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Inhibition of Rho-kinase normalizes nonspecific hyperresponsiveness in passively sensitized airway smooth muscle preparations

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Abstract

Currently, little is known about mechanisms underlying passive sensitization-induced nonspecific airway hyperresponsiveness. We sought to determine whether the nonspecific airway hyperresponsiveness observed after passive sensitization involves an increased role of Rho-kinase in airway smooth muscle contraction. In addition, the contribution of Rho-kinase to specific allergen-induced airway smooth muscle contraction was studied. Guinea pig tracheal smooth muscle preparations were incubated for 16 h, in the presence of serum obtained from nonsensitized guinea pigs or atopic serum obtained from actively ovalbumin-sensitized guinea pigs. After incubation, the contribution of Rho-kinase to histamine-, methacholine- or ovalbumin-induced isometric contractions was determined, using the specific Rho-kinase inhibitor Y-27632.

Maximal contractions induced by histamine and methacholine were significantly increased in passively sensitized preparations, without a change in potency ($-\log EC_{50}$). In control preparations, Y-27632 reduced the potency of both agonists, without affecting maximal contraction. Remarkably, the increased agonist responsiveness induced by passive sensitization was fully normalized by Y-27632. Treatment with Y-27632 also reduced ovalbumin-induced contraction in these preparations.

This study shows that the nonspecific airway smooth muscle hyperresponsiveness as well as the specific allergen responsiveness induced by passive sensitization are dependent on Rho-kinase. The complete inhibition by Y-27632 of the passive sensitization-induced increased responsiveness toward histamine and methacholine indicates a pivotal role of Rho-kinase in this process.

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1. Introduction

Passive sensitization of airway smooth muscle preparations has successfully been used as a model system to investigate mechanisms of airway hyperresponsiveness (Schmidt and Rabe, 2000). Thus, in nonsensitized human airway smooth muscle preparations it has been demonstrated that pre-incubation with atopic serum not only induced a 'specific' contractile response to the allergen, but also enhanced the responsiveness to various 'nonspecific' stimuli, including histamine, leukotriene C₄, contractile neuropeptides and KCl (Black et al., 1989; Marthan et al., 1992; Schmidt et al., 1999; Ben-Jebria et al., 1993). In addition, passive sensitization of human bronchi has been shown to increase maximal shortening velocity and max-

imal shortening capacity of the smooth muscle in response to electrical field stimulation (Mitchell et al., 1994).

The allergen-induced contractile response is fully dependent on immunoglobulin E (IgE), as no response was observed after incubation with IgE-depleted serum or serum containing anti-IgE-antibodies (Watson et al., 1998; Rabe et al., 1998). In contrast, neither the removal of IgE (Watson et al., 1998), nor the presence of anti-IgE-antibodies (Rabe et al., 1998) affected the nonspecific hyperresponsiveness toward histamine, indicating that other factors present in the serum of allergic asthmatics, possibly cytokines (Schmidt and Rabe, 2000), are likely to be major determinants of the increased airway smooth muscle responsiveness.

Remarkably, little is known about sensitization-induced alterations in signaling mechanisms of contractile agonists. Both in canine (Jiang et al., 1992) and in human airway smooth muscle (Ammit et al., 2000), it has been demonstrated that active allergic sensitization is capable of increasing myosin

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light chain kinase (MLCK)-content. This increase might be responsible for the enhanced reactivity of sensitized tissue, since agonist-induced airway smooth muscle contraction involves myosin light chain (MLC) phosphorylation induced by Ca^{2+} -calmodulin-mediated activation of MLCK. Increased MLC phosphorylation can also occur as a consequence of Ca^{2+} -sensitization (Fukata et al., 2001; Somlyo and Somlyo, 2003), which involves inhibition of myosin light chain phosphatase (MLCP) by the Rho/Rho-kinase pathway (Yoshii et al., 1999; Ito et al., 2001; Somlyo and Somlyo, 2003; Amrani et al., 2004).

