Role of Rho-Associated Protein Kinase and Histamine in Lysophosphatidic Acid-Induced Airway Hyperresponsiveness in Guinea Pigs

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ABSTRACT—Inhalation of oleoyl lysophosphatidic acid (LPA) induced airway hyperresponsiveness to acetylcholine (ACh). In contrast, palmitoyl and stearoyl LPA exerted minimal effects. Airway hyperresponsiveness was inhibited by inhalation of Y-27632, an inhibitor of Rho-associated protein kinase (ROCK). Mepyramine, an H_1 histamine receptor antagonist and ketotifen, an inhibitor of histamine release and H_1 histamine receptor antagonist, also inhibited airway hyperresponsiveness induced by LPA; however, aspirin failed to attenuate this response. The incubation of lung fragments with LPA gave rise to releases in histamine. On the other hand, LPA produced no significant changes on the smooth muscle contraction evoked by ACh. These findings suggest that LPA-induced airway hyperresponsiveness is attributable to activation of the Rho/ROCK-mediated pathway via endothelial cell differentiation gene (EDG) receptors, probably EDG 7. Moreover, histamine release may be involved.

Keywords: Lysophosphatidic acid, Airway hyperresponsiveness, Rho-associated protein kinase, Histamine

The airways of asthmatic patients are hyperresponsive to a wide array of bronchoconstrictor stimuli. Many different mediators have been implicated in this non-specific hyperresponsiveness, although the precise mechanisms are unclear. Platelet activating factor (1), thromboxane A_2 (2), leukotrienes (3) and tachykinins (4) are known to induce non-specific airway hyperresponsiveness. We previously reported that lysophosphatidic acid (LPA), a potent phospholipid mediator, induced airway hyperresponsiveness in guinea pigs and that activation of capsaicin-sensitive fiber was involved in this phenomenon (5). It was not clear whether or not LPA is released upon allergic cell activation or whether LPA was actually present in the respiratory tract. However, secretory phospholipase A_2 (sPLA$_2$), which leads to the biosynthesis of various lysophospholipids, is released during activation of inflammatory cells such as macrophages, neutrophils and mast cells (6). Increased levels of sPLA$_2$ are also found in the bronchoalveolar lavage fluid of sensitized guinea pig and of antigen-challenged allergic asthmaticus (7, 8). From these points

and our findings, it appears likely that LPA may contribute to phathomechanisms of pulmonary diseases such as bronchial asthma.

LPA stimulates the signal pathway through Rho/Rho-associated protein kinase (ROCK) to evoke a variety of responses (9, 10). In the respiratory tracts, published reports indicate that Rho activation is observed in the hyperresponsive bronchial smooth muscle (11). Rho activates a ROCK. ROCK inhibits myosin light chain dephosphorylation. Consequently, the phosphorylated form of the myosin light chain accumulates, resulting in Ca^{2+} sensitization of smooth muscle. Therefore, the Rho/ROCK-mediated pathway contributes to the development of airway hyperresponsiveness. On the other hand, LPA also exhibits nociception-producing activity on sensory neurons through substance P release from nociceptor endings as well as histamine release, probably from mast cells (12, 13). Additionally, it is reported that ROCK is involved in the phorbol-12-myristate-13-acetate (PMA)-induced superoxide production in human polymorphonuclear leukocytes (14) and the stable thromboxane analog STA$_2$-induced adenosine triphosphate (ATP) secretion in human platelets (15).

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Given these observations, this study was designed to determine the contribution, if any, of the Rho/ROCK mediated pathway and release of histamine in LPA-induced airway hyperresponsiveness. For this purpose, we evaluated the effects of Y-27632, an inhibitor of ROCK; mepyramine, an H_1 histamine receptor antagonist; and ketotifen, an inhibitor of histamine release and H_1 histamine receptor antagonist, on LPA-induced airway hyperresponsiveness. Furthermore, histamine release from lung fragments and effects on the smooth muscle contraction evoked by acetylcholine (ACh) are described.

