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Rac and Protein Kinase C-δ Regulate ERKs and Cytosolic Phospholipase A₂ in FcεRI Signaling to Cysteinyl Leukotriene Synthesis in Mast Cells

Sung-Hoon Cho,*† Hye-Jin You,*† Chang-Hoon Woo,* Yung-Joon Yoo,‡ and Jae-Hong Kim‡*

Although cysteinyl leukotrienes (cysLTs) are known to be principal inflammatory lipid mediators released from IgE-stimulated mast cells, the signaling mechanisms involved in the synthesis of cysLTs remain largely unknown. In the present study, therefore, we investigated the signaling pathway by which IgE induces cysLTs synthesis after binding to its high affinity receptor (FcεRI) in RBL-2H3 mast cells. We found that IgE-induced cysLT synthesis is completely abolished in RBL-2H3Rac-N17 cells, a stable cell line expressing RacN17, a dominant negative Rac1 mutant; conversely, synthesis was enhanced in cells expressing RacV12, a constitutively active Rac1 mutant, suggesting that Rac1 is a key mediator of IgE signaling to cysLT synthesis. Further analysis aimed at identifying mediators downstream of Rac1 revealed that pretreating cells with a protein kinase C-δ (PKC-δ) inhibitor or infection with an adenoviral vector harboring a dominant negative PKC-δ mutant significantly attenuates IgE-induced ERKs phosphorylation, cytosolic phospholipase A₂, phosphorylation/translocation, and cysLT synthesis. In addition, the expression of RacN17 blocked PKC-δ translocation and impaired the phosphorylation of ERKs and cystosolic phospholipase A₂ otherwise elicited by IgE stimulation. Taken together these results suggest that PKC-δ also plays a critical mediatory role in the IgE signaling pathway leading to cysLT synthesis, acting downstream of Rac1. Finally, the physiological significance of PKC-δ in the IgE signaling pathway was demonstrated in an Ag (OVA)-challenged in vivo mouse model, in which induced levels of cysLTs and airway responsiveness in lung airways were significantly diminished by prior i.p. injection of a PKC-δ inhibitor. The Journal of Immunology, 2004, 173: 624–631.

M ast cells are central effector and regulatory cells in Th2-dominant immune responses and innate immune defense (1–3). Ag stimulation of FcεRI on mast cells elicits allergic responses by inducing the release of three classes of proinflammatory mediators: 1) preformed granule-associated chemical mediators, such as histamine; 2) newly synthesized arachidonic acid (AA)1 metabolites, such as leukotrienes (LTs); and 3) proinflammatory TNF-α and Th2-associated cytokines (1–3). Among these mediators, the cysteinyl LTs (cysLTs; LTC₄, LTD₄, and LTE₄) exert a number of pathophysiological effects, including mucus secretion, inflammation, and vascular permeability (4–6). Indeed, cysLTs are known to be related to the pathogenesis of asthma and, ultimately, to determine the severity of asthma (7–9).

In contrast to the well-characterized signaling pathway leading to histamine degranulation, the pathways leading to LT generation from FcεRI activation are not yet well understood. It has been suggested that aggregation of multiple IgE-bearing FcεRI molecules induced by polyvalent Ag ultimately leads to activation of ERKs and cytosolic phospholipase A₂ (cPLA₂), which hydrolyzes membrane phospholipids, releasing AA (10, 11). AA is, in turn, converted to cysLTs via the actions of 5-lipoxygenase (5-LO) and its activating protein, 5-LO activation protein (FLAP). Although Lyn and Syk (two Src family protein tyrosine kinases (PTKs)), PI3K, and PLC-γ have all been suggested as potential upstream regulators of the ERK-cPLA₂ cascade, few details of the signaling mechanisms regulating ERKs and cPLA₂ are available (12, 13).

Recently, we and others were able to show that Rac, a member of the Rho family of small GTPases, acts as an upstream regulator of cPLA₂ in fibroblasts, ultimately stimulating the synthesis of LTs (14–18). In addition, evidence suggests that two Rho GTPases, Cdc42 and Rac1, regulate FcεRI signaling, leading to activation of Akt or phospholipase D₁, Ca²⁺ mobilization, and degranulation in RBL-2H3 mast cells (19–22). In contrast, the function of Rac1 in the FcεRI signaling pathway leading to cysLT synthesis in mast cells has never been examined. The aim of the present study, therefore, was to characterize the function of Rac1 in the generation of cysLTs induced by FcεRI stimulation in RBL-2H3 mast cells. Our results suggest that both Rac1 and protein kinase C-δ (PKC-δ) act upstream of ERKs and cPLA₂ to regulate IgE signaling to cysLT synthesis in RBL-2H3 mast cells. In addition, the physiological significance of PKC-δ in the IgE signaling was demonstrated in vivo in an Ag-challenged asthmatic mouse model.

