- and IgE-class antibodies in the guinea pig (Regal, 1984). However, using $Al(OH)_3$ as adjuvant, a shift toward IgE-class antibodies is induced (Van Amsterdam, 1991), resulting in relatively high IgE titers as determined by the passive cutaneous anaphylaxis test (Watanabe & Ovary, 1977). The animals were used experimentally in weeks 4–8 after sensitization. The animals were group-housed in individual cages in climate-controlled animal quarters and given water and food *ad libitum*, while a 12-h on/12-h off light cycle was maintained. All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation.

Isometric tension measurements

After a sharp blow on the head and rapid exsanguination, the trachea was removed and transferred to Krebs–Henseleit (KH) buffer solution (composition in mM: NaCl 117.5, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2$ 2.5, NaH_2PO_4 1.28, $NaHCO_3$ 25.00 and D-glucose 5.55; pregassed with 95% O_2 and 5% CO_2; pH 7.4) at 37°C. The trachea was carefully prepared free of mucosa and connective tissue. Single open-ring tracheal preparations were mounted for isometric recording, using Grass FT-03 transducers, in 20 ml water-jacketed organ baths (37°C) containing KH solution. During a 90 min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 0.5 g. Subsequently, the preparations were precontracted with 20 and 40 mM KCl. Following two washouts, maximal relaxation was established by the addition of 0.1 μM isoprenaline and tension was readjusted to 0.5 g, immediately followed by two changes of fresh KH-buffer. After another equilibration period of 30 min, cumulative concentration response curves (CRCs) were constructed to stepwise increasing concentrations of methacholine (1 nM–100 μM), histamine (1 nM–100 μM) or $PGF_{2\alpha}$ (1 nM–10 μM). When maximal agonist-induced contraction was obtained, the tracheal rings were washed several times and maximal relaxation was established using isoprenaline. When used, the Rho-kinase inhibitor Y-27632 (1 μM) was applied to the organ bath 30 min before agonist addition. This concentration has been shown to be effective and selective in smooth muscle (Uehata *et al*., 1997; Gosens *et al*., 2004).

Measurement of airway function

To investigate the contribution of Rho-kinase to airway responsiveness *in vivo*, airway function of nonsensitized (control) and actively OA-sensitized unrestrained guinea pigs was assessed, by measurement of pleural pressure (P_{pl}) as described previously (Santing *et al*., 1992). In short, a small saline-filled balloon catheter was surgically implanted (2 weeks after sensitization) inside the thoracic cavity. The free end of the catheter was driven subcutaneously to the neck of the animal, where it was exposed and attached permanently. The pleural balloon was connected *via* an external saline-filled canula to a pressure transducer (Ohmeda DTX, SpectraMed, Bilthoven, The Netherlands), enabling continuous measurement of P_{pl} changes (in cm H_2O). We have previously found that changes in P_{pl} are linearly correlated to changes in airway resistance and hence can be used as a sensitive index for bronchoconstriction (Santing *et al*., 1992).

Provocation procedure

Histamine provocations were performed by inhalation of aerosolized solutions, produced by a DeVilbiss nebulizer (type 646; DeVilbiss, Somerset, PA, U.S.A.), with an airflow of 8 l min^{-1} resulting in an output of 0.33 ml min^{-1}. Provocations were carried out in a perspex cage (internal volume, 9 l) in which the guinea pigs could move freely. Before the start of the experiment, the animals were habituated to the experimental conditions and the provocation procedures on two sequential days at least 1 week after surgery, when preoperative weight was restored. On the first day, the animals were placed in the provocation cage unconnected to the pressure transducer.

After an adaptation period of 30 min, three consecutive provocations with saline were performed, each exposure lasting 3 min and separated by a 7 min interval. The next day, this procedure was repeated with the animals connected to the measurement system. During the experimental protocol following the habituation procedure, all provocations were preceded by a 30 min adaptation period, followed by one control provocation with saline (3 min) as already described.

To assess the airway reactivity for histamine, provocations with this agonist were performed starting with a 25 $\mu g\,ml^{-1}$ solution in saline, followed by increasing dosage steps of 25 $\mu g\,ml^{-1}$. Histamine provocations lasted 3 min, separated by 7 min intervals. Histamine was aerosolized until P_{pl} was increased by more than 100% above baseline for at least 3 consecutive minutes. P_{pl} returned to baseline value within 15 min after the last histamine provocation. The concentration histamine causing a 100% increase of P_{pl} (PC_{100}-value) was derived by linear intrapolation of the concentration–P_{pl} curve and was used as an index for airway reactivity toward histamine.

