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The Functional Role of Rho and Rho-Associated Coiled-Coil Forming Protein Kinase in Eotaxin Signaling of Eosinophils

Tetsuya Adachi,* Randi Vita,† Satoshi Sannohe,* Susan Stafford,† Rafeul Alam,† Hiroyuki Kayaba,*, and Junichi Chihara*‡

The CC chemokine eotaxin plays a pivotal role in local accumulation of eosinophils. Very little is known about the eotaxin signaling in eosinophils except the activation of the mitogen-activated protein (MAP) kinase family. The p21 G protein Rho and its substrate Rho-associated coiled-coil forming protein kinase (ROCK) regulate the formation of stress fibers and focal adhesions. In the present study, we investigated whether Rho and ROCK are upstream of these MAP kinases. C3 partially inhibited eotaxin-induced phosphorylation of ERK1/2 but not p38. In contrast, neither ERK1/2 nor p38 phosphorylation was abrogated by Y-27632. Both C3 and Y-27632 reduced reactive oxygen species production from eosinophils. We conclude that both Rho and ROCK are important for eosinophil chemotaxis and reactive oxygen species production. There is a dichotomy of downstream signaling pathways of Rho, namely, Rho-ROCK and Rho-ERK pathways. Taken together, eosinophil chemotaxis is regulated by multiple signaling pathways that involve at least ROCK, ERK, and p38 MAP kinase.

Eosinophils play a pivotal role in the mechanism of allergic diseases including asthma (1). Chemotaxis of eosinophils is the single most important event in the pathogenesis of allergic inflammation. CC chemokines such as eotaxin, RANTES, monocyte chemotactic peptide-3, and monocyte chemotactic peptide-4 play a crucial role in eosinophil migration in tissue (2). The chemotactic response of eosinophils is mostly mediated by CCR3, which is also preferentially expressed on Th2 cells and basophils. CCR3 is a heterotrimeric G protein-coupled receptor that is known to transduce signals eliciting Ca2+ influx (3, 4). We and others have recently found that eotaxin activates extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen-activated protein (MAP) kinases in eosinophils, and that these kinases are indispensable for eosinophil chemotaxis and degranulation (5, 6). However, very little is known about other signaling molecules involved in CCR3 signaling in eosinophils.

The p21 G protein Rho and its targets, Rho-associated coiled-coil forming protein kinases (p160ROCK/ROCK I/ROKβ and Rho kinase/ROCK II/ROKα), play a crucial role in actin cytoskeleton reorganization (7). One example of this function could be observed in smooth muscle contraction that is regulated by the cytosolic Ca2+ concentration and by the Ca2+ sensitivity of myosin/actin filament (8). The former activates myosin light-chain kinase, whereas Rho-ROCK is responsible for the latter through the inhibition of myosin phosphatase or the direct phosphorylation of myosin light chain (9–11). Y-27632, a selective inhibitor for ROCK I and ROCK II, effectively suppresses the contraction of arterial smooth muscle and corrects hypertension in hypertensive rat models (12). The compound also inhibited guanosine 5′-O-(3-thiotriphosphate)-induced Ca2+ sensitization of rabbit trachea, suggesting that ROCK is involved in the regulation of bronchial hyperreactivity (13). Another important role of ROCK is its involvement in focal adhesion and stress fiber formation (14–16). Transfection with Rho-kinase/p160ROCK induces focal adhesions and stress fibers, whereas these phenomena are not observed in cells with the kinase-defective mutant. The assembly and disassembly of stress fibers and focal adhesions are critical processes for cell scattering. The microinjection of exoenzyme C3 transferase, a specific Rho inhibitor, or dominant negative Rho-kinase inhibitors wound-induced migration of NRK49F cells (17). The role of Rho-ROCK in cell adherence and migration has also been shown in neutrophils. Inactivation of Rho by exoenzyme C3 blocks FMLP-, IL-8-, or PMA-induced neutrophil adhesion to fibronectin (18). Furthermore, Y-27632 suppresses myosin light-chain phosphorylation and chemotactic peptide-induced chemotaxis of neutrophils (19). However, little is known about the functional role of Rho-ROCK in eosinophils.

