Protein Phosphatase 2A and Protein Kinase Ca Are Physically Associated and Are Involved in Pseudomonas aeruginosa-induced Interleukin 6 Production by Mast Cells*

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Robert T. M. Boudreau‡, Rafael Garduno‡§, and Tong-Jun Lin‡¶

From the Departments of §Microbiology and Immunology, ¶Medicine, and ¶Pediatrics, Dalhousie University, Halifax, Nova Scotia B3J 3G9, Canada

Pulmonary infection with Pseudomonas aeruginosa is characterized by massive airway inflammation, which comprises significant cytokine production. Although mast cells are abundant in the lung and are potent sources of various cytokines, a role of mast cells in P. aeruginosa infection remains undefined, and P. aeruginosa-induced signaling mechanisms in mast cells have not been studied previously. Here we demonstrate that human cord blood-derived mast cells, mouse bone marrow-derived mast cells, and the mouse mast cell line MC/9 produce significant amounts of interleukin 6 (IL-6) in response to P. aeruginosa. This response was accompanied by a stimulation of protein kinase Ca (PKCa) phosphorylation and PKC activity and was significantly blocked by the PKC inhibitors Ro 31-8220 and PKC pseudosubstrate. Interestingly, mast cells treated with P. aeruginosa had reduced protein levels of phosphatase 2A catalytic unit (PP2Ac), which prompted us to determine whether a direct association between PKC and PP2A occurs in mast cells. In mouse bone marrow-derived mast cells and MC/9 cells, as well as in the human mast cell line HMC-1, PP2A coimmunoprecipitated with PKCα either using PKCα- or PP2Ac-specific antibodies, suggesting that PKCα and PP2Ac are physically associated in mast cells. The PP2A inhibitor okadaic acid induced P. aeruginosa-like responses in mast cells including increased PKCa phosphorylation, stimulated PKC activity, and augmented IL-6 production, the last being blocked by the PKC inhibitor Ro 31-8220. Finally, okadaic acid potentiated the P. aeruginosa-induced IL-6 production. Collectively, these data provide, to our knowledge, the first evidence of both a direct physical association of PP2A and PKCα in mammalian cells and their coinvolvement in regulating mast cell activation in response to P. aeruginosa.

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Pseudomonas aeruginosa is a ubiquitous opportunistic pathogen that often colonizes the lungs of patients with cystic fibrosis or immune compromised individuals. The chronically overactive inflammatory response associated with persistent P. aeruginosa lung infections is believed to be caused by the continuous stimulation of host cells to produce cytokines (1–3). Indeed, high levels of cytokines such as interleukin 6 (IL-6) have been found in blood and sputa of cystic fibrosis patients with P. aeruginosa infection (1–3). Some studies suggest that impairment of IL-6 regulation may represent an important component of the excessive inflammatory response observed during P. aeruginosa infection (1, 4). Mast cells are recognized as sentinels in host defense against bacterial infection (5–7). Although mast cells are found in large numbers in airways and are potent sources of cytokines and chemokines, a role for mast cells in P. aeruginosa-induced dysregulation of cytokine production has not been studied previously.

Mast cells contain a series of protein serine/threonine phosphatases including protein phosphatase 2A (PP2A) (8). One recent study demonstrated that stimulation of RBL 2H3 cells, a rat mast cell line, with antigen leads to a transient translocation and activation of PP2A (9). The rate of translocation of PP2A to the membrane coincides with the kinetic pattern of degranulation (9), suggesting a link between mast cell PP2A and granule-bound mediator secretion. In addition, several studies have described in human and rodent mast cells that okadaic acid blocks IgE-dependent and IgE-independent degranulation (10–14), implicating a role for PP2A in the regulation of mast cell mediator secretion. However, the molecular target of PP2A in the regulation of mast cell function or the role that PP2A plays during cytokine production remains to be determined.

