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Leukotriene D_4-induced Rho-mediated actin reorganization in human bronchial smooth muscle cells

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Abstract

We investigated the role of cysteinyl leukotriene (CysLT) receptors on leukotriene D_4-induced actin reorganization and the signaling pathways of the response in human bronchial smooth muscle cells. The effects of leukotriene D_4 on actin reorganization in human bronchial smooth muscle cells were evaluated by dual-fluorescence labeling of filamentous (F) and monomeric (G) actin with fluorescein isothiocyanate (FITC)-labeled phalloidin and Texas Red-labeled DNase I, respectively. Leukotriene D_4 (100 nM) induced actin reorganization in the presence and absence of extracellular Ca^{2+}. The CysLT type 1 ($CysLT_1$) receptor antagonist ONO 1078 (4-oxo-8(−)[*p*-(4-phenylbutyloxy) benzoylamino]-2-(tetrazol-5-yl)-4*H*-1-benzopyran hemihydrate) inhibited leukotriene D_4-induced actin reorganization. Pretreatment with pertussis toxin, C3 exoenzyme, or tyrosine kinase inhibitors significantly reduced leukotriene D_4-induced actin reorganization. However, phosphatidylinositol-3-kinase and protein kinase C inhibitors had little effect on these responses. These results suggest that leukotriene D_4-induced actin reorganization in human bronchial smooth muscle cells is extremely dependent on the $CysLT_1$ receptor coupled with pertussis toxin-sensitive G protein, Rho GTPases and tyrosine phosphorylation pathways.

Keywords: Actin; Smooth muscle cell, bronchial; Cysteinyl leukotriene type 1 receptor; Leukotriene D_4; Pertussis toxin; Protein kinase C; Tyrosine kinase

1. Introduction

The cysteinyl leukotrienes (leukotriene C_4, leukotriene D_4 and leukotriene E_4) are synthesized de novo from membrane-associated arachidonic acid via the 5-lipoxygenase pathway (Murphy et al., 1979). The effects of cysteinyl leukotrienes mimic many of the features of asthma, including mucus secretion, inflammatory cell recruitment, edema, and neuronal dysfunction, as well as bronchoconstriction. Moreover, the cysteinyl leukotrienes have been detected after bronchospasm in the blood, bronchial alveolar lavage fluid, and urine of patients with asthma (Liu et al., 1990; Sladek et al., 1990; Wenzel et al., 1990, 1991). Leukotriene D_4 and leukotriene C_4 can contract human airway smooth muscle in vitro and have a more than 1000 times higher potency in this action than does histamine (Dahlén et al., 1980, 1983). Therefore, it is thought that the cysteinyl leukotrienes may play a significant role in the pathophysiology of asthma (Drazen and Austen, 1987; Hay et al., 1995; Henderson, 1994; Piacentini and Kaliner, 1991).

Binding and functional studies have provided evidence that the biological effects of the cysteinyl leukotrienes are mediated via G protein-coupled receptors (Crooke et al., 1990; Metters, 1995). There is pharmacological evidence for subtypes of CysLT receptors (Coleman et al., 1995; Metters, 1995), including those in human lung (Gorenne et al., 1996; Labat et al., 1992), and these receptors have been classified as CysLT type 1 ($CysLT_1$) and type 2 ($CysLT_2$) receptors (Coleman et al., 1995). CysLT receptors are located in the plasma membranes of smooth muscle cells in the airways and in other types of cells (Cristol et al., 1989; Crooke et al., 1990; Krell et al., 1990). A single class of receptors in human airway smooth muscle appears to mediate contractions induced by cys-

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teinyl leukotrienes (Buckner et al., 1986, 1990; Hay et al., 1987; Jones et al., 1989). However, there is recent information that the effect of leukotriene D_4 in human cultured tracheal smooth muscle cells may be mediated by stimulation of an atypical CysLT receptor that does not fit into the current classification ($CysLT_1$ and $CysLT_2$ receptors) (Panettieri et al., 1998).