MATERIALS AND METHODS

Animals

Male Hartley guinea pigs weighing $250 - 600$ g were obtained from Saitama Laboratory Animal, Inc. Each group consisted of five to eight animals. All experiments were according to the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Materials

Monooleoyl phosphatidic acid, monosodium (oleoyl LPA, C18:1), monopalmitoyl phosphatidic acid, monosodium (palmitoyl LPA, C16:0) and monostearoyl phosphatidic acid (stearoyl LPA, C18:0) were obtained from Avanti polar-Lipids, Inc. (Ontario, Canada). Additional reagents included the following: acetylcholine chloride (ACh, Ovisot®; Daiichi, Tokyo), ketotifen fumarate (Sigma, St. Louis, MO, USA), mepyramine maleate (Funakoshi, Tokyo), aspirin (Wako, Osaka) and urethane (Sigma). Y-27632 ((+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide) was a gift of the Welfide corporation (Osaka). o-Phtalaldehyde was obtained from Wako. All reagents were of the highest grade commercially available.

LPA was dissolved in deionized water and diluted with physiological saline. Aspirin solution was prepared with 0.15 M phosphate buffer (pH 7.4). Other reagents were dissolved in physiological saline.

Determination of airway responsiveness

Airway responsiveness was measured according to the modified method of Konzett-Rössler (16). Guinea pigs were anesthetized by intraperitoneal injection of urethane (1.5 g/kg). An endotracheal cannula was inserted and connected to a respirator (SN-480-7; Shinano Seisakujyo, Tokyo) following anesthesia. Animals were ventilated at a tidal volume of 10 ml/kg with a frequency of 70 strokes/min. The side arm of the endotracheal cannula was linked to a pressure transducer (LPU-0.1-350-0-II; Nihon Kohden, Tokyo) to monitor endotracheal pressure. A cannula was also inserted into the left jugular vein for spasmogen administration.

ACh ($2.5 - 80$ μg/kg) was administered through the cannula in the left jugular vein. Following determination of airway responsiveness to ACh, animals were inhaled with 10 μg/ml of LPA for 2 min utilizing an ultrasonic nebulizer (NE-U12; Tateishi Electrics, Kyoto) linked to the respirator. ACh was administered again at 30 min after LPA inhalation.

The change in airway responsiveness to ACh ($\Delta PD_{5\,cmH_2O}$) was evaluated as follows: $PD_{5\,cmH_2O}$ was defined as the provocative dose of ACh that increased endotracheal pressure by 5 cmH_2O above the baseline.

$$\Delta PD_{5\,cmH_2O} = (PD_{5\,cmH_2O} \text{ prior to inhalation of LPA})$$
$$/ (PD_{5\,cmH_2O} \text{ after inhalation})$$

Structure-activity relationship of LPA series with different acyl chains on airway hyperresponsiveness

The structure-activity relationship of LPAs was investigated. For this purpose, animals were treated with 10 μg/ml of oleoyl, palmitoyl or stearoyl LPA. Subsequently, airway responsiveness was determined.

Drug administration

Y-27632 (0.3 or 1 mM) was inhaled for 2 min employing an ultrasonic nebulizer linked to a respirator 5 min prior to LPA inhalation. Aspirin (20 mg/kg), mepyramine (2 mg/kg) and ketotifen (1 mg/kg) were administered intravenously 5 min before LPA inhalation.

Histamine release from lung fragments

Guinea pigs were sacrificed by bleeding from the femoral artery under anesthesia. The lung was perfused with HEPES-Tyrode's solution through the pulmonary artery and removed from the body. The isolated lung was divided into the pieces and rinsed with HEPES-Tyrode's solution. The pieces of lung were preincubated for 15 min in HEPES-Tyrode's solution at 37°C. Then the pieces were transferred to test tubes, incubated for $10 - 60$ min in the absence and presence of oleoyl LPA (10 μg/ml) in 0.5 ml of HEPES-Tyrode's solution at 37°C. After the incubation, lung pieces were removed from HEPES-Tyrode's solution, and histamine content of the solution was determined by the method of Yamatodani et al. (17) using high performance liquid chromatography (HPLC) coupled with post-column derivatization fluorometry. The amount of histamine released from the lung pieces was calculated by interpolation from a standard curve for histamine that was constructed in the range $0.5 - 5$ pmol absolute. Results were standardized on the basis of wet weight of the tissue.