Protein kinase C (PKC) is a family of serine/threonine kinases comprising at least 12 different isoforms that have been grouped into three categories: conventional PKCs (α, βI, βII, and γ), novel PKCs (δ, ε, θ, and η) and atypical PKCs (ξ, τ, λ, and μ). PKC isoform expression appears to be cell type-specific (15). PKC isoforms that have been characterized in mast cells include PKCa, βI, γ, δ, η, ε, θ, and ξ. PKC isoforms participate in signal transduction in many cell types and mediate a wide range of intracellular functions. Compared with other PKC isoforms, PKCa has distinct roles in a number of processes such as cell proliferation, apoptosis (21, 22), and bacteria- or cytokine-induced inflammatory responses (22, 23). In vivo, overexpression of PKCa in transgenic mice results in striking alterations of proinflammatory mediator production during inflammation (24). In vitro, Escherichia coli infection

1 The abbreviations used are: IL-6, interleukin 6; Ab(s), antibody(ies); BMMC, mouse bone marrow-derived mast cells; CBMC, human umbilical cord blood-derived mast cells; ELISA, enzyme-linked immunosorbent assay; FAK, focal adhesion kinase; HMC-1, human mast cell line-1; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PP2A, protein phosphatase 2A; PP2Ac, catalytic subunit of PP2A.
induces PKCa translocation from cytosol to membrane in T84 carcinoma cells (25), suggesting bacteria-induced activation of PKCa. Bacterial lipopolysaccharide-induced mediator production is enhanced significantly by overexpression of PKCa (26). Overexpression of a dominant negative version of PKCa strongly inhibits lipopolysaccharide-induced cytokine production by macrophages (27). Impaired PKCa function induced by Leishmania donovani in macrophages correlates with defective phagosome maturation and survival of the parasite in host cells (28). Thus, PKCa appears to play an important role during pathogen-induced inflammatory responses. In mast cells, PKCa has been implicated in several functions (29) such as antigen-induced hydrolysis of inositol phospholipids (16) and cytokine production (30).

PKCa kinase activity is regulated by phosphorylation of three conserved residues in its kinase domain: the activation loop site Thr-497, the autophosphorylation site Thr-638, and the hydrophobic C-terminal site Ser-657 (31). Without phosphorylation at these sites, PKCa has little or no activity (31). Phosphorylation at the C-terminal site Ser-657 plays a critical role in controlling the net phosphorylation and dephosphorylation rates (32). Without phosphorylation, PKCa can enter the cytosol to activate the dephosphorylation of PKCa (34). In intact cells, circumstantial evidence has implied that the dephosphorylation of PKCa is catalyzed by a membrane-associated PP2A (35). Consistent with a role of PP2A in the regulation of PKCa activity, okadaic acid, a potent PP2A inhibitor (36), induces numerous effects through mimicking or enhancing the actions of phorbol 12-myristate 13-acetate (PMA), a potent PKC activator (37, 38). Moreover, activation of PKC by PMA induced PP2A translocation to the membrane. Cumulatively, this evidence suggests an intimate interaction between PP2A and PKCa.

In this study, we demonstrate for the first time that PP2Ac and PKCa are physically associated in mast cells and that the associated enzymes participate in the regulation of P. aeruginosa-induced IL-6 production by mast cells.

**Materials and Methods**

**Reagents**—Rabbit anti-PKCα antibodies, aprotinin, leupeptin, pepstatin, Triton X-100, sodium deoxycholate, prostat glandulin E2, and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co. Rabbit anti-PKCα (Ser-657) antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Mouse anti-PP2A catalytic subunit (PP2Ac) antibodies were purchased from Transduction Laboratories of BD Biosciences (Mississauga, Ontario, Canada). Protein A/G PLUS-agarose immunoprecipitation beads, donkey anti-mouse IgG-horseradish peroxidase and donkey anti-rabbit IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cell culture media, okadaic acid, antibiotics, and fetal bovine serum were from Invitrogen. Ro 31-8220 and cell-permeable myristoylated PKC inhibitor peptide 19–27 were purchased from Calbiochem-Novabiochem Co. All other chemicals and reagents were of analytical grade.

**Mast Cells**—The HMC-1 SC6 human mast cells were maintained in Iscove's modified Dulbecco's medium in a 5% CO2, humidified atmosphere at 37 °C. Culture medium was supplemented with 10% fetal calf serum and 50 units/ml each of penicillin and streptomycin. Prior to experimental treatment, HMC-1 SC6 cells were starved overnight (18–24 h) in Iscove's modified Dulbecco's medium alone at a density of 0.5 × 10^6 cells/ml. For treatment, cells were resuspended in complete medium at a higher density, typically 2 × 10^6 cells/ml. After treatment cells were harvested in 50-ml conical centrifuge tubes and pelleted at 300 × g for 10 min at 4 °C.