In the present study, we investigated the activation and functional relevance of Rho and ROCK in eosinophils after eotaxin...
stimulation. We found that eotaxin stimulates activation of Rho and ROCK in eosinophils, and that there is a dichotomy of downstream signaling pathways of Rho, namely, Rho-ROCK and Rho-ERK pathways. Both Rho and ROCK are critical for eosinophil chemotaxis and production of reactive oxygen species (ROS).

Materials and Methods

Reagents

RPMI 1640 medium and Gey’s buffer were obtained from Life Technologies (Grand Island, NY) and FCS from Filitron (Brooklyn, Australia). Percoll was purchased from Pharmacia Biotech (Uppsala, Sweden). Myelin basic protein (MBP), streptolysin-O, cold ATP, and Ca ionophore A23187 were obtained from Sigma (St. Louis, MO). The mouse mAb against phospho-ERK, rabbit polyclonal anti-ERK2, anti-apt-p85 Abs, HRP-conjugated goat anti-mouse and anti-rabbit Abs, and protein A/G Plus agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal anti-ROKab was purchased from Transduction Laboratories (Lexington, KY). The polyclonal anti-phospho-p38 Ab was obtained from New England Biolabs (Beverly, MA). The source of exoenzyme C3 was Upstate Biotechnology (Lake Placid, NY). Y-27632 was a gift from Welflde (Hirakata, Japan). Anti-CD16 immunomagnetic beads were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Human eosinophils were purchased from R&D Systems (Minneapolis, MN). ECL detection system and Hybond ECL nitrocellulose membrane were obtained from Amersham (Arlington Heights, IL). [γ-32P]ATP and guanosine-5’-32P-γ-triphosphate [γ-32P]GTPγS were purchased from NEN Life Science Products (Boston, MA) and Amersham Pharmacia Biotech (Piscataway, NJ), respectively.

Eosinophil purification

Peripheral blood was obtained from subjects with mild to moderate eosinophilia. Eosinophils were isolated by sedimentation with 6% dextran followed by centrifugation on 1.088 Percoll density gradients according to the method of Hansel et al. (20). The cells were further purified by negative selection using anti-CD16 immunomagnetic beads and a MACS system (Miltenyi Biotec). Eosinophils (>99% pure) were then suspended in HBSS with 1% FCS in tubes coated with 3% human serum albumin.

Membrane isolation

Purified eosinophils were rinsed twice in cold PBS followed by incubation for 10 min on ice in Dounce homogenizing buffer containing 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5% sucrose, 0.1 mM PMSF, and 1 μg/ml aprotinin, leupeptin, and pepstatin. The cells were homogenized at 2 × 106 cells/ml with 30 strokes in a Dounce homogenizer. Intact cells were then pelleted by centrifugation at 400,000 × g for each sample) for 1 h, then incubated with 20 μl of protein A/G Plus agarose for 2 h at 4°C. The beads were washed three times with the cold lysis buffer.