Smooth muscle contraction evoked by ACh

The animal was sacrificed by bleeding from the femoral artery under anesthesia. The trachea was rapidly removed

and immersed in HEPES-Tyrode's solution. The tracheal segment was opened longitudinally through the anterior aspect and loose connective tissue. Transverse incisions were made about 2-mm-apart. These strips were suspended vertically in the organ chamber filled with HEPES-Tyrode's solution under a resting tension of 1 g, maintained at 37°C and bubbled constantly with 95% $O_2 - 5\%$ CO_2. The isometric tension was measured utilizing an isometric transducer (TB-612T, Nihon Kohden) and recorded utilizing a polygraph system (RM-6200, Nihon Kohden). After equilibration for 30 min, the dose-response curves to ACh were measured for the control. Following determination of the control response, 10 μg/ml of LPA was added to the organ chamber and incubated for 30 min. We then measured the dose-response curve to ACh again.

Statistical analyses

Values are expressed as means ± S.E.M. Student's *t*-test or Dunnett's multiple comparison test was used to calculate the statistical significance of differences between the means of the test and control groups. A *P*-value of less than 0.05 was considered significant.

RESULTS

Structure-activity relationship of LPA series with different acyl chains on airway hyperresponsiveness

In order to establish the LPA chain length demonstrating the greatest effect on airway responsiveness to ACh, three species of LPA were examined as illustrated in Fig. 1. In the series, oleoyl LPA, which possessed the longest chain evaluated, significantly increased airway responsiveness to ACh. Upon inhalation of oleoyl LPA, the $\Delta PD_{5\,cmH_2O}$ value was 1.90 ± 0.11, whereas that for saline was 1.26 ± 0.12. A significant difference was observed between oleoyl LPA and saline (*P*<0.01). In contrast, the $\Delta PD_{5\,cmH_2O}$ values of palmitoyl and stearoyl LPA were 1.26 ± 0.15 and 1.47 ± 0.12, respectively. These results were approximately 1.0 and 1.1 times, respectively, compared with the saline group. No significant differences were shown in comparison to the saline group. As a result, oleoyl LPA was utilized for the following experiments.

Effect of Y-27632 on LPA-induced airway hyperresponsiveness

The effect of Y-27632, which inhibits ROCK and relaxes airway smooth muscle, was examined as indicated in Fig. 2. The $\Delta PD_{5\,cmH_2O}$ reading of the Y-27632- (0.3 mM) and LPA-treated group was 1.76 ± 0.17. Moreover, the value was significantly different from the control (without LPA, *P*<0.05), whereas LPA-induced airway hyperresponsiveness was markedly inhibited at 1 mM. No statistically meaningful difference was observed with

respect to the control.

Effects of mepyramine, ketotifen and aspirin on LPA-induced airway hyperresponsiveness

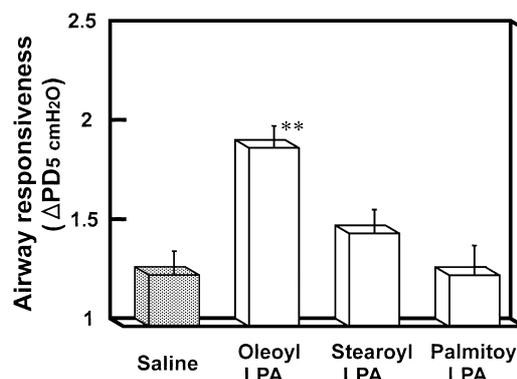Figure 3 presents the effects of ketotifen, mepyramine

Fig. 1. Structure-activity relationship of an LPA series possessing distinct acyl chains with respect to airway hyperresponsiveness in guinea pigs. LPA (10 μg/ml) was inhaled for 2 min by using an ultrasonic nebulizer. $PD_{5\,cmH_2O}$ is defined as a provocative dose of ACh which increases endotracheal pressure by 5 cmH$_2$O above the baseline. $\Delta PD_{5\,cmH_2O}$ is defined as a ratio of $PD_{5\,cmH_2O}$ obtained prior to inhalation of LPA to that obtained after inhalation of LPA. Each column represents the mean ± S.E.M. of 5 – 8 animals. **P<0.01, significantly different from saline (Dunnett's multiple comparison test). $PD_{5\,cmH_2O}$ values of ACh prior to inhalation were 17.1 – 22.7 μg/kg. No significant differences were observed between groups.
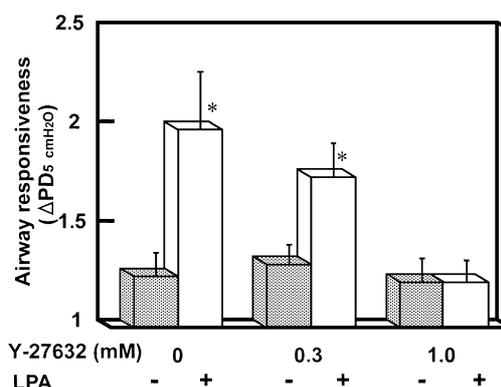