**Measurement of IL-6 by ELISA**—Human and mouse IL-6 levels in supernatants were measured using an "in-house" ELISA assay. Briefly, 96-well plates were coated with anti-human IL-6 (R & D Systems, Minneapolis) or anti-mouse IL-6 (Endogen, Woburn, MA) at 1 µg/ml for 16–20 h at 4 °C. Nonspecific binding to the plates was blocked using a 1% bovine serum albumin, 0.1% Tween 20 solution in PBS for 1 h at 37 °C. A total of 50 µl/well IL-6 standard (human rIL-6, R & D Systems; murine rIL-6, Endogen) and samples were added to the plate and incubated for 18–20 h at 4 °C. Biotinylated anti-human IL-6 (R & D Systems) and anti-murine IL-6 (Endogen) (0.2 µg/ml) were added to each well and incubated for 1 h at 37 °C. After washing, 50 µl/well of a 1/2,000 dilution of streptavidin-alkaline phosphatase (Invitrogen) was added according to the manufacturer's instructions. The minimal detectable dose was 3 pg/ml for human IL-6 and 10 pg/ml for murine IL-6 using an ELISA reader system.

**Measurement of PKC Activity**—PKC activity was measured based on the phosphorylation of a PKC substrate peptide using a radioactive PKC assay kit or a nonradioactive protein kinase assay kit according to the manufacturer's protocol (both from Calbiochem-Novabiochem Co.).

**Confocal Microscopy Imaging of PKCa and PP2Ac**—Confocal microscopy was used to demonstrate the colocalization of PP2Ac and PKCa in mast cells. HMC-1 cells (5 × 10^5 cells/test) were washed with cold PBS.
and fixed with 4% paraformaldehyde for 5 min. After washing, cells were resuspended in 10% dimethyl sulfoxide in PBS and stored at −80 °C. Thawed cells were washed and incubated with 0.1% saponin and 3% bovine serum albumin in PBS for 1 h at room temperature. After washing, cells were incubated with mouse anti-PP2Ac IgG1 and rabbit anti-PKCα IgG for 1 h at 4 °C. Then cells were incubated further for 45 min with Alexa Fluor®-594 conjugated goat anti-mouse IgG, F(ab)2, and Alexa Fluor®-488 conjugated goat anti-rabbit IgG, F(ab)2 (Molecular Probes Inc.). Cells were washed three times and resuspended in 1% formalin. Cytospins of fluorescence-labeled mast cells were made by vortexing slides in a Cytospin 3 (Shandon, U. K.) at 600 rpm for 5 min. Antibleaching solution (10 mM n-propyl galate (Sigma), 8.1 mg/ml) in Tris-buffered saline was dropped onto slides before coverslip attachment. Cells were examined with a Zeiss LSM410 confocal laser scanning microscope (Jena, Germany). PP2Ac would then be tagged in red and PKCα in green. A yellow color indicates the colocalization of these two enzymes (overlay of red and green).

RESULTS

P. aeruginosa Stimulates IL-6 Production by Mast Cells—IL-6 is a pleiotropic cytokine that is produced during the course of infectious and inflammatory disorders and plays a crucial role in both local and systemic inflammatory responses (41–43). To test whether mast cells produce the cytokine IL-6 after P. aeruginosa stimulation, the mouse mast cell line, MC/9 cells, and primary cultured mouse and human mast cells, BMMC and CBMC, were employed in this study. Mast cells at a concentration of 5 × 10^6 cells/ml were treated with cystic fibrosis-associated P. aeruginosa strain 8821 (mast cell:bacteria ratio of 1:50) for 3–48 h. IL-6 levels in cell free supernatants were determined by ELISA. P. aeruginosa treatment for 24 h stimulated IL-6 production by BMMC and MC/9 significantly (Fig. 1, a and b). In CBMC, significant IL-6 production was observed as early as 6 h after P. aeruginosa treatment (Fig. 1, c and d).

