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Protein Kinase A Inhibits Lysophosphatidic Acid-Induced Migration of Airway Smooth Muscle Cells

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ABSTRACT

Lysophosphatidic acid (LPA) is a bioactive phospholipid that is released from activated platelets and affects contractile properties of airway smooth muscle cells. However, possible roles of LPA on cell migration, one of the initial events of airway remodeling, are not clarified. This study aimed to examine the effects of LPA on migration and actin fiber formation in bovine tracheal smooth muscle cells (BTSMCs). Random and oriented cell migrations were examined with wound assay and Boyden chamber assay, respectively. Cytosolic actin fibers were stained with rhodamine-phalloidin. Membrane translocation of RhoA, a hallmark of RhoA activation, was assessed by Western blotting. LPA augmented the migration of BTSMCs from wounded confluent monolayer but did not accelerate the chemotactic migration toward LPA. LPA also induced a transient actin reorganization and RhoA activation. Dense actin fibers were observed mainly in the wound edge but not in migrated cells, thereby suggesting the role of actin reorganization in the initiation of cell migration. LPA-induced actin fiber formation was blocked by Y27632 [*R*-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexane carboxamide], an inhibitor of Rho kinase. Effects of LPA on migration and actin fiber formation were also inhibited by cAMP-elevating agents, i.e., dibutyryl cAMP, forskolin, isoproterenol, and theophylline. KT5720 (9*S*,10*S*,12*R*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid hexyl ester], a protein kinase A (PKA) inhibitor, reversed the inhibitory actins of cAMP on LPA-induced responses. These results indicate that LPA induces cAMP/PKA-sensitive, RhoA-mediated random migration of BTSMCs. Regulation of this mechanism would be beneficial for the control of airway remodeling.

Proliferation and hypertrophy of airway smooth muscle cells cause airway remodeling (Stewart, 2001), one of the major pathogenesis of bronchial asthma (Solway and Fredberg, 1997; O'Byrne and Inman, 2003). It is established in vascular smooth muscle cells that cell migration is an initial step of intimal medial thickness in atherosclerosis (Newby and Zaltsman, 2000). Therefore, although no direct evidence has been shown in vivo so far, migration of airway smooth muscle cells may be an important event in airway remodeling (Black et al., 2001; Madison, 2003). Various chemotactic agents have been demonstrated to stimulate migration of airway smooth muscle cells in vitro, including platelet-derived growth factor (Goncharova et al., 2003), interleukin-1β (Hedges et al., 1999), interleukin-6 (Govindaraju et al., 2006), TGF-α (Goncharova et al., 2003), and urokinase (Mukhina et al., 2000). It has also been suggested that $p38^{MAPK}$ (Hedges et al., 1999; Dechert et al., 2001), heat shock protein 27 (Hedges et al., 1999), phosphatidylinositol 3-kinase (Irani et al., 2002), Src (Krymskaya et al., 2005), and Rho kinase (Parameswaran et al., 2002) are involved in the signaling mechanisms underlying the migration of airway smooth muscle cells.

Lysophosphatidic acid (LPA) is a membrane-derived lysophospholipid that is generated in serum (Xie et al., 2002) or secreted from platelets (Moolenaar, 1995) and serves as an autocrine and paracrine mediator. Because increased vascular permeability with plasma leakage is a feature of asthma (Wilson, 2000), the concentration of LPA is supposed to be increased in injured and/or asthmatic airway. Potential roles of LPA in the development of respiratory diseases, especially asthma, have been suggested (Toews et al., 2002). LPA binds to its specific receptors and activates small GTPase family protein RhoA and subsequent Rho kinase (Moolenaar, 1995).

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ABBREVIATIONS: LPA, lysophosphatidic acid; BTSMC, bovine tracheal smooth muscle cell; PKA, protein kinase A; DMEM, Dulbecco's modified Eagle's medium; $[cAMP]_i$, intracellular cAMP concentration; Y27632, *R*-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexane carboxamide; KT5720, (9*S*,10*S*,12*R*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]-benzodiazocine-10-carboxylic acid hexyl ester.

It is generally accepted that cell migration requires cytoskeletal remodeling, for which small GTPase family proteins play an essential role (Ridley, 2001). Therefore, it can be hypothesized that LPA may induce airway smooth muscle migration, thereby initiating airway remodeling. Actually, LPA is reported to induce structural remodeling (Cerutis et al., 1997) as well as Ca^{2+} hypersensitivity (Toews et al., 1997; Sakai et al., 2003) in airway smooth muscle cells in vitro.

The aim of this study was to investigate the effects of LPA on cell migration and actin reorganization in bovine tracheal smooth muscle cells (BTSMCs). Our results revealed, for the first time, that LPA induces random migration of airway smooth muscle cells via RhoA-mediated, protein kinase A (PKA)-sensitive actin reorganization.