Materials and Methods

Chemicals
Mouse monoclonal anti-DNP-IgE (clone SPE-7), DMSO, PMA, wortmannin, OVA, and methacholine were all purchased from Sigma-Aldrich (St.

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3 Abbreviations used in this paper: AA, arachidonic acid; AHR, airway hyper-responsiveness; Ax, adenosine expression system; BAL, bronchoalveolar lavage; cPLA₂, cytosolic phospholipase A₂; cysLT, cysteiny1 leukotriene; DAG, diacylglycerol; DN, dominant negative; FLAP, 5-LO activation protein; HA, hemagglutinin; 5-LO, 5-lipoxygenase; LT, leukotriene; MCH, methacholine; MOI, multiplicity of infection; P₂ax, enhanced pause; PKC, protein kinase C; PLC, phospholipase C; PTK, protein tyrosine kinase; PVDF, polyvinylidene difluoride; WT, wild type; PAK, p21-activated serine/threonine protein kinase; PBD, PK-binding domain.
Louis, MO). MK886, genistein, and AACOCF3 were obtained from BIOMOL (Plymouth Meeting, PA). DNP-conjugated BSA (DNP BSA), GF109203X, Gö6976, rottlerin, and calphostin C were purchased from Calbiochem (La Jolla, CA). Rabbit polyclonal anti-ERK1/2, anti-phospho-ERK1/2, and anti-phospho-cPLA2 Abs were obtained from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti-PKC-δ Ab was purchased from BD Biosciences (Palo Alto, CA). Rabbit polyclonal anti-PKC-δ Ab and anti-hemagglutinin (anti-HA) rabbit Ab were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Tetramethylrhodamine B iso-thiocyanate-conjugated mouse Ab was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). FBS, DMEM, phenol red-free DMEM, and nonessential amino acids were obtained from Invitrogen (Carlsbad, CA). All other chemicals were purchased from standard sources and were of molecular biology grade or higher.

**Cell culture and construction of stable clones**

Rat basophilic leukemia RBL-2H3 cells were maintained as a monolayer in DMEM supplemented with 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% heat-inactivated FBS at 37°C under a humidified 95%/5% (v/v) mixture of air and CO2. Cells were transfected with pcDNA-HA-Rac1(197), encoding a dominant negative Rac1 mutant, or with pcDNA-HA-Rac V12, encoding a constitutively active form of Rac1, using the Lipofectamine transfection method. Stably transfected clones were selected with 0.4 μg/ml G418 (Invitrogen, Carlsbad, CA), after which they were screened for HA-Rac1 expression by immunoblotting the cell lysates with anti-HA rabbit Ab.

**Measurement of cysLT1/D1/E4 levels**

RBL-2H3 cells (2 × 10⁶) were plated on 60-mm dishes and incubated in DMEM supplemented with 15% FBS for 12 h. The cells were then sensitized with monoclonal anti-DNP-specific IgE (1 μg/ml) for 1 h and serum-starved in DMEM containing 0.5% FBS for an additional 6 h before being stimulated with Ag DNP-BSA (1 μg/ml) for the indicated times. LTC4/D4/E4 levels were assayed with the LTC4/D4/E4 enzyme immunoassay system (Amersham Pharmacia Biotech, Piscataway, NJ). In brief, 200 μl of culture medium was concentrated by freeze-drying for 12 h and reconstituted in assay buffer. The assay was calibrated with standard LTC4/D4/E4 ranging from 0.75 to 48 pg/well. Samples of medium and standard LTC4/D4/E4 in 96-well plates were incubated with antisemur for 2 h, followed by LTC4 peroxidase conjugate for 3 h, at 4°C. To remove unbound ligand, the wells were aspirated and washed four times with wash buffer. Substrate (tetramethylbenzidine) was then added, the reaction was stopped by adding 100 μl of 1 M sulfuric acid, and the color was read at 450 nm in a microtiter plate spectrophotometer. The sensitivity of the assay was 0.5 pg/well, which is equivalent to 10 pg/ml. Differences were assessed by ANOVA, and p < 0.01 was considered significant.