Agonist-induced Rho-mediated Ca^{2+} -sensitization of airway smooth muscle has been found to be increased in repeatedly allergen-challenged rats with airway hyperresponsiveness (Chiba et al., 1999). In addition, the specific Rho-kinase inhibitor Y-27632 suppressed allergen-induced airway hyperresponsiveness in mice repeatedly challenged with ovalbumin after active sensitization in the absence and presence of respiratory syncytial virus infection (Hashimoto et al., 2002). We have recently demonstrated that the process of active allergic sensitization by itself, without subsequent allergen exposure, is sufficient to induce an enhanced role of Rho-kinase in guinea pig airway smooth muscle contraction *ex vivo* and airway responsiveness *in vivo* (Schaafsma et al., 2004).

Based on these observations, we hypothesize that the contribution of Rho-kinase to airway smooth muscle contraction after passive sensitization is increased, which could underlie the nonspecific hyperresponsiveness in this model. Therefore, the present study focused on the role of Rho-kinase in the increased responsiveness of passively sensitized guinea pig tracheal smooth muscle preparations to histamine and methacholine. Moreover, the Rho-kinase dependency of ovalbumin-induced contraction in these preparations was investigated as well.

2. Methods

2.1. Animals

Outbred specified pathogen-free male Dunkin Hartley guinea pigs (Harlan, Heathfield, U.K.) were used. All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation.

2.2. Active sensitization procedure

Guinea pigs were actively IgE-sensitized to ovalbumin as described previously (Van Amsterdam et al., 1989). In short, 0.5 ml of an allergen solution containing 100 $\mu\text{g/ml}$ ovalbumin and 100 mg/ml $\text{Al}(\text{OH})_3$ 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 ovalbumin induces both IgG₁- and IgE-class antibodies in the guinea pig (Regal, 1984). However, using $\text{Al}(\text{OH})_3$ 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 and Ovary,

1977). The animals were used experimentally in weeks 4 to 8 after sensitization.

2.3. Passive sensitization procedure

Blood from nonsensitized and actively ovalbumin-sensitized guinea pigs was collected by heart puncture, immediately after experimental concussion. The blood was left standing to clot for 10 min at room temperature. Subsequently, the blood was centrifuged at 170 g, after which the serum was taken. Serum of 5 animals of each group was pooled.

Tracheae from nonsensitized guinea pigs were removed and transferred to Krebs–Henseleit (KH) buffer (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; continuously gassed with 95% O_2 and 5% CO_2 ; pH 7.4) at 37 °C. The trachea was carefully prepared free of serosa and connective tissue and was cut into single ring preparations. The rings were then transferred to a six-well cluster plate (3 rings/well) containing 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), gentamycin (45 $\mu\text{g/ml}$), penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$) and amphotericin B (1.5 $\mu\text{g/ml}$). The wells were supplemented with either 10% (vol/vol) control or 10% (vol/vol) atopic serum, after which the rings were incubated in an incubator shaker (37 °C, 55 rpm) for 16 h.

2.4. Isometric tension measurements

After incubation, the preparations were mounted for isometric recording 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 wash-outs, maximal relaxation was established by the addition of 0.1 μM isoproterenol and tension was re-adjusted to 0.5 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 histamine (1 nM–100 μM) or methacholine (1 nM–100 μM). When maximal histamine- or methacholine-induced contraction was obtained, the tracheal rings were washed several times and the effectiveness of passive sensitization was determined by applying 10 $\mu\text{g/ml}$ ovalbumin to each organ bath. All preparations incubated with atopic serum responded with a marked contraction after ovalbumin-application (1.56 ± 0.16 g), whereas no contraction was observed in control serum-treated preparations (0.03 ± 0.01 g). After washing the preparations, maximal relaxation was re-established using isoproterenol. When used, 1 μM of the Rho-kinase inhibitor Y-27632 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., 2004b; Schaafsma et al., 2004). In separate experiments, 10 $\mu\text{g/ml}$ ovalbumin was added to the organ bath

instead of histamine or methacholine, to establish the effect of Rho-kinase inhibition on the specific allergen-induced contraction.

2.5. Data analysis

All data represent means $\pm$ S.E.M. from n separate experiments. Statistical significance of differences was determined by the Student's t -test for paired observations. Differences were considered significant when $P < 0.05$.