Fig. 2. Effect of Y-27632 on oleoyl LPA-induced airway hyperresponsiveness in guinea pigs. LPA (10 μg/ml) was inhaled for 2 min by using an ultrasonic nebulizer. Y-27632 (0.3 or 1 mM) was inhaled for 2 min by using an ultrasonic nebulizer linked to a respirator 5 min prior to LPA inhalation. $PD_{5\,cmH_2O}$ is defined as a provocative dose of ACh that increases endotracheal pressure by 5 cmH$_2$O above the baseline. $\Delta PD_{5\,cmH_2O}$ is defined as a ratio of $PD_{5\,cmH_2O}$ obtained prior to inhalation of LPA to that obtained after inhalation of LPA. Each column represents the mean ± S.E.M. of 7 – 8 animals. *P<0.05, significantly different from saline (Student's *t*-test). $PD_{5\,cmH_2O}$ values of ACh prior to inhalation were 19.9 – 27.9 μg/kg. No significant differences were observed between groups.

and asprin on the increase in airway responsiveness as evaluated at 30 min following LPA inhalation. The $\Delta PD_{5\,cmH_2O}$ readings of the saline and LPA-treated groups were 1.39 ± 0.14 and 2.47 ± 0.27, respectively. Statistically significant differences ($P<0.01$) were shown between the saline and LPA-treated groups. Ketotifen, an inhibitor of histamine release and H_1 histamine receptor antagonist displayed a marked effect at 1 mg/kg. The $\Delta PD_{5\,cmH_2O}$ of the ketotifen-treated group was 1.40 ± 0.19. This value was nearly equal to that of the saline group; moreover, statistical significance was observed in comparison with the LPA-treated group ($P<0.01$). A histamine H_1 antagonist, mepyramine, also attenuated the increase in airway responsiveness at 2 mg/kg. The $\Delta PD_{5\,cmH_2O}$ of the mepyramine-treated group (1.60 ± 0.27) was approximately 60% of that of the LPA-treated group. Significant differences were observed between the LPA-treated and mepyramine-treated groups at $P<0.05$. In contrast, 20 mg/kg of aspirin, a cyclooxygenase inhibitor, failed to suppress airway hyperresponsiveness.

Histamine release from lung fragments

To investigate whether histamine release was induced by LPA, we performed the experiments in vitro. In agreement with the in vivo experiments, the incubation of lung fragments with 10 μg/ml of LPA gave rise to histamine release. The histamine release of LPA treated groups were
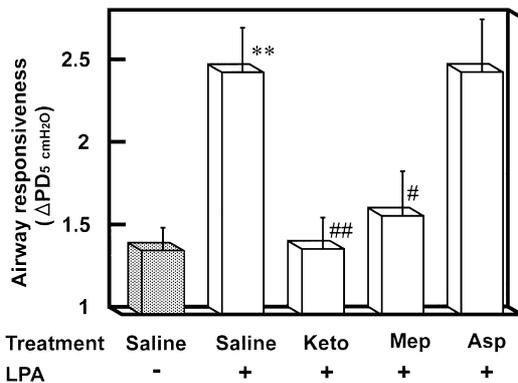

Fig. 3. Effect of ketotifen (Keto), mepyramine (Mep) and aspirin (Asp) on oleoyl LPA-induced airway hyperresponsiveness in guinea pigs. LPA (10 μg/ml) was inhaled for 2 min by using an ultrasonic nebulizer. Aspirin (20 mg/kg), mepyramine (2 mg/kg) and ketotifen (1 mg/kg) were administered intravenously 5 min before LPA inhalation. $PD_{5\,cmH_2O}$ is defined as the provocative dose of ACh that increases endotracheal pressure by 5 cmH2O above the baseline. $\Delta PD_{5\,cmH_2O}$ is defined as a ratio of $PD_{5\,cmH_2O}$ obtained prior to inhalation of LPA to that obtained after inhalation of LPA. Each column represents the mean $\pm$ S.E.M. of 7$-$8 animals. **$P<0.01$, significantly different from saline (Student's t-test). #$P<0.05$, ##$P<0.01$, significantly different from saline + LPA (Dunnett's multiple comparison test). $PD_{5\,cmH_2O}$ values of ACh prior to inhalation were 12.2$-$21.6 μg/kg. No significant differences were observed between groups.