**Rac1 activity assay**

Rac1 activity was measured as described previously (23) using a GST-p21-activated serine/threonine protein kinase (PAK)-PAK-binding domain (PBD) fusion protein that binds GAP-1 bound, activated Rac1. Briefly, the fusion protein was expressed in Escherichia coli BL21 transformed with pGEX-4T3 plasmid by incubation with isopropyl-1-thio-β-D-galactopyranoside and then purified by column chromatography using glutathione-Sepharose-4B beads. RBL-2H3 cells were sensitized with monoclonal anti-DNP-specific IgE (1 μg/ml) for 12 h, serum-starved (0.5% FBS) for 6 h, stimulated with DNP-BSA (1 μg/ml) for the indicated times, and then lysed in buffer (150 mM NaCl, 20 mM Tris-Cl (pH 7.4), 2.5 mM sodium pyrophosphate, 1% Triton X-100, 10 μg/ml leupeptin, pH 7.5). The resultant lysates were subjected to 15% SDS-PAGE, and the resolved proteins were transferred to PVDF membranes, which were then probed with an anti-Rac1 Ab. The results shown are representative of at least three independent experiments.

**Immunostaining of cPLA2 and PKC-δ proteins**

To visualize the localization of endogenous cPLA2 and PKC-δ, cells were plated on coverslips, grown for 12 h in DMEM containing 15% FBS, sensitized with anti-DNP IgE (1 μg/ml) for 12 h, serum-starved (0.5% FBS) for 6 h, and then exposed to DNP-BSA (1 μg/ml) for the indicated times. Thereafter, the cells were washed with cold PBS, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked in 1% BSA (solvilized in PBS), and labeled with mouse anti-cPLA2 (1/150) or anti-PKC-δ (1/150) Ab. The immunolabeled cells were washed with PBS and labeled with a tetramethylrhodamine B isothiocyanate-conjugated, anti-mouse secondary Ab (1/200), again washed with cold PBS, and mounted on glass slides for observation under a fluorescence microscope.

**Subcellular fractionation of cell lysates**

RBL-2H3 cells were serum-starved (0.5% FBS) for 6 h, then exposed to the appropriate agonist for the indicated times. The medium was removed, and the cells were washed twice with ice-cold PBS, scraped, harvested by microcentrifugation, and resuspended in 0.2 ml of buffer A (137 mM NaCl, 8.1 mM Na2HPO4, 2.7 mM KCl, 1.5 mM KH2PO4, 2.5 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10 μg/ml leupeptin, pH 7.5). The resuspended cells were lysed by sonication them twice for 15 s on ice or by passing them 20 times through a 21.1-gauge needle on ice. The lysates obtained were centrifuged at 65,000 × g for 1 h to prepare cytosolic and total particulate fractions. The particulate fraction, containing the membrane fraction, was washed twice and resuspended in 50 μl of buffer A. Protein concentrations were routinely determined using the Bradford procedure with Bio-Rad dye reagent (Hercules, CA), using BSA as a standard.

**FIGURE 1.** Role of Rac1 in IgE-induced cysLT synthesis in RBL-2H3 mast cells. A, Rac mutant (HA-Rac V12 or HA-Rac N17) constructs were transfected into RBL-2H3 mast cells, after which several stable clones were selected with G418; the expression of Rac mutants was confirmed by immunoblot analysis with anti-HA Ab. B, IgE-sensitized WT and mutant RBL-2H3 cells (2 × 10⁶) were stimulated with DNP-BSA (1 μg/ml) for the indicated times, after which secreted cysLTs were measured by ELISA as described in Materials and Methods. Data are expressed as the mean ± SD of three independent experiments. C, IgE-sensitized WT and mutant RBL-2H3 cells were serum-starved for 6 h and exposed to 1 μg/ml DNP-BSA for the indicated times, after which they were lysed and incubated with GST-PK-PBD coupled to glutathione-agarose beads. Bound Rac-GTPase was eluted, resolved by 15% SDS-PAGE, and transferred to a PVDF membrane, which was then probed with an anti-Rac1 Ab. The results shown are representative of at least three independent experiments.
FIGURE 2. Involvement of PI3K, PKCs, ERKs, cPLA₂, and 5-LO in IgE-induced cysLT synthesis and phosphorylation of ERKs/cPLA₂. A, IgE-sensitized RBL-2H3 cells (1 × 10⁶) were serum-starved for 6 h and then stimulated with DNP-BSA (1 µg/ml) for 30 min. In some cases cells were pretreated for 20 min with wortmannin (100 nM), AACOCF₃, (10 µM), MK886 (50 nM), PD98059 (20 µM), GF109203X (200 nM), Go6976 (100 nM), rottlerin (2 µM), or calphostin C (100 nM) before stimulation with DNP-BSA. The levels of secreted cysLTs were measured by ELISA as described in Materials and Methods. Data are expressed as the mean ± SD of three independent experiments. B and C, IgE-sensitized RBL-2H3 cells were exposed to DNP-BSA (1 µg/ml) for 5 min in the presence of 100 nM wortmannin, 20 µM PD98059, and 10 µM SB203580 (B) or 200 nM GF109203X, 100 nM Go6976, 30 nM Ro-32-0432, 2 µM rottlerin, and 100 nM calphostin C (C). Inhibitors were added 20 min before the addition of DNP-BSA. Cell lysates were probed for levels of ERKs/phospho-ERKs and cPLA₂/phospho-cPLA₂. In addition, phospho-Akt was detected using mouse monoclonal anti-phospho-Akt Ab (Cell Signaling Technology), and the control β-actin level was determined using rabbit anti-β-actin Ab (Santa Cruz Biotechnology). The results shown are representative of at least three independent experiments.

