Possible involvement of Rho kinase in Ca\(^{2+}\) sensitization and mobilization by MCh in tracheal smooth muscle

SATORU ITO, HIROAKI KUME, HARUO HONJO, HIDEKI KATOH, ITSUO KODAMA, KENICHI YAMAKI, AND HIDEHARU HAYASHI

Possible involvement of Rho kinase in Ca\(^{2+}\) sensitization and mobilization by MCh in tracheal smooth muscle. Am J Physiol Lung Cell Mol Physiol 280: L1218–L1224, 2001.—We examined the effects of Rho kinase on contraction and intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\text{i}) in guinea pig trachealis by measuring isometric force and the fura 2 signal [340- to 380-nm fluorescence ratio (F340/F380)]. A Rho kinase inhibitor, Y-27632 (1–1,000 \(\mu M\)), inhibited methacholine (MCh)-induced contraction, with a reduction in F340/F380 in a concentration-dependent manner. The values of EC\(_{50}\) for contraction and F340/F380 induced by 1 \(\mu M\) MCh with Y-27632 were 27.3 ± 5.1 and 524.1 ± 31.0 \(\mu M\), respectively. With 0.1 \(\mu M\) MCh, the values for these parameters were decreased to 1.0 ± 0.1 and 98.2 ± 6.2 \(\mu M\), respectively. Tension-F340/F380 curves for MCh indicated that Y-27632 caused an ~50% inhibition of MCh-induced contraction, without a reduction in F340/F380. These effects of Y-27632 were not inhibited by a protein kinase C inhibitor, GF-109203X. Our results indicate that inhibition of Rho kinase attenuates both Ca\(^{2+}\) sensitization and [Ca\(^{2+}\)]\text{i}.

methacholine; calcium sensitivity; fura 2; protein kinase C; small G proteins

Although an increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\text{i}) plays an important role in the contraction of airway smooth muscle (7), it is generally considered that muscarinic receptor agonists and histamine increase tension at a constant [Ca\(^{2+}\)]\text{i}. This phenomenon is referred to as Ca\(^{2+}\) sensitization (31) and is mediated by a GTP-binding (G) protein-coupled mechanism (14, 15). Because hyperreactivity to these agonists is a hallmark of bronchial asthma, an increase in sensitivity to intracellular Ca\(^{2+}\) may be involved in the pathophysiology of this disease (3). Muscarinic receptor agonists cause a transient increase in [Ca\(^{2+}\)]\text{i}, mediated by Ca\(^{2+}\) release from the sarcoplasmic reticulum by inositol 1,4,5-trisphosphate production (9), followed by a reduction in sustained [Ca\(^{2+}\)]\text{i} level that is higher than the control level (29), indicating that Ca\(^{2+}\) sensitization may be involved in tonic contraction. However, because airway contraction with carbachol is not sustained in the absence of Ca\(^{2+}\) on the extracellular side (1), it is generally considered that Ca\(^{2+}\) influx is also needed to produce tonic contraction. Not only Ca\(^{2+}\) sensitization but also Ca\(^{2+}\) mobilization may be involved in contraction induced by various receptor agonists.

It has been revealed that RhoA, a small monomeric G protein, enhances sensitivity to Ca\(^{2+}\) by affecting myosin light chain processes in smooth muscle (6, 8, 10), including that in the airways (4). Recently, it was found that Rho-associated protein kinase (Rho kinase), which is a target protein of RhoA (12, 13, 25), inhibits myosin light chain phosphatase (MLCP) activity. Y-27632, a selective Rho kinase inhibitor (34), effectively decreases Ca\(^{2+}\) sensitivity mediated by various agonists such as carbachol, histamine, phenylephrine, and endothelin-1 under the condition of a constant [Ca\(^{2+}\)]\text{i} with skinned fiber from smooth muscle (34, 35). The effects of Y-27632 were mimicked by Clostridium botulinum C3 toxin (4) and Clostridium difficile toxin B (22), direct inhibitors of Rho proteins. Therefore, it is now appreciated that the inhibition of MLCP activity through the Rho/Rho kinase pathway is the main mechanism of Ca\(^{2+}\) sensitization (32).