2.6. Materials

Ovalbumin, histamine dihydrochloride and (–)-isoproterenol hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Methacholine was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). (+)-(R)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexane carboxamide (Y-27632) was obtained from Tocris Cookson Ltd. (Bristol, U.K.). All other chemicals were of analytical grade.

3. Results

3.1. Effects of Rho-kinase inhibition on passive sensitization-induced airway smooth muscle hyperresponsiveness

No effect of passive sensitization was observed on basal myogenic tone (i.e. the resting tension compared to the maximal relaxation established with isoproterenol) (Fig. 1). In contrast, maximal contraction ($E_{\max}$) induced by histamine (Fig. 1, A) and methacholine (Fig. 1, B) was significantly increased after passive sensitization, whereas no effect on the potency ($-\log EC_{50}$) of these agonists was observed (Table 1). In control

Table 1

Effects of passive sensitization on contractile properties of guinea pig tracheal smooth muscle preparations

	Control serum		Atopic serum	
	$E_{\max}$ (g)	$-\log EC_{50}$	$E_{\max}$ (g)	$-\log EC_{50}$
Histamine				
Vehicle	0.84 ± 0.11	5.91 ± 0.18	1.44 ± 0.23^a	5.74 ± 0.08
Y-27632	0.76 ± 0.07	5.47 ± 0.08^b	0.84 ± 0.13^c	5.54 ± 0.04^b
Methacholine				
Vehicle	1.61 ± 0.14	6.66 ± 0.11	2.38 ± 0.24^a	6.57 ± 0.14
Y-27632	1.75 ± 0.14	6.19 ± 0.08^d	1.72 ± 0.14^b	6.16 ± 0.04^b

Data represent means $\pm$ S.E.M. of five experiments, each performed in duplicate.

^a $P < 0.05$, compared to control serum-treated; ^b $P < 0.05$, compared to vehicle;

^c $P < 0.01$, compared to vehicle; ^d $P < 0.001$, compared to vehicle.

serum-treated preparations a significant decrease in potency of both histamine and methacholine was observed in the presence of Y-27632, whereas no effect of Rho-kinase inhibition was observed on $E_{\max}$ (Fig. 1, Table 1). Also in atopic serum-treated preparations, treatment with Y-27632 resulted in a significant decrease in potency of both agonists, which was quantitatively similar to that observed in the control preparations (Fig. 1, Table 1). Remarkably, the passive sensitization-induced increase in $E_{\max}$ toward both histamine and methacholine was fully normalized in the presence of Y-27632 (Fig. 1, Table 1).

3.2. Effects of Rho-kinase inhibition on specific allergen-induced airway smooth muscle contraction

In both vehicle- and Y-27632-treated passively sensitized tracheal preparations, ovalbumin (10 μ g/ml) induced a rapid contractile response that reached a maximum within 10 min, followed by a gradual decline of the contraction (Fig. 2). The maximal ovalbumin-induced contraction was significantly