Materials and Methods

Cell Culture. Tracheas of 1-year-old calves were obtained from a local slaughterhouse. Smooth muscle cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum by explant method (Sakai et al., 2003). Cells grown in confluence were harvested by trypsin digestion and stored at −80°C after one-step subculture. Present study was performed with BTSMCs obtained from seven tracheas. We have confirmed with Western blotting that the BTSMCs used in the present study expressed smooth muscle marker proteins, i.e., calponin, myosin, and α-smooth muscle actin (data not shown).

Cell Migration Assays. Migration of BTSMCs was assessed with a wound assay (Kimura et al., 2001) and a Boyden chamber assay (John et al., 1997). In the wound assay, cells grown in confluent on coverslips were scraped with an edge of fine razor. The wound edge was viewed and photographed under a microscope (Eclipse TS100; Nikon, Tokyo, Japan) before and after culturing for 24 h in serum-free DMEM in the presence of 10 μM mitomycin C. The number of the cells migrated from the wound surface was then manually counted. In some experiments, actin cytoskeleton was stained in the wound edge as described below.

In the Boyden chamber assay, 10^5 cells were seeded on 6.5-mm diameter Transwell filter cups (8-μm pore; Corning Life Sciences, Acton, MA) with 100 μl of serum-free DMEM and incubated for 6 h at 37°C in the presence of 1 μM LPA either in the filter cup (i.e., upper side of the filter), in the well (i.e., lower side of the filter), or in both chambers. Control wells were incubated in the absence of LPA. The filter was removed from the cup, fixed with 70% ethanol, and stained with hematoxylin. The number of cells migrated to the lower side of the filter was manually counted under microscope.

Staining of Actin Cytoskeleton. Reorganization of actin cytoskeleton was assessed with F-actin staining (Koyama et al., 2001). Cells were fixed with 2% paraformaldehyde after each treatment and permeabilized with 0.1% Triton X-100. Rhodamine-conjugated phalloidin (Invitrogen, Carlsbad, CA) was then applied for 20 min after blocking nonspecific binding with 0.5% fetal bovine serum. F-actin fibers were then observed with fluorescence microscope (Eclipse E600; Nikon).

Measurement of Intracellular cAMP Concentration. Intracellular cAMP concentration ($[cAMP]_i$) was measured with enzyme immunoassay by using a commercial kit (Biotrak cAMP EIA system; GE Healthcare, Little Chalfont, Buckinghamshire, UK). BTSMCs were cultured overnight at a density of 5000 cells/well in 96-well culture plates, and $[cAMP]_i$ was measured according to the manufacturer's instructions.

Western Blot Analysis of RhoA Activation. Activation of RhoA was assessed with enhanced chemiluminescence Western blotting. BTSMCs were pretreated with theophylline, dibutyryl cAMP, forskolin, or vehicle for 1 h. Cells were then lysed with 1% Triton X-100 immediately or after applying 1 μM LPA for 5 min. Cell lysate was centrifuged at 100,000*g* for 1 h, and the pellet was harvested as a membrane fraction. A constant amount of membrane fraction (50 μg protein/lane) was separated with SDS-polyacrylamide gel electrophoresis, and enhanced chemiluminescence Western blotting was performed by using anti-RhoA antibody (Cytoskeleton, Inc., Denver, CO) and the antibody against β-actin (Sigma, St. Louis, MO) that was measured as an internal control. Band images were analyzed with a lumino image analyzer (FAS-1000; Toyobo, Osaka, Japan).

Drugs and Solution. The modified Krebs' solution used in the present experiment was 132.4 mM NaCl, 5.9 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11.5 mM glucose, and 11.5 mM HEPES, and pH was adjusted to 7.4 with NaOH. LPA (Sigma) was dissolved at 3 mM in chloroform/methanol. Solvent was evaporated by puffing nitrogen gas and sonicated after adding Krebs' solution or DMEM to make 20 μM LPA solution. Then, the solution was further diluted to 1 μM shortly before use. ATP, theophylline, forskolin, dibutyryl cAMP, Y27632, and KT5720 were purchased from Sigma.

Statistics. Data were expressed with mean ± S.E.M. values. Statistical significance was examined with Student's unpaired *t* test. Probability below 0.05 ($p < 0.05$) was considered as significant.

Results

LPA-Induced Migration from the Wound Edge in BTSMCs. Firstly, we examined the effects of LPA on the random migration of BTSMCs with the wound assay. LPA (1 μM) induced a marked acceleration of cell migration into the cell-free area (Fig. 1A). The number of the migrated cells after 24 h was significantly increased by LPA (control, 33 ± 3 cells/0.5-mm wound surface, $n = 6$; 1 μM LPA, 79 ± 8 cells/0.5-mm wound surface, $n = 6$, $p < 0.01$ versus control, see also Fig. 4A).