Although permeabilized preparations that allow control of [Ca\(^{2+}\)]\text{i}, have been commonly used to characterize Ca\(^{2+}\) sensitivity, a loss of proteins and ions important for contraction cannot be excluded during the permeabilization procedure by incubation with \(\alpha\)-toxin or \(\beta\)-escin. In addition to this, the Ca\(^{2+}\)-clamp technique is very beneficial in the research of Ca\(^{2+}\) sensitization, whereas this method is not appropriate for analyzing the modification in [Ca\(^{2+}\)]\text{i}. Little is currently known about the effects of Rho/Rho kinase processes on the relationships between tension and [Ca\(^{2+}\)]\text{i} induced by agonists in intact (nonpermeabilized) smooth muscle.

In this study, to determine the role of Ca\(^{2+}\) sensitization and Ca\(^{2+}\) mobilization in muscarinic receptor stimulation, we analyzed relationships between tension...
sion and \([\text{Ca}^{2+}]_i\) regulated by Rho/Rho kinase processes using intact guinea pig tracheal smooth muscle.

**METHODS**

**Tissue preparation and isometric tension records.** The methods are essentially similar to those previously described (19, 20, 30). Male guinea pigs (250–350 g) were killed by stunning and bleeding, and the tracheae were excised. The tracheal rings were opened by cutting longitudinally at the cartilaginous region, and the epithelium was dissected out. Segments containing one cartilaginous ring (for isometric tension recording) and two cartilaginous rings (for \([\text{Ca}^{2+}]_i\) measurements) were prepared. The strip was 1.5–2.0 mm in width by 4.5–5.0 mm in length and 0.3–0.4 mg in wet weight at each cartilaginous lesion. A preparation was placed horizontally in an organ bath (0.6-ml volume). One end of the segment was fixed to the chamber, and the other end was connected to a force displacement transducer to monitor isometric tension. After the initial response to 1 M methacholine (MCh) was recorded, the experiments were started, and an equimolar concentration of MCh was applied at the end of each experiment to determine whether the strips were contracted. Isometric tension and \([\text{Ca}^{2+}]_i\) measurements were made when the muscle contraction had returned to a steady state. The temperature of the muscle strip was maintained at 37°C.

**Measurement of fura 2 fluorescence.** Muscle strips were treated with 10 μM fura 2-AM for 4 h at room temperature (22–24°C). The nontoxic detergent pluronic F-127 (0.01% wt/vol) was added to increase the solubility of fura 2-AM. After the loading, the chamber was perfused with the normal solution at 37°C for 50 min to wash out the extracellular fura 2-AM before the measurements. Isometric tension and fura 2 fluorescence of the muscle strips were measured simultaneously with a displacement transducer and a spectrofluorometer (CAF-110, Japan Spectroscopic, Tokyo, Japan). The method for the measurement of \([\text{Ca}^{2+}]_i\) was as previously described (30). The mucosal side of the muscle strips was exposed to the excitation light, and the light emitted from the strip was collected in a photomultiplier through a 500-nm filter. The intensities of fluorescence due to excitation at 340 (F340) and 380 (F380) nm were measured after background subtraction. The absolute amount of \([\text{Ca}^{2+}]_i\) was not calculated because the dissociation constant of fura 2 for \([\text{Ca}^{2+}]_i\) in smooth muscle cytoplasm may be different from that obtained in vitro (16). Therefore, the ratio of F340 to F380 (F340/F380) is used as a relative indicator of \([\text{Ca}^{2+}]_i\). Because force generation with an identical concentration of MCh varied with each strip, the effects of agents are also expressed by percent normalization. Measurements of tension and F340/F380 were made when the muscle contraction had attained a steady state. Muscle tension and F340/F380 in the resting state were taken as 0%, and the values for percent contraction and F340/F380 were calculated by taking the responses to 0.1 and 1 μM MCh in the control tissues as 100%. Time-matched control tissues were treated similarly to the test tissues but were exposed to the normal bathing solution instead of Y-27632 (see Fig. 2).