Recent studies have indicated that some smooth muscle contractile agents, such as carbachol and endothelin-1, induce actin reorganization (Hirshman and Emala, 1999; Togashi et al., 1998). Actin is a cellular protein essential for the motility of both nonmuscle and muscle cells. In nonmuscle cells, dynamic actin polymerization and depolymerization is the basic mechanism of cell motility (Stossel, 1989). In smooth muscle cells, the cytoskeleton is a filamentous network consisting largely of filamentous actin (F-actin), which provides a scaffold on which motor proteins such as myosin translocate to generate internal stress and alter the mechanical properties of cells. Rho GTPases, which are a subfamily of the Ras superfamily of monomeric 20–30 kDa GTP-binding proteins, are involved in the signaling pathways mediating cytoskeleton reorganization (Chardin et al., 1989; Hirshman and Emala, 1999; Janmey, 1998; Nobes and Hall, 1995; Stossel, 1989; Togashi et al., 1998) and Ca^{2+} sensitization in intact smooth muscle preparations (Chardin et al., 1989; Croxton et al., 1998; Gong et al., 1996; Hirata et al., 1992; Otto et al., 1996). The major intermediates upstream of Rho GTPases are heterotrimeric G proteins. The signaling pathways by which the heterotrimeric G proteins couple to Rho GTPases are known to be cell specific, and little is known about the leukotriene D_4-mediated reorganization of actin in human airway smooth muscle cells including its signaling pathways.

Therefore, we investigated the role of CysLT receptors on leukotriene D_4-induced actin reorganization and the signaling pathways of the response in human bronchial smooth muscle cells.

2. Materials and methods

2.1. Bronchial smooth muscle cell culture

Cryopreserved normal human adult bronchial smooth muscle cells were obtained from Clonetics (Division of Bio Whittaker, Walkersville, MD) and grown in 25-cm^2 flasks in Clonetics modified Molecular Cellular Developmental Biology (MCDB) 131 medium containing 0.5 μg/l epidermal growth factor, 5 mg/l insulin, 2 μg/l fibroblast growth factor, 50 mg/l gentamicin, 50 mg/l amphotericin and 5% fetal bovine serum at 37°C in an atmosphere of 5% CO_2. The cells were plated on glass-bottomed microwell dishes (MatTek) and incubated until they achieved confluence. After confluence was reached, the cells were washed with Hanks' balanced salt solution (HBSS) and kept in serum-free MCDB 131 medium for 24 h. After being washed with HBSS, the cells were allowed to rest for 30 min in a *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer containing (in mM) 10 HEPES, 136.9 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 1.5 $CaCl_2$, 0.001 EDTA and 5.5 glucose (HEPES-buffered solution) before stimulation with leukotriene D_4 (100 nM) for different periods of time. In Ca^{2+}-free experiments, the same solution was used except that 1.5 mM $CaCl_2$ was omitted and 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA) was added.

2.2. Confocal microscopy

Cells were fixed in 3% phosphate-buffered saline (PBS)–formalin for 10 min, and permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature. After two washes with PBS, cells were stained with fluorescein isothiocyanate (FITC)-labeled phalloidin (5 unit/ml in PBS) to localize F-actin and Texas Red-labeled DNase I (10 μg/ml in PBS) to localize G-actin for 20 min in a dark room at room temperature (Knowles and McCulloch, 1992). Cells were washed with PBS twice and maintained in PBS. Dishes were mounted on the stage of Leica TCS NT confocal laser scanning microscope equipped with an Ar–Kr laser. The excitation and emission wavelengths for FITC-phalloidin were 490 and 525 nm, whereas the excitation and emission wavelengths for Texas Red-DNase I were 596 and 615 nm. To standardize the fluorescence intensity measurements among experiments, the time of image capture, the image intensity gain, the image enhancement, and the image black level in both channels were optimally adjusted at the outset and kept constant for all experiments. The F- to G-actin staining ratio was calculated with quantification software (Leica TCS NT confocal laser scanning microscope system) in at least 10 cells from three fields for each treatment. At least four separate experiments were performed and these values were averaged for a single data point. An increase in the F- to G-actin ratio indicated an increase in actin reorganization.

2.3. Pretreatment with pertussis toxin, C3 exoenzyme, and kinase inhibitors

To determine whether the leukotriene D_4-induced increase in stress fiber formation involved G_i proteins, cells were incubated with 100 ng/ml pertussis toxin for 4 h, after which the cells were left untreated or treated with leukotriene D_4 (100 nM) for 5 min.