Absence of Directed Migration of BTSMCs toward LPA. We then examined with a Boyden chamber assay whether BTSMCs show a directed migration toward LPA or not. When 1 μM LPA-containing medium was present in the filter cup, a significantly larger number of cells was migrated to the lower side of the filter membrane after 6 h than in control medium (Fig. 1B). Increased migration was also observed when LPA was present in both chambers. In contrast, LPA in the well alone did not increase the migration of BTSMCs (Fig. 1B).

Reorganization of Actin Cytoskeleton by LPA in BTSMCs. The above results suggest that LPA activates random migration of BTSMCs. Because cell migration is obtained by the reorganization of actin cytoskeleton, we then examined the effects of LPA on actin fiber formation in BTSMCs. LPA (1 μM) induced a formation of dense actin fibers within 5 min in subconfluent BTSMCs. Actin fibers were persisted at least for 10 min and converged into peripheral ruffles in 30 min (Fig. 2A). Thickness of actin fibers depends on the concentration of LPA (Fig. 2A), suggesting that the actin fiber formation was not due to mechanical stress artificially generated by solution exchange.

To explore the relationship between actin reorganization and cell migration, we then stained the actin cytoskeleton in the wound surface. As shown in Fig. 2B, actin fibers were not prominent in the confluent monolayer. In contrast, actin cytoskeleton was densely observed in the wound edge, and the migrated cells again showed sparse amount of actin fibers. In the higher magnification, migrated cells showed little actin fibers in cytosol, whereas the cells in the wound edge showed a denser actin fiber formation (Fig. 2C).

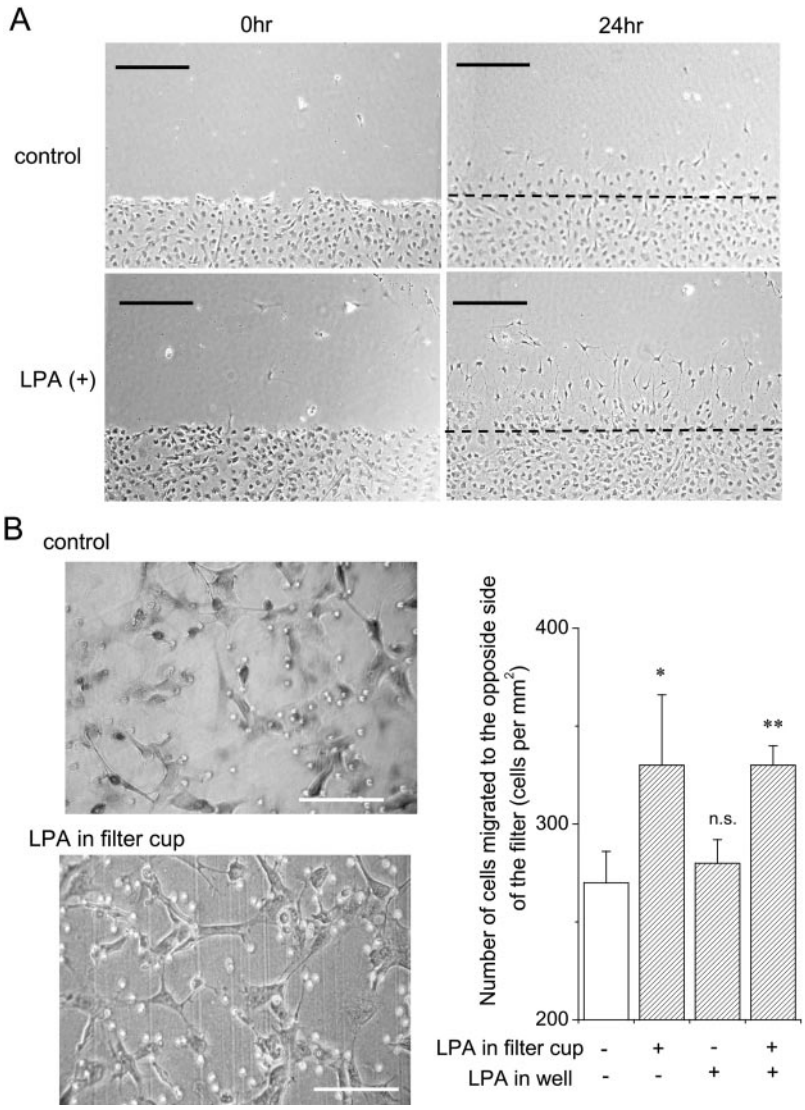

Fig. 1. Migration of BTSMCs induced by LPA. A, cell migration from wound edge after culturing for 24 h in the presence and absence of 1 μM LPA. Dotted lines, wound edge. Scales, 200 μm. B, oriented migration of BTSMCs was examined with Boyden chamber assay. BTSMCs (10^5 cells) were put in the filter cup and incubated for 6 h in the presence or absence of 1 μM LPA. LPA was applied in the filter cup, in the well, or in both chambers. The cells that migrated to the lower surface of the filter were observed after staining with hematoxylin. Representative images obtained in the absence and presence of LPA in the filter cup are shown. Note that the fibroblast-like adherent cells are the migrated cells. Scales, 50 μm. Bar graph, statistical analysis of the number of migrated cells. *, $p < 0.05$; **, $p < 0.01$; n.s., $p > 0.05$ versus control. Data were obtained from six repeated experiments for each condition.