**Materials.** MCh, indomethacin, nifedipine, pluronic F-127, and 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide (GF-109203X) were obtained from Sigma (St. Louis, MO). Phorbol-12,13-dibutyrate was from Calbiochem (La Jolla, CA). Fura 2-AM was obtained from Dojin Laboratories (Kumamoto, Japan). (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cylohexanecarboxamide dihydrochloride, monohydrate (Y-27632) was a gift from Welfide (Osaka, Japan). Fura 2-AM was dissolved in DMSO, and the final DMSO concentration did not exceed 0.5%. Y-27632 was dissolved in distilled water as stock solution (100 mM), and stored at −20°C. Neither solvent affected muscle tension or fluorescence ratio.

**Statistical analysis.** All data are expressed as means ± SE; n is the number of preparations used. Student’s unpaired t-test was used to evaluate the significance of differences between means, with P < 0.05 as the level of significance.

**RESULTS**

**Effects of Y-27632 on contraction and \([\text{Ca}^{2+}]_i\) mobilization induced by MCh.** To determine the involvement of Rho/Rho kinase processes in \([\text{Ca}^{2+}]_i\) sensitization and mobilization, the effects of Y-27632, a selective Rho kinase inhibitor, on MCh-induced contraction were investigated. Y-27632 (1–1,000 μM) was cumulatively applied to the fura 2-loaded strips precontracted with 1 μM MCh for 10 min at each concentration (Fig. 1A). Application of Y-27632 (1–1,000 μM) resulted in an inhibition of MCh-induced contraction in a concentration-dependent manner. Y-27632 (1–10 μM) inhibited 1 μM MCh-induced contraction, with little change in F340/F380. However, when concentrations of Y-27632 increased to >100 μM, a significant reduction in F340/F380 occurred, with relaxation in a concentration-dependent manner. Figure 1B shows the summarized data of the effects of Y-27632 on tension and F340/F380 for 0.1 and 1 μM MCh-induced contraction. The values of EC50 on tension and F340/F380 for 1 μM MCh with Y-27632 inhibition were 27.3 ± 5.1 and 524.1 ± 31.0 μM (n = 6). On the other hand, when the concentration of MCh was decreased to 0.1 μM, 10 μM Y-27632 caused an increase in the inhibitory effects against MCh-induced contraction, with a reduction in F340/F380. Concentration-inhibition curves for Y-27632 on MCh-induced contraction were roughly 10-fold shifted to the left, and the value of EC50 was decreased to 1.0 ± 0.1 μM (n = 6; Fig. 1B). Furthermore, concentration-inhibition curves for Y-27632 on MCh-induced contraction F340/F380 were also roughly fivefold shifted to the left, and the value of EC50 was decreased to 98.2 ± 6.2 μM (n = 6; Fig. 1B). To determine the relationship between mechanical response and \([\text{Ca}^{2+}]_i\), tension-F340/F380 curves for MCh with Y-27632 were calculated from the data shown in Fig. 1B (Fig. 1C). The curves did not shift markedly between 0.1 and 1 μM MCh (Fig. 1C). Y-27632 caused an ~50% inhibition of the MCh-induced contraction, with no change in F340/F380 at each concentration of MCh between 0.1 and 1 μM. However, this agent caused further inhibition, with a reduction in \([\text{Ca}^{2+}]_i\) (Fig. 1C).

**Effects of Rho kinase on \([\text{Ca}^{2+}]_i\) sensitization induced by MCh.** The inhibitory action of Y-27632 on contraction and \([\text{Ca}^{2+}]_i\), induced by MCh (0.01–10 μM) was examined. MCh (0.01–10 μM) was cumulatively applied, and then after exposure to the normal bathing solution for 10 min, MCh was cumulatively applied
again to the identical tissues in both the presence and absence of 10 μM Y-27632 for a time-matched control study. Under this experimental condition, we examined the effects of Y-27632 on the relationship between tension and [Ca$^{2+}$]$_{i}$ by MCh (0.01–10 μM). Y-27632 was applied 3 min before the administration of MCh. Figure 2A shows a representative trace of the cumulative effects of MCh on tension and F340/F380 at the first application in the absence of Y-27632 and at the second application in the presence of this agent. When MCh was cumulatively applied twice, before and after washout in a time-matched control study, MCh caused an increase in F340/F380, with an increase in tension in a concentration-dependent manner, and the tension-F340/F380 curves were not affected between the first and second applications in the absence of Y-27632 (data not shown; n = 6). Although both contraction and F340/F380 induced with 0.1 μM MCh were abolished in the presence of 10 μM Y-27632, this agent inhibited tension induced with 0.1–10 μM MCh, with a modest reduction in F340/F380 (Fig. 2A). The tension-F340/F380 curves for MCh (0.01–10 μM) were examined in both the absence (n = 6) and presence (n = 8) of 10 μM Y-27632, with the time-matched control study for comparison (Fig. 2B). Values of percent tension for MCh (0.01–10 μM) were attenuated in the presence of 10 μM Y-27632 at each concentration. Y-27632 shifted tension-F340/F380 curves to the right.