Since we were interested in the effect of active allergic sensitization on the contribution of Rho-kinase to airway responsiveness, we determined the effect of Y-27632 inhalation on histamine PC_{100}-values both in nonsensitized and OA-sensitized animals. To this aim, a 5 mM solution of Y-27632 in saline was aerosolized for 5 min, 30 min after the assessment of the basal histamine PC_{100}; saline inhalation was used as a control. After a resting period of 20 min, the next histamine PC_{100} measurement was started. Changes in airway responsiveness toward histamine were expressed as the ratio PC_{100} pre/post Y-27632 inhalation.

Western blotting for RhoA

Lung homogenates from control and OA-sensitized guinea pigs were prepared by pulverizing tissue under liquid nitrogen and subsequent sonification in homogenization buffer (composition in mM: NaCl 150.0, Tris HCl 10.0, 2-glycerophosphoric acid 5.0, EGTA 2.0, DTT 2.0, PMSF 1.0, Na_3VO_4 1.0, NaF 1.0 (pH 7.5), supplemented with 10 $\mu g\,ml^{-1}$ leupeptin, 10 $\mu g\,ml^{-1}$ aprotinin and 1% Triton X-100). Protein content was determined according to Bradford (1976). Protein (50 μg) per lane was separated on a 10% polyacrylamide gel. Proteins were then transferred onto nitrocellulose membranes, which were blocked overnight at 4°C in blocking buffer (composition: Tris 50.0 mM; NaCl 150.0 mM; dried milk powder 5%). After two washes in wash buffer (composition: Tris 50.0 mM; NaCl 150.0 mM; Tween-20 0.1%), membranes were incubated for 1 h at room temperature in primary antibody (anti-RhoA, Santa Cruz Biotechnology, California, U.S.A., diluted 1 : 250 in blocking buffer). After three washes, membranes were incubated in horseradish peroxidase-labelled secondary antibody (anti-rabbit IgG, Cell Signalling Technology, Beverly, U.S.A., diluted 1 : 2000 in blocking buffer) at room temperature for 1 h, followed by another three washes. Antibodies were then visualized by enhanced chemiluminescence. Photographs of blots were analysed by densitometry (Totallab™).

Data analysis

In isometric contraction experiments all curves were related to maximal histamine-induced contraction, as determined each time in at least two parallel preparations from the same animal, since no differences in maximal histamine-induced contraction were observed after active OA sensitization. All data represent means ± s.e.m. from n separate experiments. Statistical significance of difference was determined by the Student's t-test for paired observations. Differences were considered significant when $P<0.05$.

Materials

Aluminium hydroxide powder was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). OA, indomethacin, histamine and (−)-isoprenaline were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Methacholine was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.), $PGF_{2\alpha}$ from Pharmacia and Upjohn (Puurs, Belgium) and Y-27632 from Tocris Cookson Ltd (Bristol, U.K.). Enhanced chemiluminescence reagents were from Pierce (Rockford, IL, U.S.A.). All other chemicals were of analytical grade.

Results

In order to investigate the contractile properties of guinea pig ASM after active OA-sensitization, we measured both basal and agonist-induced myogenic tone in intact nonpermeabilized tracheal smooth muscle preparations. Basal tone was defined as the resting tension compared to the maximal relaxation established with isoprenaline. No effect of OA-sensitization was observed on basal myogenic tone (Figure 1). In addition, maximal contraction (E_{max}) and potency ($-\log_{10}$ of the concentration causing 50% of the effect, pEC_{50}) of methacholine were not different between preparations from control and