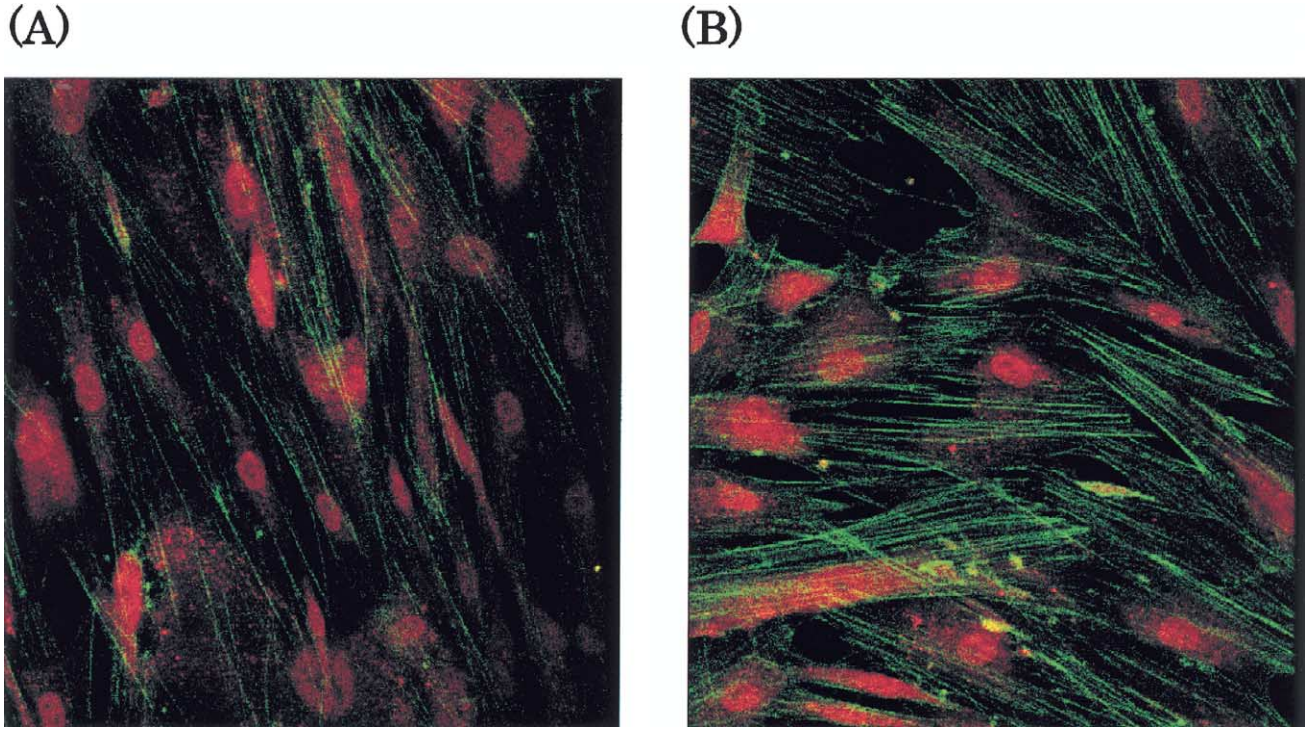

Fig. 1. Human bronchial smooth muscle cells stained with FITC-phalloidin (green) and Texas Red-DNase I (red) to illustrate F- and G-actin, respectively. Stimulation with leukotriene D_4 (100 nM) for 5 min induced an increase in F-actin and a decrease in G-actin (B), in comparison with that in untreated cells (A). The images are from at least four separate experiments.

To determine whether the leukotriene D_4-induced increase in stress fiber formation involved Rho proteins, cells were incubated with 10 μg/ml C3 exoenzyme for 72 h, after which the cells were left untreated or treated with leukotriene D_4 (100 nM) for 5 min.

To determine whether phosphatidylinositol-3-kinase (PI-3-kinase), protein kinase C and tyrosine kinase were involved in the leukotriene D_4-induced increase in stress fiber formation, each cells were incubated with 500 nM wortmannin, 100 nM GF-109203X (3-[*N*-(Dimethylamino)propyl-3-indolyl]-4-[3-indolyl]maleimide) and 20 μM genistein for 20 min, after which the cells were left untreated or treated with leukotriene D_4 (100 nM) for 5 min.

2.4. Statistical analysis

All data are presented as means ± S.E.M. Statistical analysis was performed using an analysis of variance (ANOVA). A value of $P < 0.05$ was considered significant.

2.5. Drugs

Leukotriene D_4 and pertussis toxin were purchased from Sigma, St. Louis, MO. GF-109203X, wortmannin, genistein and C3 exoenzyme were purchased from BIOMOL Research Laboratories, Plymouth Meeting, PA. FITC-phalloidin and Texas Red-DNase I were purchased from Molecular Probes, Eugene, OR. ONO 1078 (4-oxo-8(−)[*p*-(4-phenylbutyloxy) benzoylamino]-2-(tetrazol-5-yl)-4*H*-1-benzopyran hemihydrate) was a kind gift from Ono Pharmaceutical, Osaka, Japan.