Infection with recombinant PKC-δ adenovirus

Adenoviral expression vectors for wild-type and dominant negative (DN) mutant PKC-δ were provided by Dr. J. S. Chun (Kwangju Institute of Science and Technology, Kwangju, Korea). Subconfluent RBL-2H3 cells in a 60-mm dish were incubated with control virus (Ax-lacZ) or virus harboring wild-type (Ax-PKC-δ-WT) or DN PKC-δ (Ax-PKC-δ-DN) for 1 h at various multiplicities of infection (MOIs) in DMEM containing 0.1% FBS. After removing the virus, cells were cultured for an additional 3 h in DMEM supplemented with 15% heat-inactivated FBS and then serum-starved (0.5% FBS) for 12 h.

PKC-δ assay

RBL-2H3 cells were harvested, then lysed in lysis buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 25 mM glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM NaF, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS. Cell lysates were subjected to centrifugation at 12,000 rpm for 10 min at 4°C, and the resultant soluble fraction was subjected to immunoprecipitation using rabbit polyclonal anti-PKC-δ Ab (Santa Cruz Biotechnology, Santa Cruz, CA). The immunopellets were resuspended in 15 µl of kinase assay buffer (25 mM Tris-Cl (pH 7.5), 5 mM β-glycerol phosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 10 mM MgCl₂, and [γ⁻³²P]ATP) and incubated with 1 µg of myelin basic protein for 15 min at room temperature. Substrate phosphorylation was detected by autoradiography.

Sensitization and mouse challenge

The sensitization and mouse challenge were achieved using a modified version of the method described by Kanehiro et al. (24). Briefly, female C57BL/6j mice (6 wk of age) were injected i.p. with 200 µg of OVA (Sigma-Aldrich) and 2.25 mg of aluminum hydroxide gel (Pierce, Rockford, IL). A second i.p. injection of 20 µg of OVA adsorbed to aluminum hydroxide gel was administered 10 days later. After an additional 10 days, mice were exposed to an aerosol of 1% OVA in PBS for 30 min daily for 3 consecutive days. Nebulization was performed in a plastic chamber connected to an ultrasonic nebulizer (Omron, Vernon Hills, IL) that allowed OVA aerosol entry. Two days after final exposure to the 1% OVA aerosol, the animals were intranasally challenged with 100 µg of OVA to induce the asthmatic phenotype. In some cases, GF109203X (20 µg) or rottlerin (5 µg) was injected into the peritoneum of mice 1 and 24 h before OVA challenge.