A Role of PKC in P. aeruginosa-induced IL-6 Production by Mast Cells—To determine the role of PKCα in P. aeruginosa-induced mast cell activation, PKCα phosphorylation and PKC activity were determined in MC/9 cells after P. aeruginosa treatment. MC/9 cells were treated with P. aeruginosa strain 8821 for 3 h or 12 h and lysed in extraction buffer. Cell lysates were subjected to SDS-PAGE and probed with Ab to phosphorylated PKCα on serine 657. Increased phosphorylation of PKCα on serine 657 was seen in mast cells after treatment with P. aeruginosa (Fig. 2a). Interestingly, significant PKCα phosphorylation was seen in both the shorter (3 h) and longer (12 h) exposures to P. aeruginosa, suggesting a sustained stimulation of PKCα. Treatment of mast cells with P. aeruginosa did not affect the total PKCα levels, suggesting that the increase of phosphorylated PKCα is not the result of the increase of total PKCα levels. It is noteworthy that no degradation of PKCα protein was observed after sustained stimulation of mast cells with P. aeruginosa for 12 h.

To determine the effect of P. aeruginosa treatment on mast cell PKC activity, MC/9 cells were incubated with P. aeruginosa for 1, 5, or 3 h and lysed in extraction buffer. PKC activity was determined in cell lysates. As shown in Fig. 2b, treatment with P. aeruginosa for 1 h stimulated PKC activity in mast cells significantly. Similar stimulatory effects on PKC activities were observed when mast cells were treated with P. aeruginosa for 0 or 3 h, suggesting a sustained PKC activation. No PKC activity was observed in P. aeruginosa lysates (data not shown).

The involvement of PKC in P. aeruginosa-induced mast cell activation was confirmed further by using PKC inhibitors, Ro 31-8220 and PKC inhibitor peptide. BMMC and CBMC were treated with Ro 31-8220 at a dose of 10 μM during the course of P. aeruginosa stimulation. Treatment of mast cells with Ro 31-8220 dramatically blocked P. aeruginosa-induced IL-6 production by BMMC (Fig. 2c) and CBMC (Fig. 2d). To confirm further the specific effect of PKCα on IL-6 production, a cell-permeable PKC pseudosubstrate sequence from PKCα (IC50 = 8 μM in fibroblasts according to the manufacturer) was incubated with CBMC during P. aeruginosa stimulation. P. aeruginosa-induced IL-6 production by CBMC was inhibited significantly by PKC peptide at the dose of 20 μM (Fig. 2e).

P. aeruginosa Treatment Decreased PP2Ac Levels—Given that PP2A has been shown in vitro to regulate PKC activity and phosphorylation (33), the effect of P. aeruginosa treatment on mast cell PP2Ac was assessed. MC/9 cell and BMMC were treated with P. aeruginosa strain 8821 for 18 h and lysed in RIPA buffer. Total cell lysates were used for Western blot analysis and probed with Ab to PP2Ac. P. aeruginosa treatment induced a decrease of PP2Ac protein in both BMMC (Fig. 3a) and MC/9 cells (Fig. 3b).
PP2A and PKC