Effects of Rho Kinase Inhibitor on LPA-Induced Responses in BTSMCs. LPA is known to activate small G-protein RhoA and subsequent Rho kinase (Moolenaar, 1995), so we then examined the effects of Rho kinase inhibitor Y27632 on LPA-induced migration and actin reorganization. In the wound assay, the LPA-induced increase in migration was significantly reversed by 10 μM Y27632 (37 $\pm$ 3 cells/0.5-mm wound surface, $p < 0.01$ versus LPA alone, $n = 6$, see also Fig. 4A). LPA-induced actin reorganization was also inhibited by the treatment with 10 μM Y27632 for 1 h (Fig. 3A). These suggest the involvement of RhoA/Rho kinase pathway in LPA-induced migration and actin formation.

Effects of cAMP-Elevating Agents in LPA-Induced Responses in BTSMCs. We have previously shown in BTSMCs that cAMP-elevating agents inhibit the LPA-induced activation of RhoA (Sakai et al., 2003). So, we then examined the effects of cAMP-elevating agents, i.e., dibutyryl cAMP, forskolin, theophylline, and isoproterenol, on LPA-induced migration and actin reorganization.

LPA-induced increase in the migration of BTSMCs was significantly suppressed by 300 μM dibutyryl cAMP, 30 μM forskolin, 10 μM theophylline, and 10 μM isoproterenol (Fig. 4A). Furthermore, these agents also abolished LPA-induced actin reorganization (Fig. 3B). These agents, however, did not affect the migration (Fig. 4A) and actin cytoskeleton (not shown) of control cells.

Measurement of the Intracellular cAMP Concentration in BTSMCs. Next, we measured $[cAMP]_i$ in the presence or absence of dibutyryl cAMP, forskolin, and theophylline. $[cAMP]_i$ was 94 $\pm$ 14 fmol/well in control BTSMCs and was elevated to 3100 $\pm$ 310 and 700 $\pm$ 130 fmol/well by forskolin (30 μM) and theophylline (10 μM), respectively ($n =$ 6 for each condition). Membrane-permeated dibutyryl cAMP was also detected as "cAMP" by the antibody in the kit and showed 4500 $\pm$ 500 fmol/well after the treatment with dibutyryl cAMP (300 μM, $n = 6$).

Effects of KT5720 on LPA-Induced Migration and Actin Reorganization. Because these suggest that cAMP inhibits LPA-induced migration and actin reorganization in BTSMCs, we examined whether this was obtained directly with cAMP or via PKA. Pretreatment with a PKA inhibitor, KT5720 (1 μM), alone for 1 h did not affect LPA-induced actin fiber formation in BTSMCs (Fig. 3C). The inhibitory effects of Y27632 on LPA-induced actin reorganization were also not affected by KT5720 (Fig. 3C). In contrast, dibutyryl cAMP, forskolin, theophylline, and isoproterenol did not suppress