**Mechanisms of reduction in [Ca$^{2+}$]$_{i}$ induced by Rho kinase inhibition.** To determine whether Y-27632 inhibits Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels (VDCCs), tension and F340/F380 with Y-27632 were measured in the presence of nifedipine, an inhibitor of VDCCs. Nifedipine (1 μM) was applied to the fura 2-loaded strips precontracted with 1 μM MCh for 10 min. Nifedipine reduced F340/F380, with a small decrease in tension; however, application of Y-27632 (100 μM) caused a further decrease in tension, with a modest reduction in F340/F380 (Fig. 3A). Figure 3B shows that the percent contraction and percent F340/F380 for MCh with nifedipine were 90.5 ± 4.1 and 66.7 ± 5.2%, respectively. In the presence of 1 μM nifedipine, those values for MCh with 100 μM Y-27632 were 20.5 ± 4.1 and 49.4 ± 6.5%, respectively (n = 5; Fig. 3B). On the other hand, those values for MCh with 100 μM Y-27632 were 28.2 ± 5.1 and 78.5 ± 4.5%, respectively (n = 6; Fig. 1B). The inhibitory effects of nifedipine and Y-27632 on the change in F340/F380...
were additive, indicating that a reduction in \([\text{Ca}^{2+}]_i\) with Y-27632 was not affected by nifedipine.

Effects of protein kinase C on MCh-induced contraction and on Rho-induced \(\text{Ca}^{2+}\) sensitization. To determine the involvement of protein kinase (PK) C on MCh-induced \(\text{Ca}^{2+}\) sensitization, the inhibitory action of Y-27632 on MCh-induced contraction was examined in the presence of GF-109203X, a membrane-permeable inhibitor of PKC (33). MCh (1 \(\mu\)M) was applied to the fura 2-loaded strips for 10 min, and after washout for 5 min, 5 \(\mu\)M GF-109203X was perfused. After incubation with GF-109203X for 30 min, 1 \(\mu\)M MCh was again applied to the identical tissues, and then 10 \(\mu\)M Y-27632 was applied (Fig. 4A). A significant reduction in tension and F340/F380 with 1 \(\mu\)M MCh did not occur in the presence of 5 \(\mu\)M GF-109203X (Fig. 4, B and C). Moreover, subsequent application of 10 \(\mu\)M Y-27632 caused a reduction in tension produced with 1 \(\mu\)M MCh in the presence of 5 \(\mu\)M GF-109203X (Fig. 4A). The percent contraction for MCh with Y-27632 in the presence and absence of GF-109203X was 63.0 \(\pm\) 4.0 (\(n = 5\)) and 60.5 \(\pm\) 6.0% (not significant; \(n = 5\)), respectively (Fig. 4B), whereas the percent F340/F380 for MCh with Y-27632 in the presence and absence of GF-109203X was 91.5 \(\pm\) 5.6 (\(n = 5\)) and 90.6 \(\pm\) 7.1% (not significant; \(n = 5\)), respectively (Fig. 4C). GF-109203X did not affect tension or F340/F380 regulated with Y-27632.