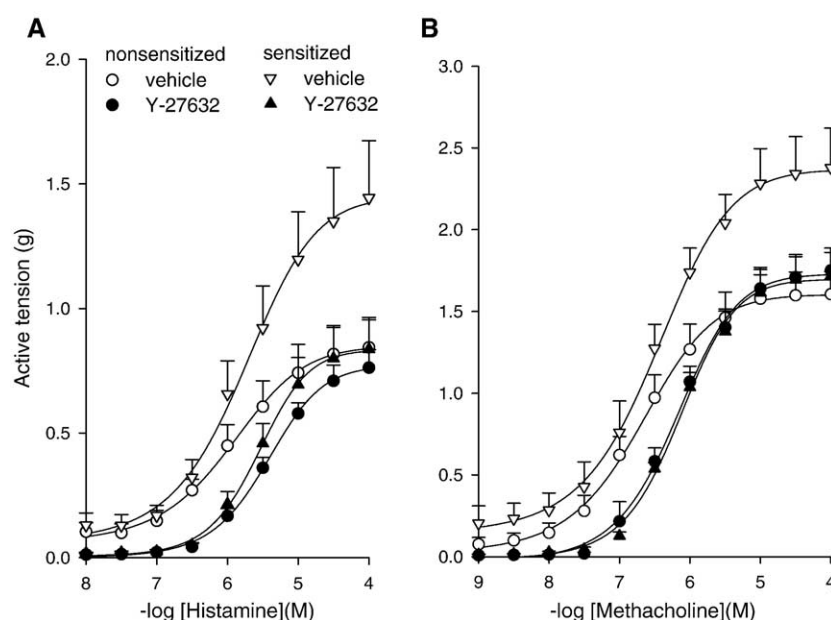


Fig. 1. Effects of Rho-kinase inhibition on nonspecific airway smooth muscle hyperresponsiveness after passive sensitization. Histamine (A)- and methacholine (B)-induced contraction in the absence (open symbols) and presence (closed symbols) of 1 μ M Y-27632 of nonsensitized (circles) and passively sensitized (triangles) guinea pig open-ring tracheal smooth muscle preparations. Data represent means $\pm$ S.E.M. of five experiments, each performed in duplicate.

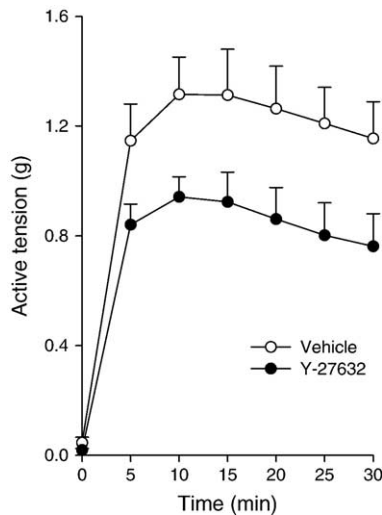


Fig. 2. Effects of Rho-kinase inhibition on passive sensitization-induced allergen responsiveness. Ovalbumin (10 μ g/ml)-induced contraction in the absence (open symbols) and presence (closed symbols) of 1 μ M Y-27632 of passively sensitized guinea pig open-ring tracheal smooth muscle preparations. Data represents means $\pm$ S.E.M. of 4 experiments each performed in duplicate.

reduced by $28 \pm 4\%$ ($P < 0.05$) in the Y-27632-treated preparations (0.96 ± 0.08 g) as compared to vehicle-treated preparations (1.34 ± 0.13 g).

4. Discussion

In the present study, we demonstrated that passive sensitization-induced nonspecific hyperresponsiveness of guinea pig tracheal preparations toward histamine and methacholine, is fully normalized in the presence of Y-27632, indicating a crucial role for Rho-kinase in this process. This finding corresponds well with our previous study, which demonstrated that the contribution of Rho-kinase to contractile airway responsiveness, both in vivo and ex vivo, is augmented after active allergic sensitization (Schaafsma et al., 2004).

In accordance with our previous findings, it was demonstrated that Rho-kinase inhibition had no effect on histamine- and methacholine-induced maximal contractions of nonsensitized preparations, indicating that under normal conditions this parameter is independent of Rho-kinase. In freshly isolated guinea pig tracheal preparations (Schaafsma et al., 2004) and in human bronchial smooth muscle preparations (Gosens et al., 2004a), Y-27632 does not affect potency of histamine-induced contraction. However, we found that preparations treated with nonsensitized control serum demonstrated a slight but significant rightward shift of the concentration response curve to both agonists after treatment with Y-27632. This may suggest that incubation of guinea pig tracheal preparations with normal serum ex vivo induces some role for Rho-kinase in the sensitivity to the agonist or that there might be species or airway generation related differences in Rho-kinase dependency of histamine-induced contractions. Notably, in the actively (Schaafsma et al., 2004) as well as in the passively sensitized (present study) preparations, inhibition of Rho-kinase caused a significant reduction of both $E_{\max}$ and

sensitivity to the agonists, indicating common effects on the Rho-kinase signaling pathway by either route of allergic sensitization. Importantly, this may imply that one or more systemic factors present in the atopic serum increase the involvement of Rho-kinase to agonist-induced airway smooth muscle contraction. This process could involve increased expression of RhoA, as found after active allergic sensitization (Schaafsma et al., 2004).