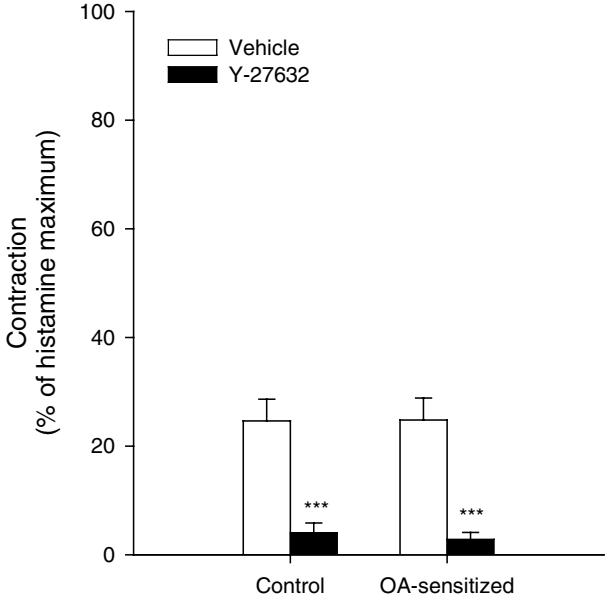

Figure 1 Effect of OA-sensitization on basal myogenic tone of open-ring tracheal smooth muscle preparations from control and OA-sensitized guinea pigs in the absence (white bars) and presence (black bars) of 1 μM Y-27632. Data represent means ± s.e.m. of 10 to 14 experiments each performed in duplicate. ***$P<0.001$ compared to vehicle.

OA-sensitized animals ($E_{max} = 2.21 \pm 0.18$ and 2.14 ± 0.16 g, respectively; $pEC_{50} = 6.62 \pm 0.12$ and 6.61 ± 0.09, respectively). However, potency of histamine and $PGF_{2\alpha}$ increased significantly after OA sensitization, whereas no effect on E_{max} was observed (Figure 2; Table 1). Similar effects of active allergic sensitization on histamine-induced contraction were found in epithelium-denuded preparations, indicating that OA-sensitization-induced differences have not been caused by epithelium-derived factors/mediators (Table 2).

Considering the effect of OA-sensitization on histamine and $PGF_{2\alpha}$-mediated contraction, we further focused on these two agonists.

To determine the contribution of Rho-kinase, we used the selective Rho-kinase inhibitor Y-27632 (1 μM). In preparations from both control and OA-sensitized animals, basal tone was almost fully inhibited by Y-27632 (Figure 1). In control preparations, treatment with Y-27632 had no significant effect on E_{max} or pEC_{50} of histamine (Figure 3a; Tables 1 and 2). In contrast, treatment with Y-27632 significantly decreased E_{max} and potency of $PGF_{2\alpha}$ in control tracheal preparations (Figure 3c; Table 1).

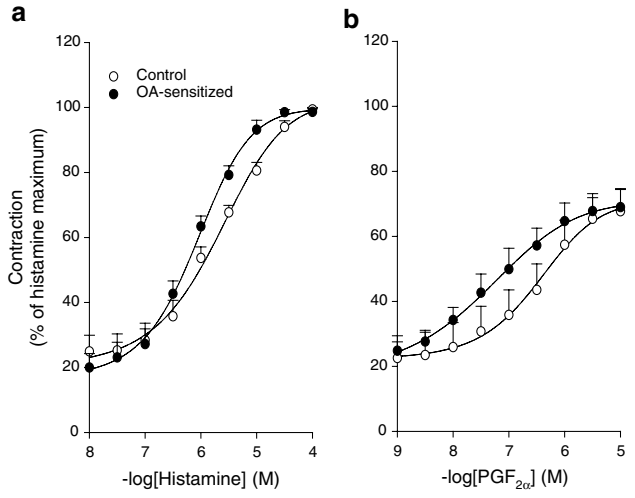

Figure 2 Effects of OA-sensitization on agonist-induced contraction. (a) Histamine- and (b) $PGF_{2\alpha}$-induced contraction of guinea pig open-ring tracheal smooth muscle preparations from control (open circles) and OA-sensitized (closed circles) guinea pigs. Data represent means ± s.e.m. of five to six experiments each performed in duplicate.

Table 1 Effects of OA-sensitization on contractile properties of guinea pig tracheal smooth muscle preparations

	Control		*OA-sensitized*	
	pEC_{50} ($-log$ M)	E_{max} (%)	pEC_{50} ($-log$ M)	E_{max} (%)
Histamine	5.68 ± 0.05	100	6.09 ± 0.05*	100
+ Y-27632	5.63 ± 0.15	104.5 ± 11.3	5.73 ± 0.09§	66.5 ± 4.8‡
$PGF_{2\alpha}$	6.54 ± 0.12	67.7 ± 6.7	7.21 ± 0.22†	69.3 ± 5.7
+ Y-27632	6.23 ± 0.14§	41.8 ± 9.3§	6.39 ± 0.11‡	35.8 ± 5.4‡

E_{max} is expressed as a percentage of the maximal histamine-induced contraction (see Methods). Data represent means ± s.e.m. of five to six experiments each performed in duplicate. *$P<0.01$ vs control; †$P<0.05$ vs control; ‡$P<0.01$ vs untreated; §$P<0.05$ vs untreated.