Determination of airway hyper-responsiveness (AHR) to methacholine (MCh)

AHR to increasing doses of nebulized MCh was assessed in unrestrained, conscious female C57BL/6j mice 2 days after the last challenge, as previously described (25). Mice were placed in a barometric whole-body plethysmographic chamber (All Medicus, Seoul, Korea), and baseline readings were taken and averaged for 3 min. Aerosolized MCh, a cholinergic agonist with an inhaled bronchoconstrictor agent, at increasing concentrations (6.25–50 mg/ml) was nebulized for 3 min. Readings were taken and averaged for 3 min after each of the nebulizations, which were administered at 5-min intervals. Signals were analyzed to derive whole-body flow parameters, including respiratory rate, tidal volume, inspiratory time, expiratory time (Te), peak inspiratory flow (PIF), peak expiratory flow (PEF), and relaxation time (RT). These parameters were used to calculate enhanced pause (Pₑₑₑₑ), a unitless parameter that is used as a measure of airway responsiveness to MCh. Pₑₑₑₑ reflects changes in pulmonary resistance during bronchoconstriction according to the following equation: Pₑₑₑₑ = ((Te − RT) × RT) × (PEF ÷ PIF). Baseline Pₑₑₑₑ measurements for each animal were recorded for 3 min and averaged. Results are expressed as the percent increase in Pₑₑₑₑ after challenge with each concentration of MCh, where the baseline Pₑₑₑₑ (after saline challenge) is expressed as 100%. Pₑₑₑₑ values averaged for 3 min after each nebulization were evaluated.

Penh measurements for 3 min after each nebulization were evaluated. The result for each animal was recorded for 3 min and averaged. Results are expressed as the percent increase in Penh after challenge with each concentration of MCh, where the baseline Penh (after saline challenge) is expressed as 100%. Penh values averaged for 3 min after each nebulization were evaluated.
FIGURE 3. Role of PKC-δ in IgE-induced phosphorylation of ERKs and cPLA₂. A, Down-regulation of PKC-δ by long term PMA treatment was assessed (top panel). After down-regulating PKC levels through prolonged exposure to PMA, RBL-2H3 cells were sensitized for 12 h with DNP-specific IgE (1 μg/ml) in the presence of DMSO (control) or PMA (50 nM), then stimulated for 5 min with DNP-BSA (1 μg/ml). The cell lysates were then immunoblotted with anti-phospho-ERK and anti-phospho-cPLA₂ Abs, after which the blots were stripped and reprobed with anti-total ERK and cPLA₂ Abs. B, Involvement of PKC-δ in the phosphorylation of ERKs and cPLA₂. RBL-2H3 cells were infected with a control adenoviral vector (Ax-lacZ), Ax-PKC-δ-WT, or Ax-PKC-δ-DN at an MOI of 100. After 30 h the cells were sensitized with IgE (1 μg/ml) for 12 h and stimulated with DNP-BSA (1 μg/ml) for 5 min. Cell lysates were immunoblotted with anti-phospho-ERKs and anti-phospho-cPLA₂ Abs, after which the blot was stripped and reprobed with anti-total ERKs and cPLA₂ Abs. Additionally, PKC-δ kinase activity was measured by the γ-32P phosphorylation of myelin basic protein, and the levels of PKC-δ and β-actin were detected as a control. Data are representative of three separate experiments. C, IgE-sensitized RBL-2H3 cells were exposed to DNP-BSA (1 μg/ml) for 10 min in the presence or the absence of 200 nM GF109203X or 2 μM rottlerin, after which they were lysed, and cytosolic and particulate fractions were prepared. Translocation of cPLA₂ was analyzed by Western analysis of each fraction. The results shown are representative of at least three independent experiments.

SDS-PAGE and immunoblot analysis

Protein samples were heated at 95°C for 5 min, resolved by SDS-PAGE using 8% (for cPLA₂ or PKC-δ) or 10% (for HA or ERKs) acrylamide gels, and transferred to PVDF membranes using a wet transfer unit (for 2 h at 100 V; NOVEX, San Diego, CA). The membranes were then blocked for 1 h with TBS containing 0.05% (v/v) Tween 20 plus 5% (w/v) nonfat dry milk, and incubated first for 2 h with primary Ab (1/1000 dilution) in TBS containing 0.05% (v/v) Tween 20 plus 3% (w/v) BSA, then for 1 h with HRP-conjugated secondary Ab before development using an ECL kit.

Data analysis and statistics

Data are expressed as the mean ± SD or as the percentage ± SD of the control value. Statistical comparisons between groups were made using Student’s t tests. Values of p < 0.01 were considered significant.