[γ-32P]GTPγSA labeling

The labeling of cell membranes with [γ-32P]GTPγS was performed as described elsewhere, with slight modifications (22). Frozen membrane samples were thawed and pelleted by centrifugation at 14,000 × g for 10 min at room temperature. The membranes were then incubated in TE buffer (20 mM Tris-HCl, 1 mM EDTA, pH 7.4) containing 50 μM GDP, 0.2 mM EGTA, 2 mM MgCl2, and 50 mM CH3COONa for 30 min at room temperature. The sample was stimulated with eotaxin in the presence of 2 μCi of [γ-32P]GTPγS. The reaction was terminated by the addition of ice-cold buffer and anesole placement of samples on ice for 10 min. After centrifugation at 14,000 × g for 10 min, the pellets were resuspended in TE buffer with 1 mM DTT. The suspension was then UV irradiated using a hand-held UV lamp at 254 nm for 10 min in a 24-well plate. The samples were collected and the wells were rinsed with buffer followed by centrifugation at 14,000 × g for 10 min. The pellets were dissolved in 20 μl of 2% SDS, and then 400 μl of the lysis buffer was added to each sample. After measuring the protein concentration using bicinchoninic acid assay (Pierce, Rockford, IL) to standardize the samples, the lysates were subjected to immunoprecipitation.

Immunocomplex kinase assay

The immunoprecipitates were assayed for ROCK (ROKa) by measuring the phosphotransferase activity for MBP. The kinase reaction was performed by incubating the immunoprecipitates in 40 μl of kinase buffer (10 mM HEPES, 50 mM NaCl, 10 mM MgCl2, 100 μM Na2VO3, 500 μM DTT, 25 mM β-glycerophosphate) containing 2.5 μM ATP, 10 μl of [γ-32P]ATP, and 50 μg/ml MBP for 30 min at 30°C. After centrifugation, the reaction was stopped by boiling the supernatant with an equal amount of 2× Laemmli buffer. The kinase reaction products were then applied to SDS-PAGE and autoradiography.

Gel electrophoresis and Western blotting

SDS-polyacrylamide gels were prepared according to the Laemmli protocol and used for Western blotting. In some experiments, gel electrophoresis was performed using Ready Gels J (Bio-Rad, Hercules, CA). The concentration of polyacrylamide was 10–15% depending on the m.w. of the protein in which we were interested. Gels were blotted onto Hybond membranes for Western blotting using the ECL system. Blots were incubated in a blocking buffer containing 10% BSA in TBST buffer (20 mM Tris-HCl, 137 mM NaCl, pH 7.6, 0.05% Tween 20) for 1 h followed by incubation in the primary Ab (0.1 μg/ml) for 1–2 h. After washing three times in TBST buffer, blots were incubated for 30 min with a HRP-conjugated secondary Ab (0.04 μg/ml) directed against the primary Ab. The blots were developed with the ECL substrate according to the manufacturer’s instructions. In some experiments, blots were reprobed with another Ab after stripping in a buffer of 62.5 mM Tris-HCl (pH 6.7), 100 mM 2-βME, and 2% SDS at 50°C for 30 min.

Chemotaxis assay

The chemotaxis assay was performed in a 48-well Boyden microchamber (NeuroProbe, Gaithersburg, MD) as described previously (6). Briefly, eotaxin was diluted in Gey’s buffer with 0.02% BSA and placed in the lower wells (100 μl) at a 10 nM concentration. After incubation of eotaxin at 37°C with and without the inhibitors for indicated times, 100 μl of the cell suspension at 2 × 106 cells/ml was added to the upper well of the chamber, which was separated from the lower well by a 5-μm pore size polycarbonate, polynylpyrolidone-free membrane (Nuclepore, Pleasanton, CA). The chamber was incubated for 60 min at 37°C. Then, the membrane was removed following by fixation and staining for 5 min in May-Grünwald solution. The cells that migrated and adhered to the lower surface of the membrane were counted from 10 fields by light microscopy. The chemotactic response to buffer (<40/10 fields) was subtracted from that induced with eotaxin with or without the inhibitors.

ROS production from eosinophils

ROS production from eosinophils was analyzed by luminol-dependent chemiluminescence. The stimulation of eosinophils was performed by adding 50 μl of 30 μM Ca ionophore A23187 to 100 μl of eosinophil suspension (5 × 106 cells) containing 0.25 mM luminol. The luminol–dependent maximal and integral intensity counts were determined for 60 min using an ARGUS-502D lumimeter/MP (Hamamatsu Photonics, Hamamatsu, Japan). All experiments were performed in duplicate or triplicate.
Statistical analysis

Data were analyzed for statistical significance using ANOVA and paired Student’s t test.