1.3 to 1.7 times higher than that of the vehicle-treated group. There was significant differences between the LPA-treated and saline-treated groups for 60 min ($P<0.05$) (Fig. 4).

Effects of LPA on the smooth muscle contraction evoked by ACh

The effect of LPA on the dose-response curves to ACh is shown in Fig. 5. Although incubation of strips with 10 μg/ml of LPA scarcely increased contraction, within a range of 10% to 90%, and no statistically significant differences were observed between LPA-treated and saline-treated groups.

DISCUSSION

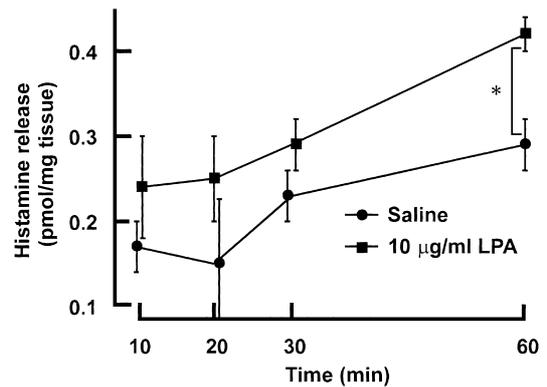In the present study, the mechanisms of LPA-induced

Fig. 4. Histamine release induced by oleoyl LPA from guinea pig lung fragments. After preincubation at 37°C for 15 min in HEPES-Tyrode's solution, the suspended lung flugments were stimulated with 10 μg/ml of oleoyl LPA at 37°C for 10, 20, 30 and 60 min. Each point represents the mean $\pm$ S.E.M. of 3 experiments. *$P<0.05$, significantly different from saline (Student's t-test).
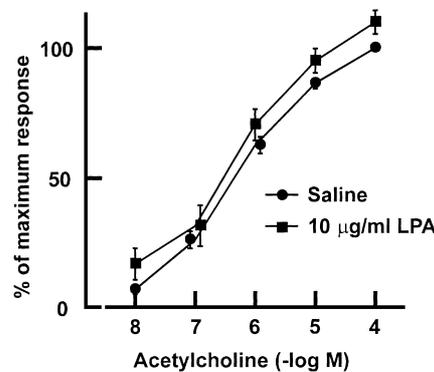

Fig. 5. Effects of LPA on the smooth muscle contraction evoked by ACh in guinea pig tracheal strips. Tracheal strips were pretreated with saline or 10 μg/ml of LPA for 30 min. Each point represents the mean $\pm$ S.E.M. of 6 experiment.

airway hyperresponsiveness were investigated. We confirmed that 10 μg/ml of LPA induced airway hyperresponsiveness; moreover, the maximum effect was observed 30 min following inhalation (5). Consequently, airway responsiveness was determined using 10 μg/ml of LPA at 30 min after inhalation. Doses of all drugs examined and its administrative routes were also selected for sufficient inhibition of in vivo experiments from preliminary examination in our laboratory.

LPA exhibits various biological activities through the G protein-coupled, seven transmembrane domain receptors. To date, several subtypes of LPA receptors, which are G protein-coupled receptors, have been identified. At least three are G protein-coupled receptors belonging to the endothelial cell differentiation gene (EDG) family. EDG 2, EDG 4 and EDG 7 have been identified as cellular receptors for LPA. Each LPA receptor can be activated differentially by LPA with various acyl chains bound at either the *sn*-1 or the *sn*-2 position of the glycerol backbone. In the case of EDG 7, the highest reactivity was observed with oleoyl LPA. In contrast, EDG 2 and EDG 4 showed broad ligand specificity (18). The results of the present study indicated that differential activation by LPA species was evident in airway responsiveness to ACh. Inhalation of oleoyl LPA induced airway hyperresponsiveness to ACh; however, palmitoyl and stearoyl LPA scarcely affected this responsiveness. These data suggested that, at least in part, LPA-induced airway hyperresponsiveness was mediated via the EDG receptor, probably EDG 7.