Results

FcεRI-mediated cysLT synthesis in mast cells is dependent on Rac1 activation

We previously showed that in fibroblasts Rac1 plays a crucial role in LT synthesis elicited by various stimuli, including TNF-α (15–18). To assess whether Rac1 is also involved in the IgE signaling to cysLT synthesis in mast cells, we generated several cell lines stably expressing an HA-tagged DN or constitutive-active Rac1 mutant (HA-RacN17 or HA-RacV12, respectively) in RBL-2H3 mast cells (Fig. 1A). To investigate the role of Rac1 in FcεRI-mediated cysLT generation, groups of cells were sensitized with anti-DNP-IgE (1 μg/ml), serum-starved, and then stimulated with Ag (1 μg/ml DNP-BSA) for the indicated times before measurement of cysLT levels (Fig. 1B). Ag stimulation of WT RBL-2H3 cells induced cysLT synthesis that reached a maximum within 30 min. Notably, cysLT synthesis was much greater in the RacV12-expressing cells than in WT cells, whereas the production of cys-LTs was completely blocked in cells expressing RacN17 (Fig. 1B), suggesting that Rac is a key mediator of FcεRI signaling to cysLT synthesis.

This result prompted us to test directly the extent to which exposure to Ag (DNP-BSA) stimulates cellular Rac1 activity. Using in vitro PAK binding assays to assess Rac1 activity, we found that, consistent with the proposed mediatory role of Rac1 in FcεRI signaling, Ag induced a significant increase in Rac1 activity within 5 min in WT RBL-2H3 cells (Fig. 1C). However, in DNP-BSA-stimulated RBL-2H3 RacN17 stable cells, any activation of Rac1 was not detected, whereas enhanced activation of Rac1 was observed in RBL-2H3 Rac-V12 cells (Fig. 1C).

IgE-induced cysLT synthesis requires PI3K and PKC

To identify additional mediators contributing to IgE-induced cysLT generation, we tested the effects of inhibitors of several candidate molecules, including wortmannin, a PI3K inhibitor with an IC50 of 5 nM (26); GF109203X, a general PKC inhibitor with an IC50 of 0.2 μM (27); Gö6976, a potent inhibitor of Ca2+-dependent PKC isozymes with an IC50 of 6.2 nM (27); rottlerin, a PKC-δ inhibitor with an IC50 of 3–6 μM (28); calphostin C, a diacylglycerol (DAG)-dependent PKC inhibitor with an IC50 of 60 nM (29); PD98059, a MEK inhibitor with an IC50 of 2 μM (30); A2058, a cPLA₂ inhibitor with an IC50 of 20 μM (31); and MK886, a 5-LO/FLAP inhibitor with an IC50 of 102 nM (32). Consistent with the well-known mediatory roles played by ERKs, cPLA₂, and 5-LO in LT synthesis (18), pretreatment with PD98059, A2058, or MK886 attenuated IgE-induced cysLT synthesis in RBL-2H3 cells (Fig. 2A). That wortmannin (100 nM)
also inhibited cysLT synthesis was consistent with our earlier reports suggesting the involvement of PI3K in the regulation of Rac and cPLA2 in IgE signaling. For example, pretreatments with GF109203X, rottlerin, and calphostin C were each able to significantly inhibit the production of cysLTs, G66976 (a PKC-α, -β, and -γ inhibitor) did not (Fig. 2A). This strongly suggests that PKC-δ is also involved in IgE-induced cysLT synthesis in RBL-2H3 mast cells.

We next used immunoblot analysis to investigate the extent to which PI3K and PKC-δ serve as upstream modulators of ERKs and cPLA2 activation in IgE signaling. For example, pretreating cells with wortmannin (100 nM) blocked FceRI-mediated phosphorylation of ERKs and cPLA2, as did PD98059 (Fig. 2B). At the same concentration (100 nM) of wortmannin, phosphorylation of Akt induced by DNP-BSA was also selectively blocked, demonstrating a specific inhibitory action of wortmannin toward PI3K in this experimental condition (Fig. 2B). By contrast, a p38 MAPK inhibitor had no effect on the phosphorylation of ERKs or cPLA2, suggesting that IgE induces cysLT synthesis via a PI3K-ERK-cPLA2-linked cascade. In addition, whereas G06976 and Ro-320432 (a PKC-α, -β, -γ, and -ε inhibitor) had no effect on levels of ERKs and cPLA2 activation, a general PKC inhibitor (GF109203X), a DAG-dependent PKC inhibitor (calphostin C), and a PKC-δ specific inhibitor (rottlerin) each significantly inhibited IgE-induced ERKs and cPLA2 phosphorylation (Fig. 2C), consistent with several recent reports that PKC regulates the activation of ERKs and cPLA2 in various cell lines (33, 34).