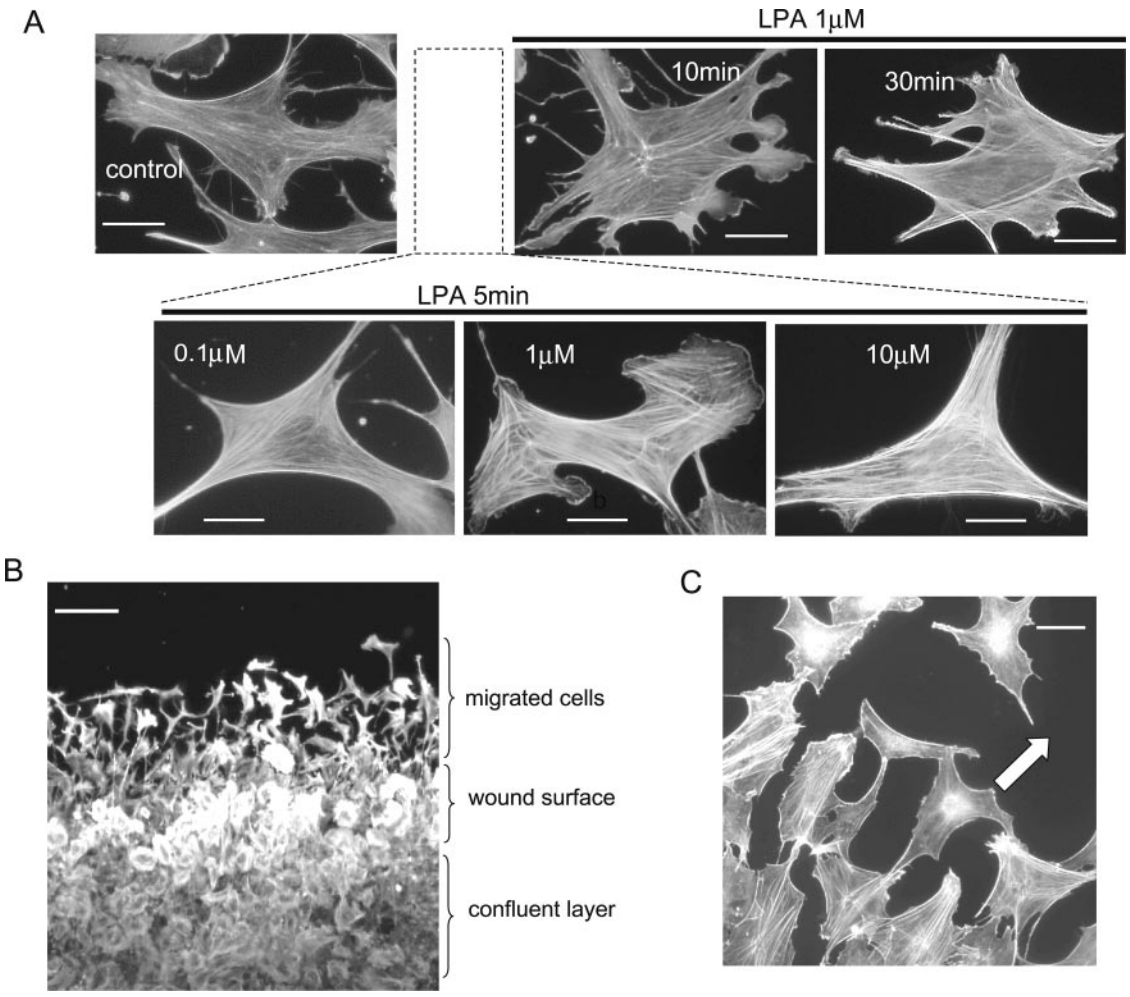

Fig. 2. Effects of LPA on actin cytoskeleton in BTSMCs. A, LPA (0.1, 1, or 10 μM) was applied to nonconfluent control BTSMCs for 5, 10, or 30 min, and actin fibers were stained with rhodamine-phalloidin. Representative images from 4 to 12 repeated experiments are shown for each condition. Scales, 20 μm. B, actin fibers were stained in the wound assay. Note that dense actin fibers were observed in the wound surface but not in confluent monolayer or migrated cells. Scale, 100 μm. C, higher magnification of actin fibers of the migrated cells and the wound surface. Arrow, direction of migration. Scale, 20 μm.

the LPA-induced actin fiber formation in KT5720-treated BTSMCs (Fig. 3C), indicating the PKA-mediated rather than direct effects of cAMP on LPA-induced actin reorganization.

KT5720 also reversed the inhibitory effects of dibutyryl cAMP, forskolin, theophylline, and isoproterenol on LPA-induced migration of BTSMCs from the wound surface (Fig. 4B). Effects of KT5720 were concentration-dependent, and 10 μM KT5720 almost completely reversed the effects of cAMP-elevating agents on LPA-induced migration of BTSMCs. In contrast, 1 μM KT5720 did not reverse the effects of Y27632, indicating that a nonspecific augmentation of cell migration by KT5720 is negligible (Fig. 4B).

LPA-Induced Membrane Translocation of RhoA in BTSMCs. We finally examined the effects of cAMP-elevating agents and KT5720 on LPA-induced RhoA membrane translocation, which is a reflection of RhoA activation. LPA-induced membrane translocation of RhoA was inhibited by dibutyryl cAMP (Fig. 5A) and theophylline (Fig. 5B). KT5720 did not affect either the resting level of RhoA in the membrane fraction or LPA-induced RhoA membrane translocation. On the other hand, KT5720 reversed the inhibitory effects of dibutyryl cAMP (300 μM) and theophylline (10 μM) on LPA-induced membrane translocation of RhoA (Fig. 5).

Discussion

We have shown in the present study that LPA accelerates the random migration of BTSMCs (Fig. 1). The increased cell number in the cell-free area was not due to proliferation but to migration of the cells from the wound edge because we performed the migration assay in the presence of mitomycin C in serum-free medium. In the Boyden chamber assay, acceleration of cell migration was observed when LPA was present in the upper chamber but not when LPA was only in the lower chamber (Fig. 1B). Thus, we have concluded that the effects of LPA are not chemotactic but chemokinetic.