Remarkably, although treatment with Y-27632 had no effect on histamine-induced contraction in control preparations, Y-27632 induced a significant decrease in potency and E_{max} of histamine in preparations from OA-sensitized animals (Figure 3b; Tables 1 and 2). Treatment with the Rho-kinase inhibitor also markedly decreased potency and E_{max} of $PGF_{2\alpha}$ (Figure 3d; Table 1). Note that the OA-sensitization-induced increase in potency of histamine and $PGF_{2\alpha}$ was normalized in the presence of Y-27632.

As shown in Figure 1, Y-27632 strongly reduced basal tone. Since it could be imagined that reduction of basal tone by itself might affect the maximal response or potency of contractile agonists, separate experiments were performed using preparations from nonsensitized animals in which basal tone was decreased using indomethacin (3 μM). This treatment did not at all affect E_{max} or potency of histamine. If original basal tone was then re-established using small additions of $PGF_{2\alpha}$, potency or maximal contraction of histamine were not affected either (Figure 4).

To establish whether the contribution of Rho-kinase to airway responsiveness is enhanced *in vivo*, we investigated the effects of treatment with Y-27632, given by inhalation (5 mM, nebulizer concentration) in permanently instrumented, freely moving guinea pigs. Y-27632 inhalation decreased the histamine responsiveness both of nonsensitized and OA-sensitized animals. The histamine PC_{100} ratio pre/post Y-27632 inhalation, however, was significantly smaller in OA-sensitized animals (0.47) as compared to control animals (0.60; Figure 5).

To study the effects of OA-sensitization on RhoA protein levels, we assessed the expression of RhoA in lung homogenates by Western analysis. As shown in Figure 6, RhoA was expressed in lungs from both control and OA-sensitized animals. RhoA expression was markedly increased (2.6-fold) after OA-sensitization (Figure 6), suggesting that the enhanced role of Rho-kinase to airway responsiveness after OA-sensitization might be caused by augmented levels of RhoA.

Discussion

Rho-mediated Ca^{2+}-sensitization contributes to smooth muscle contraction under normal conditions (Gong *et al*., 1996; Ito *et al*., 2001; Sakurada *et al*., 2003). However, evidence exists that Ca^{2+}-sensitizing mechanisms may be primed by pathophysiological conditions. In vascular smooth

Table 2 Effects of OA-sensitization on contractile properties of epithelium-denuded guinea pig tracheal smooth muscle preparations

	Control		*OA-sensitized*	
	pEC_{50} ($-log$ M)	E_{max} (%)	pEC_{50} ($-log$ M)	E_{max} (%)
Histamine	5.56 ± 0.02	100	5.91 ± 0.12†	100
+ Y-27632	5.45 ± 0.07	97.1 ± 1.5	5.45 ± 0.09‡	72.23 ± 2.5‡

E_{max} is expressed as a percentage of the maximal histamine-induced contraction (see Methods). Data represent means ± s.e.m. of four to six experiments each performed in duplicate. †$P<0.05$ vs control; ‡$P<0.01$ vs untreated.