In passively sensitized airway smooth muscle preparations, it has previously been established that factors other than IgE present in atopic serum are likely to be the major determinants of this hyperresponsiveness (Watson et al., 1998; Rabe et al., 1998; Schmidt and Rabe, 2000). However, the nature of these factors is presently unclear. Possible factors involved might include growth factors, including angiotensin II. Thus, it has been reported that serum levels of angiotensin II are increased in patients with asthma (Millar et al., 1994), while angiotensin II has been shown to increase the expression (both mRNA and protein) and function of Rho-kinase in human vascular smooth muscle (Hiroki et al., 2004). In addition, several growth factors, including angiotensin II, platelet-derived growth factor, epidermal growth factor and insulin like growth factor-1, were shown to induce sustained guinea pig (Schaafsma et al., 2005) and human (Gosens et al., 2004a) airway smooth muscle contractions, that are fully dependent on Rho-kinase activity.

Several cytokines, including tumor necrosis factor- α (TNF- α) (Hunter et al., 2003) and the T_H2 -type cytokines interleukin-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Hakonarson et al., 1999), have been associated with airway hyperresponsiveness in asthma. It has been demonstrated that TNF- α is capable of inducing Rho/Rho-kinase-mediated Ca^{2+} -sensitization both in guinea pig and in human airway smooth muscle (Hunter et al., 2003). In addition, GM-CSF has been shown to activate RhoA in human monocytic cells (Kohno et al., 2004). However, further investigations are warranted to reveal the precise mechanisms responsible for the augmented role of Rho-kinase in airway smooth muscle contraction after either passive and active sensitization.

In addition to its effects on nonspecific airway hyperresponsiveness, we also studied the effects of Y-27632 on specific allergen responsiveness. As expected, no response to ovalbumin was observed in nonsensitized preparations, whereas a marked contraction after ovalbumin-application was observed in the passively sensitized preparations. These findings correspond to previous studies using human isolated bronchial smooth muscle, in which passive sensitization has been demonstrated to induce a response to the specific allergen and to anti-IgE (Dunlop et al., 1977; Tunon de Lara et al., 1995).

Ovalbumin-induced contractions were partially inhibited by Y-27632, indicating a role for Rho-kinase in specific allergen responsiveness. These observations are in agreement with findings that intravenously administered ovalbumin in passively sensitized guinea pigs induced an increase in lung resistance, which was partially inhibited by Y-27632 inhalation (Iizuka et al., 2000). It could be envisaged however, that these effects of Y-27632 reflect an involvement of Rho-kinase in allergen-induced mediator release. However, the ovalbumin (10 μ g/ml)

and the histamine (10–30 μ M) induced contractions of the sensitized preparations, which reach the same magnitude, are reduced to a similar extent by Y-27632. Furthermore, it has been demonstrated in rat peritoneal mast cells that secretion is dependent on Rho, but not on Rho-kinase (Sullivan et al., 1999). Presumably, the effects of Y-27632 on specific allergen-induced responsiveness are explained by the fact that mediators released by mast cell degranulation, such as leukotrienes, growth factors and histamine (Robinson, 2004), depend on Rho-kinase for their contractile effects (Setoguchi et al., 2001; Tokuyama et al., 2002; Gosens et al., 2004a; Schaafsma et al., 2004).

In conclusion, in guinea pig tracheal preparations it was demonstrated that passive sensitization-induced nonspecific airway smooth muscle hyperresponsiveness as well as specific allergen responsiveness are dependent on Rho-kinase. Moreover, it was shown that the nonspecific hyperresponsiveness toward histamine and methacholine is fully normalized by Rho-kinase inhibition, indicating that Rho-kinase plays a pivotal role in the increased agonist-induced contraction after passive sensitization. Since increased airway responsiveness to allergic and nonallergic stimuli is a major determinant of allergic asthma (Schmidt and Rabe, 2000), inhibition of Rho-kinase may be considered useful as pharmacotherapeutic target in this disease.

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