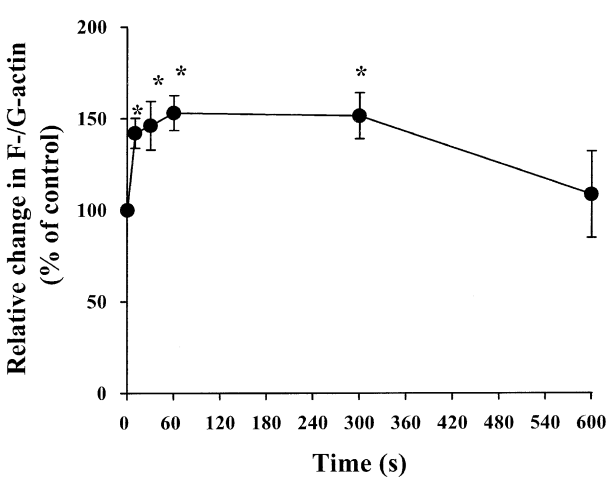

Fig. 2. Time course of the F- to G-actin fluorescence intensity ratio after leukotriene D_4 stimulation. The cells were stimulated with 100 nM leukotriene D_4 for different periods of time (10, 30, 60, 300, and 600 s). Values are expressed as percentages of control and are means ± S.E.M. from four separate experiments. $^{*}P < 0.01$, significant difference from untreated cells.

3. Results

3.1. Actin reorganization induced by leukotriene D_4

Exposure of serum-deprived human airway smooth muscle cells to 100 nM leukotriene D_4 for 5 min resulted in an increase in the FITC-phalloidin staining intensity of F-actin and a decrease in the Texas Red-DNase I staining intensity of G-actin compared with that in the untreated cells (Fig. 1). The F- to G-actin fluorescence-staining ratio, which is indicative of actin fiber reorganization, significantly increased to 151.4 ± 12.5% of that of untreated cells.

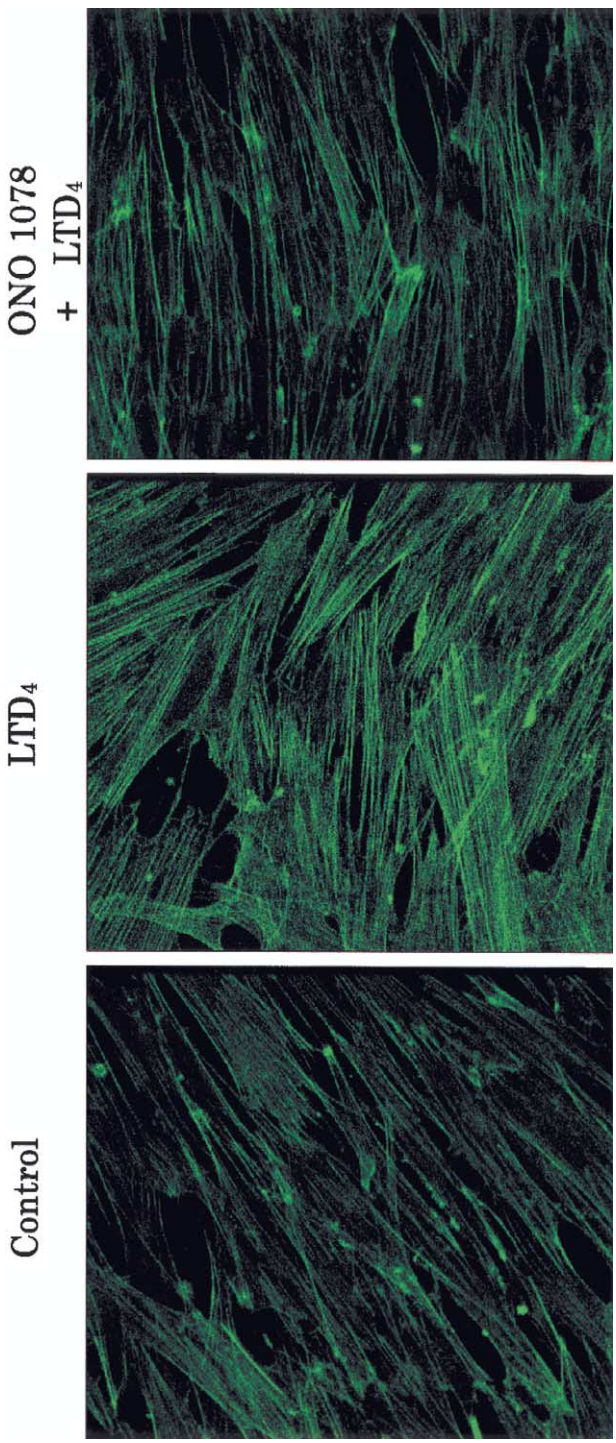

Fig. 3. Effect of the $CysLT_1$ receptor antagonist ONO 1078 on leukotriene D_4 (LTD_4)-induced F-actin reorganization. Pretreatment with ONO 1078 (10 μM) for 20 min inhibited the leukotriene D_4-induced increase in F-actin reorganization. The images are from at least four separate experiments.