Results

Rho activation using eosinophil membranes

To study the activation of Rho in CCR3 signaling, we first determined the presence of Rho in eosinophils. Using Western blotting, we demonstrated that the p21 Rho A is present in the lysates of eosinophils (Fig. 1A). The Rho activation was analyzed based on the level of GTP binding by the activated G proteins. With the difficulties in internalizing a nonhydrolyzable form of GTP and maintaining the ability to stimulate eosinophils with chemokines, we used the cell membranes that do not require the internalization. The study of membrane-bound G proteins using cell membranes has been demonstrated (22). Prepared eosinophil membranes were labeled with [γ-32P]GTPγAA, a nonhydrolyzable form of GTP, to detect Rho activity. Additionally, [γ-32P]GTPγAA forms a covalent bond when UV irradiated, resulting in a strong binding between the GTP and the G protein. Immunoprecipitation for Rho A was performed after eotaxin stimulation of the labeled membranes. The bound GTP was examined by SDS-PAGE and autoradiography. We found that the Rho A activity was enhanced by eotaxin in a dose-dependent manner (Fig. 1B). The densitometric analysis revealed that 10 nM eotaxin stimulates Rho activity (n = 3) (Fig. 1C).

Kinase activity of ROCK by eotaxin

Next, the expression of ROCK in eosinophils was studied. We found that eosinophils express ROCK II (ROKα) (Fig. 2A) but not ROCK I (data not shown). To investigate the ROCK activity, eosinophils were stimulated with eotaxin (10 nM) and then immunoprecipitated with the anti-ROKα Ab. The kinase activity of ROCK II was then determined by measuring the phosphotransferase activity for MBP. As shown in Fig. 2B, the ROCK activity in eosinophils was enhanced by eotaxin. The pretreatment of exoenzyme C3 (25 μg/ml) inhibited the ROCK activity, suggesting that ROCK II is exactly downstream of Rho A in eotaxin signaling. As expected, Y-27632 (1–100 μM) blocked the phosphorylation of MBP by ROCK in a dose-dependent manner (Fig. 2C).

Effect of exoenzyme C3 and Y-27632 on eosinophil chemotaxis

Rho and ROCK are critical for the formation of stress fibers and focal adhesions (14–16), and consequently regulate the cell scattering (17). Therefore, we studied their involvement in eotaxin-induced eosinophil chemotaxis. Eosinophils were incubated with

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**FIGURE 1.** A, Presence of Rho A in eosinophils. Purified eosinophils were lysed and immunoprecipitated with anti-Rho Ab. The immunoprecipitate was pelleted and separated from the supernatant. The pellet and the supernatant were electrophoresed and Western blotted with the same Ab. The supernatant were electrophoresed and Western blotted with the same Ab. B, Activation of Rho A in eosinophil membranes. Isolated eosinophil membranes were labeled with [γ-32P]GTPγAA and stimulated with eotaxin for 1 min. The cell membranes were immunoprecipitated with the anti-Rho A Ab, and the immunoprecipitates were run on 14% acrylamide gel. The activation of Rho A was examined by autoradiography. Eotaxin induced dose-dependent activation of Rho A in eosinophil membranes. C, Densitometric analysis showed the activation of Rho A by eotaxin (n = 3). *, p < 0.05 vs without eotaxin (paired Student’s t test).