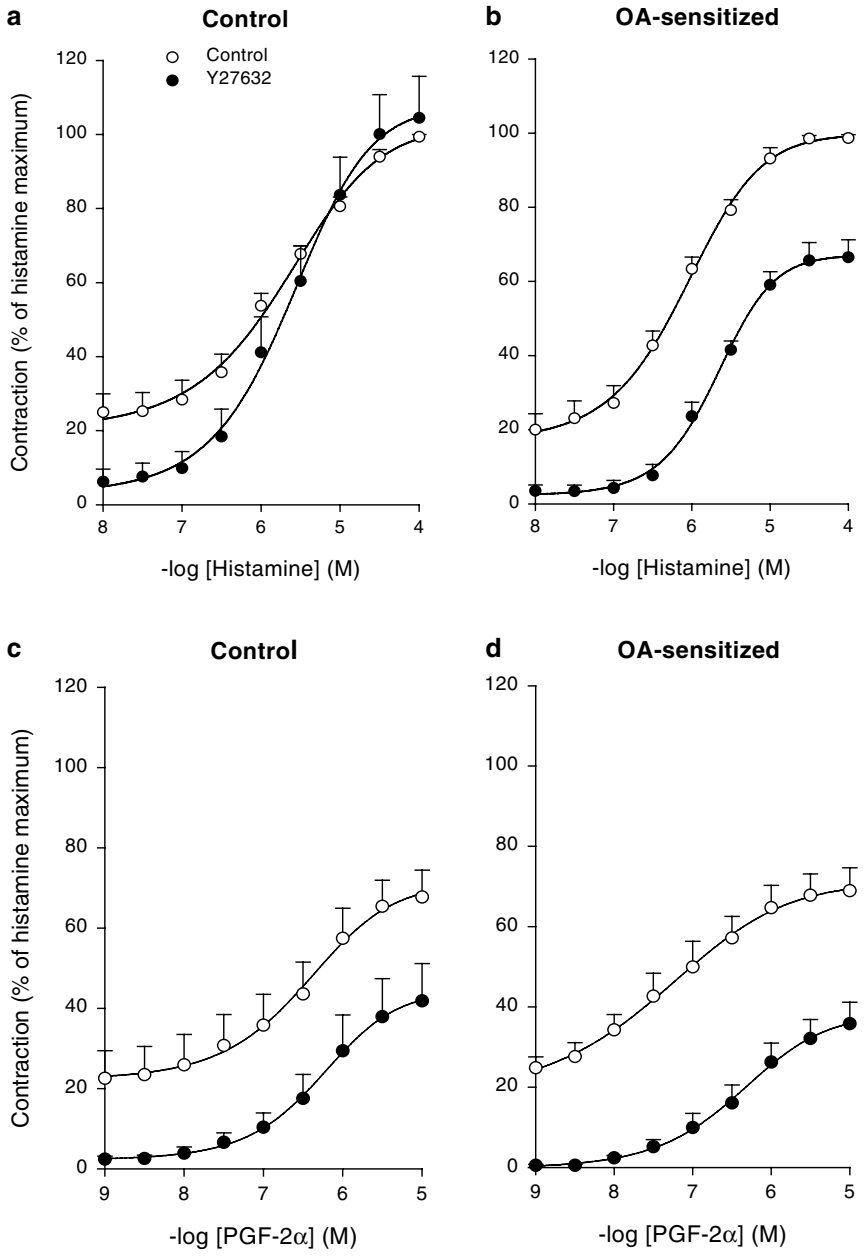

Figure 3 Effects of Rho-kinase inhibition on agonist-induced contraction. Histamine (a, b)- and $PGF_{2\alpha}$ (c, d)-induced contraction in the absence (open circles) and presence (black circles) of 1 μM Y-27632 of open-ring tracheal smooth muscle preparations of control (a, c) and OA-sensitized (b, d) guinea pigs. Data represent means$\pm$s.e.m. of five to six experiments each performed in duplicate.

muscle, increased activity of the Rho-kinase-mediated pathway has been implicated in the genesis of enhanced vasoconstriction in spontaneously hypertensive rats (Mukai *et al*., 2001). Also, in humans it has been shown that Rho-kinase could be involved substantially in the pathogenesis of increased peripheral vascular resistance in hypertension (Masumoto *et al*., 2001). In addition, in patients with vasospastic angina, acetylcholine-induced coronary artery spasm was effectively prevented by the Rho-kinase inhibitor fasudil, whereas fasudil had a minimal effect on acetylcholine-induced vasoconstriction in nonspastic segments in these patients (Masumoto *et al*., 2002).

This pathophysiology-primed role for Rho-kinase may also be important for airway diseases, since previous studies showed an augmented role of Rho-kinase in acetylcholine-induced bronchial smooth muscle contraction after repeated allergen challenge in rats (Chiba *et al*., 1999a, b). Moreover, using the specific Rho-kinase inhibitor Y-27632, we currently demonstrate that the process of active allergic sensitization by itself is sufficient to induce an enhanced role of Rho-kinase to contraction *ex vivo* and airway resistance *in vivo*. In addition, we observed a difference in Rho-kinase dependency for histamine- and $PGF_{2\alpha}$-induced contraction. Rho-kinase inhibition affected potency and E_{max} of $PGF_{2\alpha}$-induced contraction in control preparations, which corresponds to observations by Ito *et al*. (2003) in vascular smooth muscle, indicating that Rho-kinase plays an essential role in prostaglandin $F_{2\alpha}$-induced contraction. Treatment with Y-27632 had