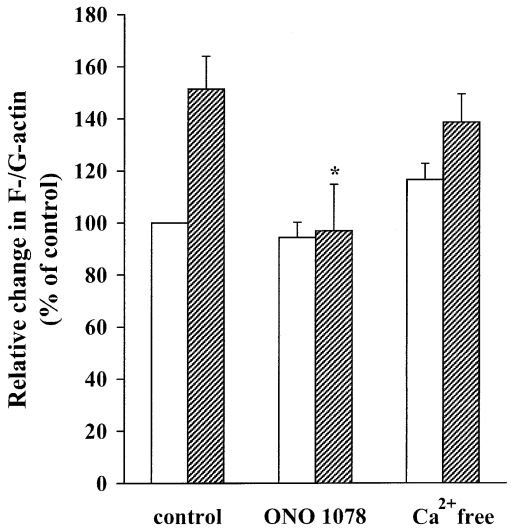

Fig. 4. Effects of the $CysLT_1$ receptor antagonist ONO 1078 (10 μM) and the absence of extracellular Ca^{2+} on the 100 nM leukotriene D_4-induced F- to G-actin fluorescence intensity ratio. Open bars = control; hatched bars = treated with leukotriene D_4. Values are expressed as percentages of control and are means ± S.E.M. from four separate experiments. $^{*}P < 0.05$, significant difference from each control.

The cells were stimulated with 100 nM leukotriene D_4 for different periods of time. Fig. 2 shows the percentage change in the F- to G-actin staining ratio following stimulation with 100 nM leukotriene D_4. The F- to G-actin staining ratio increased rapidly (142.1 ± 8.1% after 10 s) and this increase lasted at least 5 min. After 10 min, it returned to 108.4 ± 23.6% of that of untreated cells.

3.2. Effect of the $CysLT_1$ receptor antagonist ONO 1078 on actin reorganization induced by leukotriene D_4

Treatment with the specific $CysLT_1$ receptor antagonist ONO 1078 (10 μM) alone for 20 min did not affect F-actin staining and the F- to G-actin ratio (94.3 ± 5.9%). A 20-min preincubation with the specific $CysLT_1$ receptor antagonist ONO 1078 (10 μM) reduced the increase in F-actin staining (Fig. 3) and in the F- to G-actin ratio induced by leukotriene D_4 from 151.4 ± 12.5% to 96.9 ± 17.8% ($P < 0.05$, Fig. 4).

3.3. Effects of extracellular Ca^{2+} on actin reorganization induced by leukotriene D_4

To determine the role of extracellular Ca^{2+} in actin reorganization, the cells were incubated with Ca^{2+}-free HEPES buffer containing 0.1 mM EGTA for 30 min