**FIGURE 2.** A, Presence of ROCK II in eosinophils. Eosinophils were lysed followed by electrophoresis and Western blotting with the anti-ROKα Ab. Left lane, Contains a positive control and shows the position of ROCK II. ROCK II was present in eosinophils regardless of eotaxin stimulation. B and C, Activation of ROCK II in eotaxin-stimulated eosinophils. Permeabilized eosinophils were incubated with and without 25 μg/ml exoenzyme C3 for 24 h at 37°C. In some experiments, purified eosinophils were incubated with and without Y-27632 for 1 h. The cultured eosinophils were stimulated with 10 nM eotaxin for 3 min. The lysates of the cells were immunoprecipitated with the anti-ROKα Ab. The immunocomplex was visualized by autoradiography. The ROCK II activity was enhanced after eotaxin stimulation. The pretreatment of C3 (B) or Y-27632 (C) inhibited the activity of ROCK II.
the inhibitors and then applied in the upper chambers. The lower chambers contained 10 nM eotaxin. The cells that migrated and adhered to the lower surface of the membrane were counted by light microscopy. The pretreatment of exoenzyme C3 (25 μg/ml) significantly reduced the number of migrated eosinophils stimulated with eotaxin (n = 3) (Fig. 3A). Y-27632 (0.3–100 μM) also inhibited eosinophil chemotaxis in a dose-dependent manner (n = 3) (Fig. 3B).

**Effect of exoenzyme C3 and Y-27632 on MAP kinase phosphorylation**

We have previously found that ERK1/2 and p38 MAP kinases are activated by eotaxin, and that these kinases are critical for eosinophil chemotaxis (5, 6). Because Rho and ROCK are also important for the function, we investigated whether the MAP kinases are downstream of Rho or ROCK. After incubation with exoenzyme C3 or Y-27632, eosinophils were stimulated with eotaxin followed by electrophoresis and Western blotting with the anti-p-ERK or anti-p-p38 Ab. Exoenzyme C3 (25 μg/ml) partially inhibited eotaxin-induced phosphorylation of ERK1/2 (Fig. 4A), but not p38 (Fig. 4B). In contrast, neither ERK1/2 nor p38 phosphorylation was abrogated by Y-27632 (Fig. 5, A and B). Reprobing the membranes with the anti-ERK2 or anti-p38 Ab indicated that the same amounts of protein were loaded on the gels. Taken together, ERK1/2 appears to be downstream molecules of Rho in eotaxin signaling.

**Effect of exoenzyme C3 and Y-27632 on ROS production**

We studied the functional relevance of Rho and ROCK in ROS production from eosinophils. Eotaxin alone has a minimal effect on ROS production (23). As eotaxin induces Ca2+ influx in eosinophils (24, 25), we stimulated eosinophils with Ca ionophore A23187. ROS production from eosinophils was analyzed by luminol-dependent chemiluminescence. Both exoenzyme C3 (25 μg/ml) and Y-27632 (100 μM) reduced ROS production by 45% (n = 3) and 58% (n = 5), respectively (Fig. 6, A and B). We next performed similar experiments using eotaxin-primed eosinophils because eotaxin has been shown to possess a priming effect on eosinophil chemotaxis. Pretreatment with exoenzyme C3 (A) and Y-27632 (B) inhibited eotaxin-induced eosinophil migration (n = 3). *, p < 0.05 vs without inhibitors (paired Student’s t test and ANOVA).
Discussion

In the present work, we studied the involvement and the functional relevance of Rho and ROCK in eotaxin signaling. Rho A and ROCK II were activated in eosinophils stimulated with eotaxin. We found that these molecules are critical for eosinophil chemotaxis and ROS production. There was a dichotomy of downstream signaling pathways of Rho, namely, Rho-ROCK and Rho-ERK pathways. This is the first report that showed essential roles of Rho and ROCK in eosinophil function.