LPA-induced airway hyperresponsiveness was markedly inhibited by 1 mM of Y-27632, an inhibitor of ROCK. This finding indicated the involvement of the Rho/ROCK-mediated pathway in LPA-induced airway hyperresponsiveness. The Rho/ROCK-mediated Ca^{2+} sensitization occurs in airway smooth muscle in vitro and Y-27632 relaxes airway smooth muscle through inhibition of Ca^{2+} sensitization (19). In the asthma model, ACh-induced Ca^{2+} sensitization occurs where Rho protein expression has increased (11). Tumor necrosis factor-α has been reported to enhance smooth muscle responsiveness to ACh through agonist-mediated Ca^{2+} sensitization (20). Thus, it is considered that the agonist-mediated Ca^{2+} sensitization plays an important role in airway hyperresponsiveness. However, LPA produced no statistically significant changes in the contractile response evoked by ACh in the present study. These result suggests that LPA does not alter Ca^{2+} sensitivity of intracellular contractile elements.

In the present study, mepyramine and ketotifen were also shown to exert an inhibitory effect on LPA-induced airway hyperresponsiveness. Additionally, incubation of lung fragments with LPA showed the rise in histamine release in vitro. Lloret and Moreno (21) reported that histamine release from purified mouse mast cells was induced by several lysophospholipids such as lysophosphatidylcholine, lysophosphatidylethanolamine and lysophosphatidylserine. Although it was considered that the effect of LPA was weaker than that of these lysophospholipids, the fact that histamine was released by LPA treatment was in agreement with the in vivo experiments. In contrast, aspirin scarcely affected hyperresponsiveness. Arachidonic acid metabolites, especially thromboxane A_2, have been reported to play a key role in the development of airway hyperresponsiveness (1, 2); however, airway hyperresponsiveness induced by LPA is independent of release of arachidonic acid metabolites. Antihistaminics have been a cornerstone in the treatment of allergic diseases; however, these substances have been of no benefit in the treatment of asthma. Twentyman et al. (22) documented that histamine is not an important mediator of the late asthmatic response or allergen-induced increases in bronchial responsiveness. On the other hand, Santing et al. (23) described the contribution of histamine to the development of the late asthmatic reaction as well as to early and late bronchial hyperreactivity. Histamine does not directly increase the responsiveness of guinea pig trachea; however, histamine may be involved in a cascade of events leading to airway hyperresponsiveness (24). Although a clear explanation has not been reported regarding the involvement of histamine in the development of airway hyperresponsiveness, the present study indicates that histamine contributes to LPA-induced airway hyperresponsiveness based on the effectiveness of antihistaminics and the in vitro experiment. The fact that LPA-induced nociception was weakened by treatment with diphenhydramine (13) also suggests that histamine release occurred on LPA treatment.

The difference between the in vivo and in vitro findings is that in vivo ones, there are significant differences between LPA-treated and saline-treated groups at 30 min. The discrepancy between the in vivo and in vitro findings may be explained by the contribution of other mediators such as tachykinins, because we previously reported that activation of capsaicin-sensitive fiber was involved in LPA-induced airway hyperresponsiveness (5).

It is not clear from present studies whether the Rho/ROCK-mediated pathway is identified with the histamine-mediated pathway. However, Kawaguchi et al. (14) reported that ROCK was involved in the PMA-induced superoxide production in human polymorphonuclear leukocytes and this kinase might be located downstream of protein kinase C. Sando and Chertinin (25) also reported that a physiologically or pathologically relevant concentration of LPA could contribute to protein kinase C activation. Kurosawa and Kobayashi (26) documented that activation of protein kinase C was observed during histamine release from rat mast cells stimulated with compound 48/80 or Ca^{2+} ionophore A23187. From these reports, we speculate

that the Rho/ROCK-mediated pathway may be part of the histamine-mediated pathway.

In summary, we conclude that LPA-induced airway hyperresponsiveness is attributable to the activation of the Rho/ROCK-mediated pathway via the EDG 7 receptor. Moreover, our findings suggest that histamine release may be involved. In view of these effects on the airway, LPA has been advanced as a possible mediator of pulmonary diseases such as bronchial asthma.

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