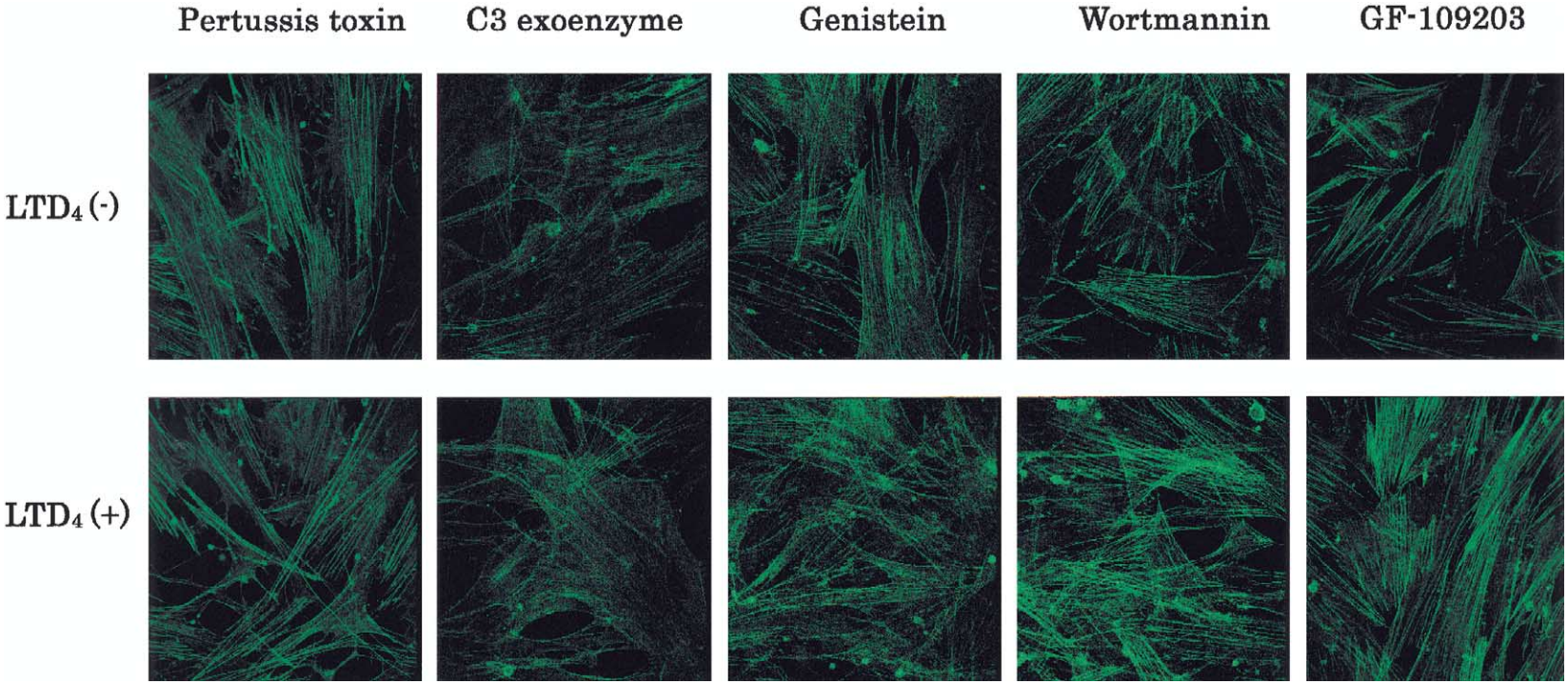

Fig. 5. Effects of pretreatment with 100 ng/ml pertussis toxin, 10 μg/ml C3 exoenzyme, 20 μM genistein, 500 nM wortmannin, and 100 nM GF-109203 on leukotriene D_4 (LTD_4)-induced increases in F-actin staining. Untreated cells are shown in the upper panel, and 100 nM leukotriene D_4-treated cells are shown in the lower panel. Pretreatment with pertussis toxin, C3 exoenzyme and genistein blocked leukotriene D_4-induced increases in F-actin staining. The images are from at least four separate experiments.

before stimulation with 100 nM leukotriene D_4. Under this condition, the leukotriene D_4-induced increases in F-actin staining and in the F- to G-actin ratio (138.5 ± 10.8% of control, Fig. 4) were not significantly different from those in the presence of extracellular Ca^{2+}.

3.4. Inhibition of leukotriene D_4-induced actin reorganization by pertussis toxin and C3 exoenzyme

To determine whether pertussis toxin-sensitive heterotrimeric G protein (G_i) and/or Rho proteins are intermediates in the signaling pathway in the actin reorganization induced by leukotriene D_4 and mediated via $CysLT_1$ receptor, the cells were pretreated with pertussis toxin for 4 h and C3 exoenzyme for 72 h. Pertussis toxin pretreatment alone had no effect on F-actin staining (Fig. 5) or on the F- to G-actin ratio (92.8 ± 6.9%) (Fig. 6). In the cells pretreated with pertussis toxin and then challenged with leukotriene D_4, F-actin staining did not increase (Fig. 5) and the increase in the F- to G-actin ratio induced by leukotriene D_4 was reduced from 151.4 ± 12.5% to 80.9 ± 4.7% ($P < 0.01$, Fig. 6).

C3 exoenzyme pretreatment alone had no effect on F-actin staining (Fig. 5) or on the F- to G-actin ratio (103.7 ± 6.0%) (Fig. 6). In the cells pretreated with C3 exoenzyme and then challenged with leukotriene D_4, F-actin staining did not increase (Fig. 5). Moreover, the increase in the F- to G-actin ratio induced by leukotriene D_4 was reduced from 151.4 ± 12.5% to 103.3 ± 1.0% ($P < 0.05$, Fig. 6).