The Rho GTPase family, including Rho, Rac, and Cdc42, regulates actomyosin-based cellular function such as cell adhesion and migration (26). Rac regulates the assembly of actin filaments at the cell periphery to form lamellipodia and membrane ruffles, whereas Cdc42 induces actin-rich surface protrusions called filopodia. The activation of Rho leads to the formation of stress fibers and associated focal adhesion complexes. Although several targets of Rho have been identified, the details of Rho downstream signaling are complicated. Ishizaki et al. (16) have initially shown that the phenotype of stress fibers induced by ROKα is different from that induced by Rho A. The dominant active mutant of RhoA induces the formation of parallel stress fibers, whereas the dominant active mutant of ROCK induces the formation of stellate stress fibers. Furthermore, the transfection of HeLa cells with Rho A and p160ROCK defective in kinase and Rho-binding activities exhibits an increase in the amount of F-actin along with the reduction of stress fiber formation, indicating that Rho-induced actin polymerization is mediated by different effectors other than ROCK. mDia has subsequently been identified as another target protein for Rho that regulates stress fiber formation through profilin (27). Interestingly, however, the roles of ROCK and mDia in the regulation of actin cytoskeleton appear to be different (28, 29).

The mDia1-induced formation of thin actin stress fibers is disorganized in the absence of ROCK activity. Moreover, active mDia transforms ROCK-induced condensed actin fibers into structures similar to those regulated by Rho. According to these results, the thickness and density of Rho-induced stress fibers depends on the balance between ROCK and mDia signals. Although ROCK regulates actin cytoskeleton by inhibiting myosin phosphatase activity, two groups have identified an alternative pathway that transduces signals through ROCK-LIM kinase-cofilin (30, 31). In the present study, we showed that the Rho-ROCK pathway is activated by eotaxin in eosinophils. Several studies have shown that the actin polymerization is induced by eotaxin (5, 24, 25, 32). Thus it is possible that the eotaxin signaling pathway involves molecules other than Rho-ROCK, e.g., mDia and profilin.

The involvement of MAP kinases in the downstream of the small GTPase Rho family has been well studied (33). Frost et al. (34) have investigated the molecular action of Rho family proteins on MAP kinase activation using a kidney epithelial cell line, 293 cells. The expression of either activated Cdc42 or Rho A does not increase ERK activity. However, the ERK2 activity, which can be stimulated with the active form of Raf, is greatly enhanced when the constitutively active Rac2, Cdc42, or Rho A is coexpressed with Raf. Consistent with the results, our data revealed that ERK1/2 activity is partially regulated by Rho in eotaxin signaling. Angkachatchai et al. (35) have also shown that the pretreatment of T cells with exoenzyme C3 partially reduced the ERK activity following TCR ligation, which supports our results. We did not see any inhibition of p38 MAP kinase phosphorylation by exoenzyme C3. One possible reason for this observation is that the activation of p38 MAP kinase is regulated by Rac or Cdc42 (36) through p21-activated kinase (PAK) activation (34, 37). Although the details remain to be clarified, we preliminarily found that Rac and

ROS production evoked by Ca ionophore (23). However, after culturing eosinophils for 24 h in case of the experiments using exoenzyme C3, the Ca ionophore-induced ROS production was much less than that of freshly purified eosinophils, and the priming effect of eotaxin was barely observed. For this reason, we simply investigated the effect of Y-27632 on ROS production from eosinophils primed with eotaxin. As shown in Fig. 6C, the priming of eosinophils with 100 nM eotaxin augmented the Ca ionophore-induced ROS production that was inhibited by Y-27632 (1–100 μM) in a dose-dependent manner (n = 3).

FIGURE 6. Effect of exoenzyme C3 and Y-27632 on Ca ionophore-induced ROS production from eosinophils. Permeabilized eosinophils were incubated with and without 25 μg/ml exoenzyme C3 for 24 h at 37°C. Alternatively, purified eosinophils were incubated with and without incremental concentration of Y-27632 for 1 h. In some experiments, the eosinophils were primed with or without eotaxin (100 nM) for 15 min. After stimulation of the cultured eosinophils with 10 μM Ca ionophore, the luminol-dependent maximal and integral intensity counts were determined. The Ca ionophore-induced ROS production was inhibited by exoenzyme C3 (A) (n = 3) and Y-27632 (B) (n = 5). C, Y-27632 also blocked the ROS production from eosinophils primed with eotaxin (n = 3). *, p < 0.05 vs without inhibitors (paired Student’s t test and ANOVA).
PAK are activated by eotaxin in eosinophils (R. Vita, S. Stafford, and R. Alam, unpublished data).