We have also shown that LPA increases the actin fiber formation in nonconfluent BTSMCs (Fig. 2A). Actin cytoskeleton was markedly augmented in the wound edge, whereas cytosolic actin fibers were not prominent in the migrated cells (Fig. 2, B and C). Therefore, although detailed relationship between actin formation and cell migration has yet to be elucidated, this suggests that the formation of dense actin fibers is essential for the initiation of random cell migration in BTSMCs. Y27632, an inhibitor of Rho kinase, inhibited LPA-induced migration (Fig. 4A) and actin reorganization (Fig. 3A). Furthermore, LPA induced a membrane transloca-

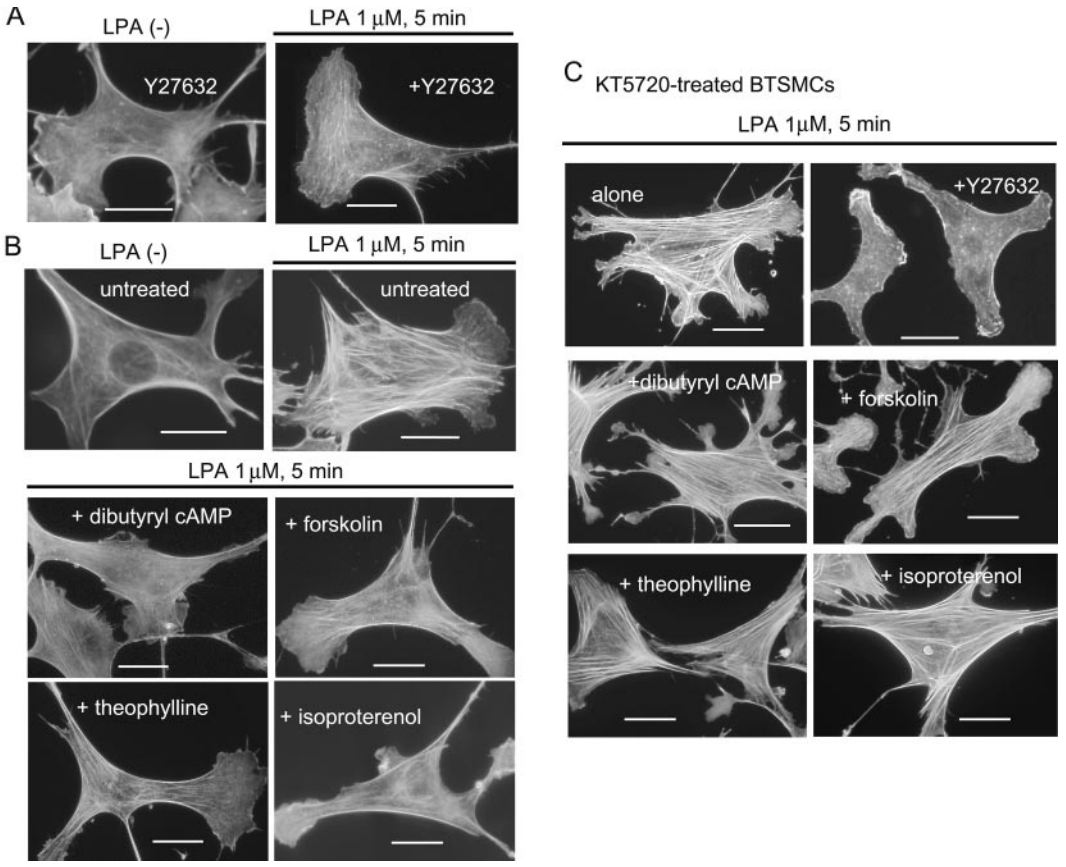

Fig. 3. Effects of Rho kinase inhibitor, cAMP, and protein kinase A inhibitor on LPA-induced actin fiber formation in BTSMCs. A, cells were treated with 10 μM Y27632, and actin fibers were stained before or after the application of 1 μM LPA for 5 min. B, LPA (1 μM) was applied for 5 min to the cells treated with dibutyryl cAMP (300 μM), forskolin (30 μM), theophylline (10 μM), or isoproterenol (10 μM) for 1 h. LPA-induced actin formation in untreated cells is also demonstrated. C, BTSMCs were pretreated with 1 μM KT5720 for 1 h in the absence or presence of dibutyryl cAMP (300 μM), forskolin (30 μM), theophylline (10 μM), or isoproterenol (10 μM), and the LPA (1 μM)-induced actin fiber formations were examined. Representative images from four to six repeated experiments are shown for each condition. Scales, 20 μm.

tion of RhoA (Fig. 5), which is a hallmark of RhoA activation (Kranenburg et al., 1997). A previous report also showed that Y27632 inhibited the platelet-derived growth factor-induced migration of cultured human airway smooth muscle cells (Parameswaran et al., 2002). It is well established that the activation of RhoA/Rho kinase cascade induces actin reorganization (Kaibuchi et al., 1999); thus, we hypothesize that RhoA activation and subsequent actin reorganization play a central role in the LPA-induced migration of BTSMCs. Activation of peroxisome proliferator-activated receptor γ, another intracellular signal evoked by LPA (McIntyre et al., 2003), is probably not involved in the LPA-induced actin reorganization because peroxisome proliferator-activated receptor γ activator troglitazone could not mimic the effects of LPA on actin fibers (M. Watanabe and M. Oike, unpublished data).