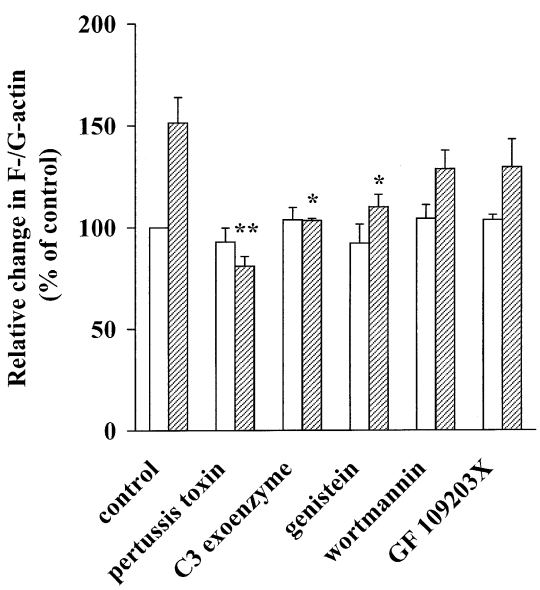

Fig. 6. Effects of pertussis toxin, C3 exoenzyme, genistein, wortmannin, and GF-109203 on the F- to G-actin fluorescence intensity ratio in the absence (open bars) or the presence (hatched bars) of 100 nM leukotriene D_4. Values are expressed as percentages of control and are means ± S.E.M. from four separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, significant difference from each control.

3.5. Effects of genistein, wortmannin and GF-109203X on actin reorganization induced by leukotriene D_4

To determine whether tyrosine phosphorylation, PI-3-kinases, or protein kinase C is required for the actin reorganization induced by leukotriene D_4, the cells were pretreated with the tyrosine kinase inhibitor, genistein (20 μM), the PI-3-kinase inhibitor, wortmannin (500 nM), or the protein kinase C inhibitor, GF-109203X (100 nM), for 20 min before stimulation with 100 nM leukotriene D_4. The inhibitors alone had no effect on F-actin staining or on the F- to G-actin ratio. Pretreatment with genistein significantly inhibited the increase in F-actin staining (Fig. 5) and reduced the F- to G-actin ratio from 151.4 ± 12.5% to 109.9 ± 6.1% ($P < 0.05$, Fig. 6). Pretreatment with wortmannin or GF-109203X did not significantly inhibit the increase in F-actin staining (Fig. 5) and in the F- to G-actin ratio (Fig. 6). In the cells pretreated with wortmannin, the F- to G-actin ratio was increased by leukotriene D_4 to 128.5 ± 9.1%, and in the cells pretreated with GF-109203X, the F- to G-actin ratio was increased by leukotriene D_4 to 129.4 ± 13.7%.

4. Discussion

This study demonstrates that activation of the $CysLT_1$ receptor with leukotriene D_4 led to a rapid and sustained reorganization of the actin cytoskeleton in human bronchial smooth muscle cells. We determined the intermediate proteins involved in this signaling pathway by using bacterial toxins and exoenzymes specific for GTP-binding proteins and inhibitors of protein kinases. Pretreatment with pertussis toxin or C3 exoenzyme inhibited the $CysLT_1$ receptor activation to induce stress fiber formation. An inhibitor of tyrosine kinase blocked the leukotriene D_4-induced actin reorganization, whereas inhibitors of PI-3-kinase and protein kinase C had no effect. Moreover, this response was independent of Ca^{2+} influx. These results indicated that pertussis toxin-sensitive heterotrimeric G proteins and Rho GTPases are involved in this pathway. Further, tyrosine phosphorylation may be important in the actin reorganization induced by leukotriene D_4 in human bronchial smooth muscle cells.

The CysLT receptors have been classified as $CysLT_1$ and $CysLT_2$ receptors in binding and functional studies (Coleman et al., 1995). Recently, two groups of investigators reported on the molecular cloning, expression, localization, and pharmacological characterization of the $CysLT_1$ receptor (Lynch et al., 1999; Sarau et al., 1999). Similar findings were obtained in pharmacological and localization experiments in the two separate studies. This receptor was identified by ligand fishing with orphan seven-transmembrane-spanning, G protein-coupled receptors. Leukotriene D_4-induced Ca^{2+} mobilization in human embryonic kidney (HEK)-293 cells expressing the CysLT