DISCUSSION

This study demonstrates for the first time the effects of inhibition of Rho kinase on tension and \([\text{Ca}^{2+}]_i\) induced by a muscarinic receptor agonist in airway smooth muscle. Previous reports (6, 13, 34, 35) have demonstrated that inactivation of MLCP by Rho kinase leads to agonist-induced \(\text{Ca}^{2+}\) sensitization. When Y-27632 is cumulatively applied to permeabilized tissues under the condition of \(\text{Ca}^{2+}\) clamp, this agent inhibited agonist- and guanosine 5'-O-(2-thiotriphospho-
phate)-induced Ca\(^{2+}\) sensitization in various types of smooth muscle in a concentration-dependent manner (5, 11, 34, 35). Based on a previous report (29) with intact fura 2-loaded smooth muscle, we examined the relationship between tension and [Ca\(^{2+}\)]\(_i\) induced by MCh to evaluate the change in Ca\(^{2+}\) sensitization by Y-27632 (Fig. 2). The tension-F340/F380 curves for MCh (0.01–10 \(\mu\)M) were shifted to the right in the presence of 10 \(\mu\)M Y-27632, indicating that an inhibition of Rho kinase inhibits MCh-induced Ca\(^{2+}\) sensitization (Fig. 2B). However, when the concentration of MCh was lowered to 0.01 \(\mu\)M, 10 \(\mu\)M Y-27632 caused a reduction in [Ca\(^{2+}\)]\(_i\) with relaxation, indicating that inhibition of Rho kinase may affect Ca\(^{2+}\) mobilization. In intact smooth muscle, Y-27632 also caused a roughly complete relaxation of agonist-induced contraction of vascular (34) and tracheal (35) smooth muscle in rabbits. In this study, Y-27632 inhibited MCh-induced contraction in guinea pig tracheal smooth muscle similar to these previous observations (Fig. 1). However, Y-27632 caused a reduction in F340/F380, with relaxant effects in a concentration-dependent manner (Fig. 1). Our results not only support the idea that inhibition of Rho kinase activity leads to inhibition of agonist-induced contraction via a reduction in Ca\(^{2+}\) sensitivity, but they also suggest that the Rho/Rho kinase pathway affects intracellular Ca\(^{2+}\) mobilization.

To determine the involvement of Rho kinase in Ca\(^{2+}\) mobilization, tension-[Ca\(^{2+}\)]\(_i\) curves were examined under the condition that Y-27632 was cumulatively applied to the tissues in the presence of 0.1 and 1 \(\mu\)M MCh (Fig. 1, B and C). In guinea pig tracheal smooth muscle, 10 \(\mu\)M MCh produces a roughly 100% contraction, and the percent contraction for 0.1 and 1 \(\mu\)M MCh was \(~40\) and 80%, respectively (19). These concentrations are appropriate to investigate muscarinic stimulation without side effects. Because concentrations of Y-27632 \(<10 \mu\)M caused up to an \(~50\)% inhibition of 1 \(\mu\)M MCh-induced contraction, with little change in [Ca\(^{2+}\)]\(_i\), a reduction in Ca\(^{2+}\) sensitivity by the Rho/Rho kinase pathway is involved in the relaxant effects of this agent (Fig. 1, B and C). On the other hand, higher concentrations of Y-27632 caused further relaxation, with a reduction in [Ca\(^{2+}\)]\(_i\) in a concentration-dependent manner. The inhibition constant (\(K_i\)) value of Y-27632 for inhibiting Rho kinase is generally considered to be 0.14 \(\mu\)M in vitro (34). Y-27632 at 10 \(\mu\)M, a concentration close to the \(K_i\) value for Rho kinase inhibition, caused relaxation, with a reduction in F340/F380 in guinea pig tracheal smooth muscle when MCh was lowered to 0.1 \(\mu\)M (Fig. 1, B and C). Moreover, application of HA-1077, another inhibitor of Rho kinase (34), caused inhibition of MCh-induced contraction, with a reduction in F340/F380 in guinea pig tracheal smooth muscle similar to that with Y-27632.
(Ito, unpublished observations). These results indicate that Rho kinase inhibition may lead to a reduction in [Ca^{2+}]_i. However, it is still unclear whether a reduction in [Ca^{2+}]_i with concentrations of Y-27632 > 100 μM is mediated by Rho kinase inhibition. Higher concentrations of Y-27632 may cause an inhibition in PKA and PKC because Ki values of these kinases are 25 and 26 μM, respectively (34). Because isoproterenol and forskolin reduce [Ca^{2+}]_i in guinea pig tracheal smooth muscle (30), an activation of PKA leads to a reduction in [Ca^{2+}]_i. This result indicates that inhibition in PKA activity may not lead to a reduction in [Ca^{2+}]_i in guinea pig tracheal smooth muscle. As shown in this study, inhibition of PKC mediated by GF-109203X did not reduce [Ca^{2+}]_i (Fig. 4). These results indicate that PKA and PKC are not involved in Ca^{2+} mobilization induced with Y-27632. A reduction in [Ca^{2+}]_i by concentrations of Y-27632 > 100 μM may be mediated by nonspecific effects.