Goncharova et al. (2003) reported that the migration of human tracheal smooth muscle cells induced by various growth factors is inhibited by cAMP. They speculated that cAMP-responsive element promoter activity was involved in the inhibition of migration (Goncharova et al., 2003). Possible involvement of cAMP-responsive element binding proteins was also suggested in the migration of vascular smooth muscle cells (Klemm et al., 2001). The authors, therefore, speculated that the inhibition of cell migration with cAMP was determined at the level of gene transcription regulation, and the relationship between cAMP-responsive element binding and RhoA-mediated actin cytoskeleton was not considered in their reports. In contrast, another group reported in hepatoma cells that cAMP suppressed the migration due to the inhibition of RhoA (Mukai et al., 2000). In the present study, dibutyryl cAMP, forskolin, and theophylline, all of which were confirmed to induce $[cAMP]_i$ elevation, as well as isoproterenol inhibited LPA-induced migration (Fig. 4A) and actin fiber formation (Fig. 3B) in BTSMCs. Furthermore, dibutyryl cAMP and theophylline inhibited the LPA-induced RhoA activation (Fig. 5). Therefore, we have concluded that the elevation of $[cAMP]_i$ suppresses LPA-induced actin reorganization and subsequent cell migration by inhibiting RhoA activation. KT5720 reversed the inhibitory effects of cAMP on LPA-induced actin reorganization (Fig. 3C), migration (Fig. 4B), and RhoA activation (Fig. 5). However, KT5720 but did not affect the Y27632-induced inhibition of migration (Fig. 4B) and actin reorganization (Fig. 3C). Therefore, the changes in RhoA and actin shown here seem to be a more likely explanation for the effects on cell migration than the transcriptional mechanisms proposed previously.

Previous studies have suggested at least two possibilities for the mechanism of action of PKA on RhoA, i.e., direct phosphorylation of RhoA and the alteration of Dbl proteins. It was reported that PKA specifically phosphorylated RhoA at Ser188 (Lang et al., 1996; Ellerbroek et al., 2003), which