receptor was not affected by pertussis toxin, and the signal appears to be the result of the release of Ca^{2+} from intracellular stores. This result suggests that the G protein involved in transfected cells is of the $G_{q/11}$ rather than the G_i class. However, the leukotriene D_4-induced Ca^{2+} response of the endogenous receptor expressed in differentiated human U937 cells is partially blocked by pertussis toxin, suggesting that this receptor is coupled to pertussis toxin-sensitive and -insensitive G proteins (Saussy et al., 1989). Our study showed that leukotriene D_4-induced actin reorganization was dependent on pertussis toxin-sensitive G protein (G_i). Moreover, leukotriene D_4-induced actin reorganization was not affected by the absence of extracellular Ca^{2+}. Although Sarau et al. (1999) have explained these discrepancies in terms of different sensitivities to pertussis toxin and extracellular Ca^{2+}, further studies will be needed to identify the G-protein coupled to the $CysLT_1$ receptor.

Activation of $CysLT_1$ receptors with leukotriene D_4 led to reorganization of the actin cytoskeleton in human bronchial smooth muscle cells. Pertussis toxin-sensitive heterotrimeric G proteins and Rho GTPases mediated this response. Moreover, tyrosine phosphorylation is necessary for this reorganization of actin. Because it has become obvious recently that Rho GTPases are involved in the cytoskeletal reorganization and Ca^{2+} sensitization induced by extracellular stimuli in many cell types, a downstream effector pathway linking Rho GTPases to the actin cytoskeleton and Ca^{2+} sensitization has extensively been studied. Moreover, the signaling pathways upstream from Rho GTPases are cell-type specific and poorly understood, particularly in airway smooth muscle. Currently, there is only information about the carbachol, endothelin-1, and lysophosphatidic acid mediated pathway in airway smooth muscle cells (Hirshman and Emala, 1999; Togashi et al., 1998). This study provides the first evidence linking $CysLT_1$ receptors to Rho GTPases and to actin reorganization in airway smooth muscle cells.

A signaling role for RhoA in cytoskeletal organization has been suggested since the early 1990s (Paterson et al., 1990; Ridley and Hall, 1992, 1994). In serum-starved Swiss 3T3 fibroblasts activated RhoA induced stress fiber formation (Paterson et al., 1990; Ridley and Hall, 1992). In human airway smooth muscle cells, actin reorganization induced by carbachol is linked to Rho GTPases via a pertussis toxin-sensitive G protein (G_i) (Togashi et al., 1998), and that induced by endothelin-1 and lysophosphatidic acid is linked to Rho GTPases via involved G_q and G_{i-2} activation (Hirshman and Emala, 1999). Our study suggests that leukotriene D_4-induced actin reorganization involves Rho via a pertussis toxin-sensitive G protein. These results indicate that the $CysLT_1$ receptor is linked at least to the G_i protein. However, it seems that there are some types of receptors in human airway smooth muscle cells in which both pertussis toxin-sensitive and -insensitive G proteins are linked to Rho GTPases.

Tyrosine phosphorylation induced by tyrosine kinase was necessary for actin reorganization in human bronchial smooth muscle cells, although we could not determine whether tyrosine kinases were upstream or downstream of Rho protein. Tyrosine phosphorylation is also an important mechanism for regulating smooth muscle contraction. Tyrosine kinase inhibitors suppress agonist-induced contraction in many smooth muscle preparations (Di Salvo et al., 1993; Ohanian et al., 1997). Pretreatment with a PI-3-kinase inhibitor and a protein kinase C inhibitor induced a small reduction in stress fiber formation induced by leukotriene D_4. PI-3-kinase, and thus protein kinase C may also be involved in the pathway linked to actin reorganization.

Under extracellular Ca^{2+}-free conditions, to block Ca^{2+} influx, leukotriene D_4 induced actin reorganization in human bronchial smooth muscle cells. In human intestinal epithelial cells loaded with the intracellular calcium chelator MAPT/AM (1,2-bis-5-methyl-aminophenoxylethane-*N*,*N*,*N*′-tetra-acetoxymethyl acetate), leukotriene D_4 induced a normal actin polymerization response, but the subsequent depolymerization was completely inhibited (Massoumi and Sjölander, 1998). Therefore, an intracellular Ca^{2+} signal, involved in leukotriene D_4-induced actin reorganization, may be important for F-actin depolymerization rather than for its polymerization.

5. Conclusion

We have shown that the $CysLT_1$ receptor is linked to pertussis toxin-sensitive G proteins and Rho GTPases in the pathway to induce actin reorganization in human smooth muscle cells. The importance of tyrosine phosphorylation in this pathway was also indicated in this study.

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