PKC-δ is specifically involved in IgE signaling to ERKs and cPLA2 phosphorylation

To further confirm that PKC-δ is responsible for Ag-stimulated cysLT synthesis, we initially tested the effects of long term (12-h) PMA treatment, which is known to down-regulate PKC-δ (Fig. 3A) as well as the PKC-α, -β, -βII, -γ, and -ε isoforms in RBL-2H3 mast cells (data not shown). As shown in Fig. 3A, down-regulation of PKC-δ with long term PMA treatment attenuated IgE-induced ERKs and cPLA2 phosphorylation. We then used an adenoviral expression system (Ax) to introduce WT or DN mutant PKC-δ into RBL-2H3 cells. As shown in Fig. 3B, infection with DN PKC-δ blocked FceRI-mediated ERKs and cPLA2 activation, whereas infection with WT PKC-δ had no effect on IgE-induced ERKs or cPLA2 phosphorylation. In this DN PKC-δ infection system, no activation of PKC-δ kinase was observed in the infected cells, verifying the action of our Ax-PKC-δ-DN (Fig. 3B). Inhibition of PKC-δ by GF109203X or rottlerin also blocked DNP-BSA-induced translocation of cPLA2 to the membrane compartment in RBL-2H3 cells (Fig. 3C).

Rac1 acts upstream of PKC-δ in IgE signaling

Collectively, the results presented above strongly suggest that PKC-δ and Rac1 are specifically required for IgE-induced ERKs and cPLA2 phosphorylation as well as for subsequent cysLT synthesis in RBL-2H3 mast cells. In a later experiment aimed at determining whether Rac1 activation is situated up- or downstream
Rac1 is necessary for IgE-induced ERKs and cPLA₂ activation

That Rac1 acts upstream of PKC-δ in IgE signaling suggests that it also lies upstream of ERKs and cPLA₂. This was confirmed by comparing the effects of Ag stimulation in RBL-2H3 and RBL-2H3\(^{Rac-N17}\) cells. Although substantial significant phosphorylation of ERKs and cPLA₂ was observed within 5 min of exposing RBL-2H3 cells to DNP-BSA, little or no phosphorylation was observed in RBL-2H3\(^{Rac-N17}\) cells under the same conditions (Fig. 5A). Moreover, translocation of cPLA₂ to the membrane compartment was completely blocked in RBL-2H3\(^{Rac-N17}\) cells (Fig. 5, B and C).

Effect of PKC-δ inhibition on cysLT synthesis in a murine OVA-induced asthma model

Our findings on the involvement of PKC-δ in cysLT synthesis in mast cells prompted us to investigate its physiological significance in the synthesis of cysLTs in vivo using an OVA-induced asthmatic mouse model. The protocol used to induce the asthmatic phenotype is shown in Fig. 6A (see Materials and Methods). Two days after the OVA challenge, analysis of bronchoalveolar lavage (BAL) fluid revealed significantly elevated levels of cysLTs, an effect that was markedly inhibited by prior i.p. administration of GF109203X (20 μg) or rottlerin (5 μg; Fig. 6B). Moreover, the BAL fluid showed a marked infiltration of inflammatory cells, especially eosinophils (Fig. 6C, upper panels), and this, too, was significantly inhibited by pretreating mice with GF109203X or rottlerin (Fig. 6C, lower panels). In addition, cysLTs are the most prominent inducer of bronchoconstriction (5–7). Airway responsiveness was assessed as the percent increase in P\(_{\text{rea}}\), in response to increasing doses of MCh, and the percent P\(_{\text{rea}}\) produced by MCh administration (at doses from 6.25 to 50 mg/ml) increased significantly in OVA-challenged mice compared with controls (~3-fold). As shown in Fig. 6D, however, pretreatment with GF109203X or rottlerin produced a significantly reduced AHR in OVA-challenged mice, suggesting that in vivo the major effect of cysLTs is indeed prevented by PKC-δ inhibitors. PKC-δ thus appears to be a key mediator of cysLT synthesis in vivo as well as in vitro, making it a potential target for the development of anti-asthma therapy.

Discussion

Although the function of Rac1 in IgE/FcεRI-evoked degranulation of mast cells has been described previously (19–22), the present study provides the first direct evidence of the pivotal roles played by Rac1 and PKC-δ in the signaling by which IgE evokes the production of cysLTs in RBL-2H3 mast cells. Acting together with PI3K and ERKs, these two mediators serve as integral components in a cascade regulating the activity of cPLA₂ and ultimately the synthesis of cysLTs.