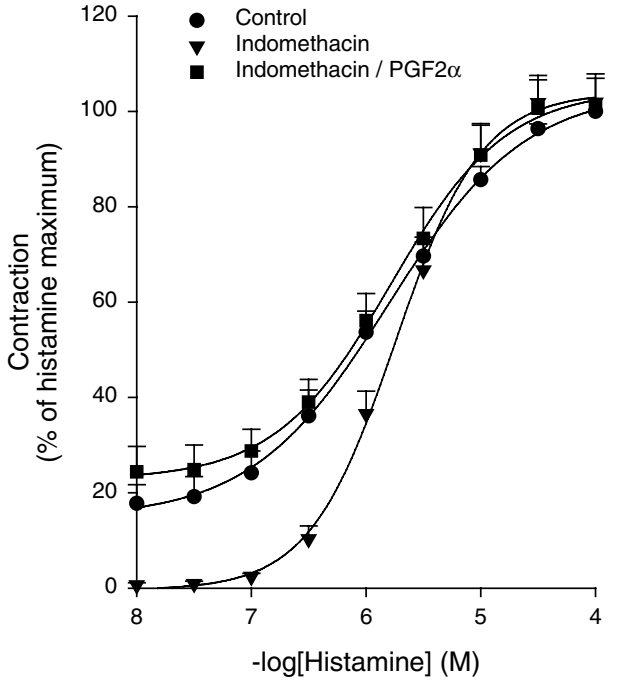

Figure 4 Effects of basal tone on histamine-induced contraction. Histamine-induced contraction of open-ring tracheal smooth muscle preparations in the absence (circles) or presence of 3 μM indomethacin (triangles) or 3 μM indomethacin plus $PGF_{2\alpha}$ (squares). Data represent means$\pm$s.e.m. of four experiments each performed in duplicate.

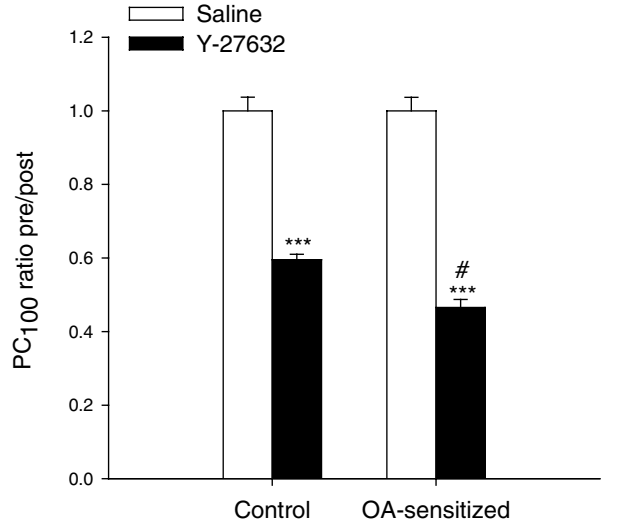

Figure 5 Effects of Y-27632 inhalation (5 mM) on airway responsiveness toward histamine in control and actively OA-sensitized guinea pigs. Effects were expressed as the histamine PC_{100} ratio pre/post saline (white bars) or pre/post Y-27632 (black bars) inhalation. Data represent means$\pm$s.e.m. of five animals. $^{***}P<0.001$ compared to saline treated; $^{\#}P<0.01$ compared to Y-27632-treated controls.

no effect on potency and E_{max} of histamine-induced contraction in control preparations, suggesting that under control situations these parameters are independent of Rho-kinase. However, treatment with Y-27632 did decrease contraction at