The mechanism of cell migration involves dynamic and coordinate disassembly and reassembly of stress fibers and focal adhesions. The contribution of Rho family members to cell migration has been studied in the macrophage chemotaxis assay (38). The migration of macrophages by colony-stimulating factor-1 was completely inhibited when the cells were injected with exoenzyme C3, an inhibitor of Rho, or with the dominant-negative Rac mutant, suggesting the critical role of Rho and Rac in the function. In contrast, macrophages injected with the dominant-negative Cdc42 mutant were able to migrate but did not polarize in the direction of the gradient. Rac is required for the formation of protrusions at the front of the migrating cells and provides the major driving force for movement. Cdc42 provides a polarity signal that is required for directed migration. The activity of Rho appears to be restricted to the rear of the cells to generate the retraction force that is necessary to pull the cell body. Another study has shown that chemotactic peptide-induced neutrophil polarity and locomotion were inhibited by Y-27632, an inhibitor of ROCK (19). In support of these findings, we found that both Rho and ROCK are involved in eosinophil chemotaxis.

The role of ROCK in superoxide production from neutrophils has been studied (39). Y-27632 reduced superoxide production from neutrophils stimulated with PMA, but not with C3 exoenzyme A23187. The discrepancy of the responsiveness between neutrophils and eosinophils is unclear. Although our data indicate the contribution of Rho and ROCK to ROS production from eosinophils, one cannot rule out the possibility that other candidates are involved. A major component of superoxide generation in inflammatory cells is the membrane-associated NADPH oxidase complex that contains cytochrome b_{558}, p47phox, p67phox, and the GTPase Rac2 (40). Chemoattractants, such as fMLP and platelet-activating factor, induce rapid and transient Rac activation in neutrophils (41). The stimulation of neutrophils with PMA elicits a respiratory burst without Rac activation. However, in bone marrow-derived Rac2^{−/−} neutrophils, the superoxide production in response to IgG-coated SRBC or fMLP was significantly abrogated, suggesting the important role of Rac2 in the function downstream of chemoattractant and FcγR (42). In addition, Lacy et al. (43) have shown that Rac2 translocates from cytosol to plasma membrane-associated fractions along with p47phox and p67phox in PMA-stimulated eosinophils.

We showed that the Ca ionophore-induced ROS production from eosinophils was blocked by exoenzyme C3 and a relatively high concentration of Y-27632. In addition, a similar effect of Y-27632 was observed in eotaxin-primed eosinophils. Sullivan et al. (21) have demonstrated that Rho is downstream of Ca signaling and independent of calmodulin in mast cells. Furthermore, Rho, but not ROCK, is essential for hexosaminidase secretion despite of the ROCK activation through Rho. Our data are consistent with the results of Sullivan et al., except that both Rho and ROCK are involved in ROS production from eosinophils. Although reasons for the discrepancy between eosinophils and mast cells are unclear, signaling pathways responsible for mediator release and ROS production may be slightly different.

In conclusion, we have defined the activation of Rho and ROCK in eotaxin signaling and its essential role in eosinophil chemotaxis and ROS production. Fig. 7 summarizes the evidence and shows our current model. Recruitment of eosinophils from the bloodstream into the airways is the most characteristic feature of asthma and allergic diseases. Chemokines, particularly eotaxin, are important regulators of eosinophils in the foregoing conditions. Therefore, the delineation of the signaling pathway of chemokines and its role in various functions of eosinophils will help elucidate the mechanism of allergic diseases and may shed light on developing a new strategy to treat eosinophilic disorders.

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