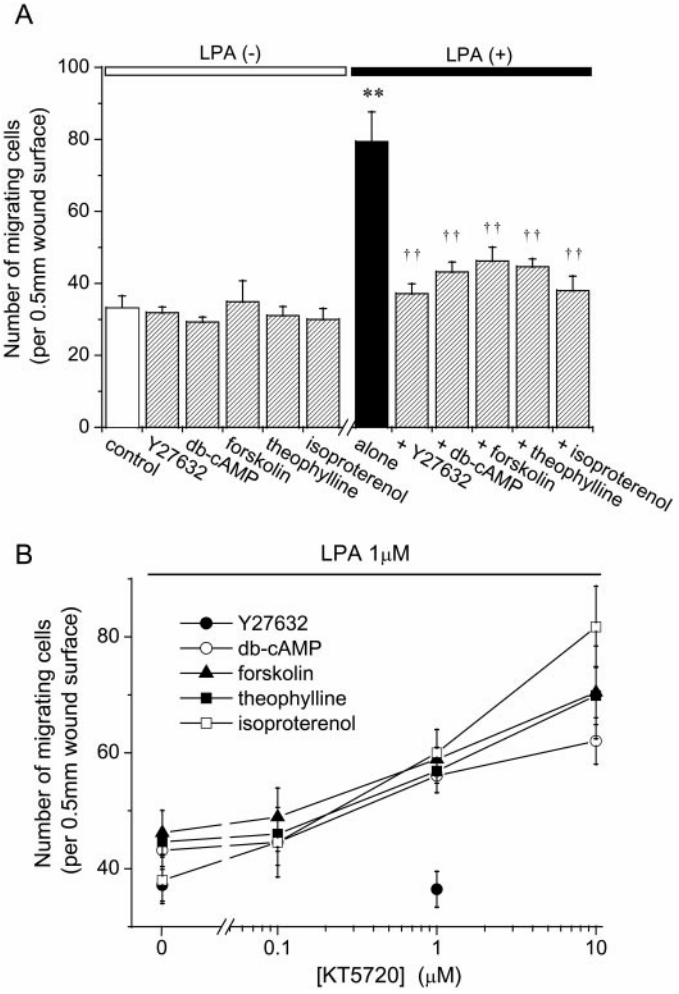

Fig. 4. Statistical analysis of LPA-induced migration of BTSMCs. A, confluent BTSMCs monolayer was wounded and cultured in the presence or absence of 1 μM LPA, and the effects of Y27632 (10 μM), dibutyryl cAMP (300 μM), forskolin (30 μM), theophylline (10 μM), or isoproterenol (10 μM) were examined. The number of cells migrated to the cell-free area was counted after 24 h ($n = 6$ for each condition). **, $p < 0.01$ versus control. ††, $p < 0.01$ versus LPA alone. B, effects of KT5720 on the inhibition of LPA-induced migration by cAMP-elevating agents and Y27632 were examined. LPA (1 μM) was applied to the wounded monolayer in the presence of 0.1, 1, or 10 μM KT5720, and the number of migrated cells was counted after 24 h. Data were obtained from six repeated measurements.

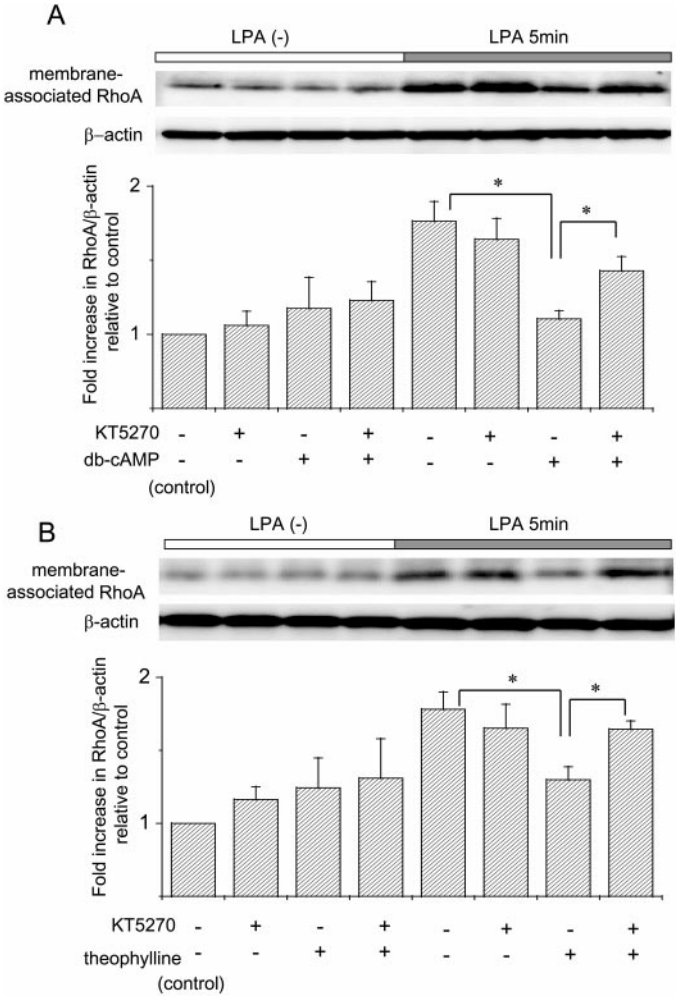

Fig. 5. LPA-induced membrane translocation of RhoA in BTSMCs. BTSMCs were pretreated with or without dibutyryl cAMP (300 μM, A) and theophylline (10 μM, B) for 1 h in the presence or absence of KT5720 (1 μM). Control or LPA (1 μM, 5 min)-treated BTSMCs were lysed, and the membrane fraction was obtained by centrifugation at 100,000*g* for 1 h. Expressions of RhoA and β-actin in the membrane fraction were then examined with Western blotting, and the representative band images are shown in the upper panels. Densitometric analysis of the bands is shown in the lower panels. Each bar represents mean ± S.E.M. value obtained from four repeated experiments. *, $p < 0.05$.

would lead to the dissociation of RhoA from the membrane toward the cytosol (Lang et al., 1996). Another possibility is the alteration of Dbl family proteins, which convert inactive RhoA-GDP into active RhoA-GTP (Whitehead et al., 1997). AKAP-Lbc, a Dbl family protein in cardiomyocytes, has an anchoring site for PKA and was activated by LPA (Diviani et al., 2001) and inactivated by the anchored PKA (Diviani et al., 2006). Although it is not known yet whether such PKA-anchoring Dbl proteins are expressed in airway smooth muscle cells or not, PKA may negatively control the functions of Dbl proteins in BTSMCs.

Involvement of LPA in the pathogenesis of airway diseases has recently been considered from various aspects, including smooth muscle mitosis (Cerutis et al., 1997), contractility (Toews et al., 1997), and Ca^{2+} sensitization (Sakai et al., 2003). The present study has revealed, for the first time, that LPA also induces smooth muscle migration that would lead to airway remodeling. Furthermore, this study provides a novel possible mechanism of action of theophylline and probably other cAMP-elevating agents on airway diseases, i.e., the inhibition of smooth muscle migration.

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