Aggregation of multiple IgE-bearing FcεRI molecules by polyvalent Ag is known to lead to activation of Lyn and Syk, two Src family tyrosine kinases, and subsequent phosphorylation/activation of PI3K, and several studies have suggested that PI3K functionally interacts with Rho GTPases in IgE signaling (22). Nevertheless, the detailed relationship between Rac1 and PI3K in FcεRI signaling to cysLT synthesis remains unclear. However, given that both Rac1 activation and cysLT synthesis via FcεRI stimulation are blocked by wortmannin, we suspect that Rac1 lies downstream of PI3K in the cysLT generation cascade. The mechanism by which PI3K stimulates Rac1 is not yet known, but the guanine nucleotide exchange factor, Vav, reportedly mediates the interaction between PI 3-kinase and Rho GTPases (35), which suggests the following scenario. The PI3K product, phosphoinositol 3,4,5-trisphosphate, stimulates the tyrosine phosphorylation/activation of Vav, which, in turn, catalyzes GDP/GTP exchange on Rac1, causing this G protein to switch from its inactive to its active state. In any event, Rac1 appears to be an essential mediator of IgE/FcεRI signaling leading to cysLT synthesis.
PKC-δ is a member of the novel PKC subfamily and is a DAG-dependent, Ca^{2+}-independent PKC isoform. An earlier report showed that FceRI stimulation triggers the phosphorylation and translocation of PKC-δ, leading to degradation of RBL-2H3 mast cells (36). In bone marrow-derived mast cells, by contrast, PKC-δ appears to function as a negative regulator of Ag-induced degranulation (37). There have been several recent reports showing that inhibition of PKC reduces LT synthesis and histamine release in various mast cell types (38, 39), but the role of PKC-δ in IgE-induced cysLT synthesis had not been examined until now. Our results clearly demonstrate that PKC-δ plays a crucial role in IgE signaling to cysLT synthesis in mast cells, acting as a downstream mediator of Rac1. Although the signaling pathway by which Rac1 activation leads to PKC-δ activation is not well understood, several lines of evidence suggest that Rac1 and cdc42 associate with some PKC isotypes, regulating stress fiber formation, cell movement, transcriptional activation, and degranulation (40–42).

RBL-2H3 mast cells express PKC-α, -β1, -β2, -δ, -ε, and -ζ (S.-H. Cho and J.-H. Kim, unpublished observation), which are all known to be activated by Ca^{2+} and/or DAG. During our work we found that a PLC-γ inhibitor blocked IgE-induced PKC-δ translocation as well as phosphorylation of ERKs and cPLA₂ in RBL-2H3 cells (unpublished observations). Consistent with that result, recent evidence suggests that activated Rac1 participates in PLC-γ activation, and the DAG produced may function in PKC-δ activation, leading to activation of ERKs and cPLA₂. In support of this hypothesis, several reports have shown that PMA, a DAG analog, stimulates cPLA₂ activation and AA release in macrophages and epithelial cells (43–45). We therefore suspect that PLC-γ may also function in IgE signaling, although its precise role as a linker of Rac1-mediated PKC-δ stimulation in IgE signaling remains unclear.

There are also several studies implying that PKC is involved in the regulation of cPLA₂ (45–47). For example, the presence of a consensus phosphorylation site for MAPKs at Ser^{505} of cPLA₂ has led to studies of the role of MAPKs (e.g., ERKs and p38 MAPK) in the phosphorylation and activation of this enzyme (48–50). The precise relationship between PKCs and MAPKs in the activation of cPLA₂ remains largely unknown, however.

Finally, murine models of allergic inflammation have proven extremely useful for examining the basic mechanisms of allergic inflammation and the underlying immunologic response. To evaluate the physiological significance of the involvement of PKC-δ in cysLT synthesis, we developed a murine OVA-induced asthma model that exhibits elevated cysLT levels in BAL fluid. Given that cysLTs are known to contribute to asthmatic attacks in several ways, we hypothesized that inhibition of cysLT synthesis should alleviate those symptoms. Indeed, treating OVA-sensitized mice with a PKC-δ inhibitor (GF109203X or rottlerin) before provocation diminished the cysLT production, eosinophil infiltration, and AHR elicited by an OVA challenge. Certainly inhibition of PKC-δ might inhibit other signaling pathways related to airway inflammation, but, together with our in vitro results, these findings suggest blocking cysLT generation by mast cells may be a therapeutically useful new strategy for substantially reducing asthmatic airway inflammation.

References
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