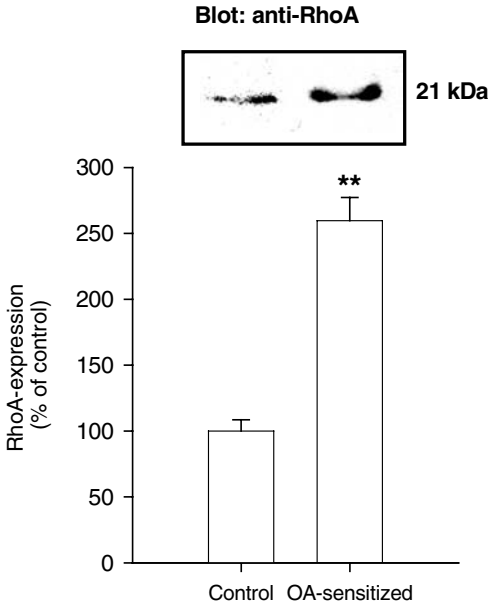

Figure 6 Western analysis of RhoA expression in lung homogenates of control ($n=4$) and OA-sensitized ($n=3$) guinea pigs. Upper panel: representative Western blot. Lane 1: control; lane 2: OA-sensitized. Lower panel: Densitometric analysis. Data represent means$\pm$s.e.m. $^{**}P<0.01$ compared to controls.

the lower part of the CRC, indicating a contribution of Rho-kinase to contraction induced by low concentrations of histamine. This fully corresponds with our finding that Rho-kinase inhibition results in a decreased airway responsiveness to histamine *in vivo*, both in control and OA-sensitized animals, since the lower part of the CRC most likely represents the relevant *in vivo* concentrations of histamine.

Mansour & Daniel (1987) reported that in guinea pig tracheal smooth muscle LTD_4 induced a significantly increased maximal contraction after sensitization, while contractile responsiveness toward other agonists tested did not alter markedly after OA-sensitization. This supports our findings that the Rho-kinase-dependent component of contraction is increased after sensitization, since leukotrienes are strongly dependent on Rho-kinase for contraction (Setoguchi *et al*., 2001).

The OA-sensitization-induced increase in potency of histamine and $PGF_{2\alpha}$ was normalized in the presence of Y-27632, indicating that this increased potency is caused by an enhanced contribution of Rho-kinase. Since MLCK is upregulated after sensitization (Jiang *et al*., 1992; Stephens *et al*., 1998; Ammit *et al*., 2000), suppression of Rho-kinase-mediated inhibition of MLCP activity would result in a more pronounced effect, because the level of MLC phosphorylation is governed by the ratio of MLCK and MLCP activities (Somlyo & Somlyo, 2003).

An increased functional role of Rho-kinase might involve the small G-protein RhoA, which has been established to be one of the main upstream activators of Rho-kinase in (airway) smooth muscle (Yoshii *et al*., 1999; Fukata *et al*., 2001; Wettschureck & Offermanns, 2002; Somlyo & Somlyo, 2003). Others have shown elevated bronchial protein levels of RhoA (Chiba *et al*., 1999b) and increased RhoA translocation (Chiba *et al*., 2001) in repeatedly challenged airway hyperreactive rats.

We observed that active allergic sensitization by itself is sufficient to induce an increase in RhoA expression, which might explain the enhanced functional contribution of Rho-kinase to airway responsiveness after OA-sensitization both *in vitro* and *in vivo*. The mechanisms underlying this augmented contribution of the Rho/Rho-kinase signalling pathway after active allergic sensitization are currently unknown.

Recent work from our laboratory already showed that Rho-kinase is a major regulator involved in the basal maintenance of contractility in bovine tracheal smooth muscle (Gosens *et al*., 2004). We concluded that long-term inhibition of Rho-kinase might be beneficial for the treatment of airway diseases, as this will result in a less contractile ASM state without the induction of a proliferative phenotype. In addition, Y-27632 inhalation inhibited acetylcholine- and OA-induced increases in lung resistance in passively sensitized guinea-pigs (Iizuka *et al*., 2000). These findings suggest that there might be an essential role for Rho-kinase in airway hyperreactivity. Furthermore, our present findings indicate that Rho-kinase might be involved in the degree (and perhaps the development) of airway hyperreactivity, in view of the observation that the sensitization-induced increase in potency of histamine and $PGF_{2\alpha}$ is reverted in the presence of Y-27632.

In conclusion, this study shows that the process of active allergic sensitization by itself without subsequent exposure to allergen is capable of altering the contractile mechanism in guinea pig ASM. This is explained by an enhanced contribution of Rho-kinase to ASM contraction, presumably involving an increased expression of RhoA. Since increased airway responsiveness to bronchospasmogenic stimuli is a characteristic feature of asthma (Hirst, 2000), inhibition of the RhoA/Rho-kinase pathway may be considered useful as pharmacotherapeutical target in allergy and asthma.

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