Because the physiological role of PKC in agonist-induced Ca^{2+} sensitization is still controversial in permeabilized tissues of tracheal smooth muscle (2, 11), we examined the involvement of PKC in Ca^{2+} sensitization in intact tissues. PKC represents a family of various lipid-dependent serine/threonine kinases that can be divided into three categories: conventional PKC (cPKC), novel PKC (nPKC) and atypical PKC (aPKC) (28). As shown in Fig. 4, the inhibitory effects of 10 μM Y-27632 on tension and F340/F380 by 1 μM MCh were not affected in the presence of 5 μM GF-109203X, an inhibitor of cPKC and nPKC (33). Moreover, 5 μM GF-109203X did not change tension and F340/F380 with 1 μM MCh. Because incubation with 5 μM GF-109203X for 30 min completely the blocked contraction with 2.5 μM phorbol-12,13-dibutyrate, GF-109203X acts as a PKC inhibitor (Ito, unpublished observations). Under the condition of intracellular application, GF-109203X inhibits cPKC and nPKC, with an EC_{50} value of 0.2 μM, and this agent also inhibits aPKC, with an EC_{50} value of 5.7 μM (23). This agent at 5 μM is considered to be sufficient to inhibit cPKC and nPKC. Our results indicate that these PKC subtypes (cPKC and nPKC) are involved neither in Ca^{2+} sensitization by Rho kinase nor in the contraction induced by muscarinic stimulation in intact guinea pig tracheal smooth muscle. However, PKC-induced contraction is considered to be mediated by many pathways such as Ca^{2+} sensitization, myosin light chain phosphorylation (24), Ca^{2+} influx (27), and the inhibition of MLCP (21). PKC inhibitors do not necessarily antagonize all the mechanisms. A possible role of aPKC in Ca^{2+} sensitization could not be excluded by the results of this study. Further experiments are required to determine the involvement of PKC in contraction and Ca^{2+} sensitization by muscarinic stimulation.

It is well known that tension development is not always consistent with an increase in [Ca^{2+}]_i between high K^- and carbachol-induced contraction (29). Because high K^-induced contraction is completely abolished by an inhibitor of VDCCs, this contraction may be mediated by an increase in [Ca^{2+}]_i via Ca^{2+} influx through VDCCs. On the other hand, because a VDCC inhibitor does not cause a marked inhibition against contraction induced by agonists such as MCh, histamine, and eicosanoids, contraction induced by these agents may be involved in mechanisms other than VDCCs (26). As shown in Fig. 3A, 1 μM nifedipine caused a modest inhibition of 1 μM MCh-induced contraction, with a marked reduction in F340/F380. MCh caused an increase in VDCCs via membrane depolarization by an inactivation of large-conductance Ca^{2+}-activated K^+ channels (18); however, this process makes a modest contribution to the contraction induced by 1 μM MCh. In single-channel records, VDCCs are completely inhibited by 0.1–10 μM nifedipine (17). Because 10 μM Y-27632 did not attenuate [Ca^{2+}]_i induced by 1 μM MCh (Fig. 1A), 100 μM (10-fold concentration) was applied in the presence of 1 μM nifedipine to examine involvement of VDCCs in the reduction in [Ca^{2+}]_i with Y-27632 (Fig. 3). Y-27632 caused a reduction in [Ca^{2+}]_i, even though VDCCs were fully suppressed by 1 μM nifedipine (Fig. 3B). These results indicate that a reduction in [Ca^{2+}]_i with Y-27632 may not be mediated by VDCCs and an increase in sensitivity to Ca^{2+} with Rho kinase may play an important role in agonist-induced contraction.

In conclusion, the Rho/Rho kinase process is involved in Ca^{2+} sensitization and may be involved in Ca^{2+} mobilization induced by muscarinic activation in intact airway smooth muscle. Our results provide evidence that inhibition of Rho kinase may beneficial to improvement of bronchial hyperreactivity.

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