Are Physically Associated in Human and Mouse Mast Cells

Based on circumstantial evidence, it has been proposed that activation of PP2A by stimuli will lead to dephosphorylation and inactivation of PKC and subsequent responses in smooth muscle cells and Molt-4 human leukemia cells (44, 45). The stimulation of PKC phosphorylation and PKC activity and reduction of PP2Ac protein by P. aeruginosa treatment of mast cells, together with the in vitro functional inter-regulation between PKC and PP2Ac (33), suggest that PKC and PP2Ac may interact closely in the regulation of P. aeruginosa-induced mast cell responses. However, interactions between these two enzymes in mast cells have not been previously described. To determine whether PP2Ac and PKC are physically associated in mast cells, human mast cell line HMC-1, mouse primary cultured BMMC, and mouse mast cell line MC/9 were used in our study. Immunoprecipitation and Western blot analysis showed the constitutive expression of both PKC and PP2Ac in unstimulated mast cells (Fig. 4, b and d). As seen in Fig. 4a, immunoprecipitates of PKC, when probed with anti-PP2Ac Ab, demonstrated the presence of PP2Ac. To confirm further the association of PP2Ac and PKC, p < 0.05 compared with group without bacterial treatment. c, BMMC (5 × 10⁵ cells/ml) were treated with P. aeruginosa (Ps.a) for 24 h in the presence or absence of PKC inhibitor Ro 31-8220 (Ro). Cell-free supernatants were used to determine IL-6 production using ELISA. Results are the means ± S.E. of four independent experiments. d, CBMC (5 × 10⁵ cells/ml) were treated with P. aeruginosa for 24 h in the presence or absence of Ro 31-8220. IL-6 protein was determined in cell-free supernatants using ELISA. Results are the means ± S.E. of triplicate determinations (*, p < 0.05 compared with group without bacterial treatment). e, CBMC (5 × 10⁵ cells/ml) were treated with a cell-permeable PKC pseudosubstrate, a sequence derived from PKC (PKC peptide). Treatment of mast cells with PKC peptide significantly inhibited P. aeruginosa-induced IL-6 production. Results are the means ± S.E. of triplicate determinations (*, p < 0.05 compared with bacterial treatment alone).
mast cell lysates were immunoprecipitated with Ab to PP2Ac and then blotted with Ab to PKCα. The presence of PKCα in the immunoprecipitates of PP2Ac (Fig. 4c) and the presence of PP2Ac in the PKCα immunoprecipitates (c) demonstrate the physical association between these two enzymes. PKCα does not associate with FAK. HMC-1 cells were immunoprecipitated with Ab to FAK and probed with Abs to PKCα and PP2Ac, showing no PP2Ac in FAK immunoprecipitates. In addition, PP2Ac immunoprecipitates or total cell lysates were Western blotted (WB) with Ab to FAK, showing no FAK in PP2Ac immunoprecipitates, although mast cells express FAK proteins (Fig. 4e).

Confocal microscopy was used to demonstrate the colocalization of PP2Ac and PKCα in mast cells. Unstimulated HMC-1 cells were permeabilized and stained with Abs to PP2Ac (mouse IgG1) and PKCα (rabbit IgG). Fluorescence-labeled second Abs to mouse IgG (Alexa Fluor® 594, red) and to rabbit IgG (Alexa Fluor® 488, green) were used to visualize the distribution of PP2Ac (red) and PKCα (green) in mast cells. The yellow color indicates the colocalization of these two enzymes (overlay of red and green). As shown in Fig. 4, f–h, PKCα was mainly located in the cell cytosol, whereas PP2A was distributed in both cytosol and nuclear fractions. Colocalization of PKCα and PP2Ac was observed in cytosols of mast cells (Fig. 4h). Although PP2A has long been considered as a predominantly cytosolic enzyme, the presence of PP2Ac in fibroblast nuclei and other cellular compartments has also been reported (46), Fig. 4h indicates that mast cells express two populations of PP2Ac, a PKCα-associated PP2Ac distributed in the cytosol and a PKCα-unassociated population located mainly in the nuclear fraction.

**PKCα Phosphorylation and PKC Activity in Mast Cells Are Enhanced by the PP2A Inhibitor Okadaic Acid**—The physical association between PKCα and PP2Ac suggests a functional interaction between these two enzymes. The phosphorylation of PKCα on serine 657 controls accumulation of active enzyme and contributes to the maintenance of the phosphatase-resistant conformation (32). To test whether inhibition of PP2Ac by okadaic acid modulates mast cell PKCα phosphorylation, BMMC and MC/9 cells were treated with okadaic acid at various doses (10, 100, and 1,000 nM) for 1 h and lysed in RIPA buffer. Total cell lysates were analyzed by SDS-PAGE and probed with an Ab that recognizes phosphorylated PKCα on serine 657. As shown in Fig. 5a, phosphorylation of PKCα in both BMMC and MC/9 was enhanced by okadaic acid, an effect...
similar to *P. aeruginosa* treatment. The increase of phosphorylated PKCα was not the result of changes in total PKCα levels because total cell lysates, when probed with Abs to nonphosphorylated PKCα, showed similar PKCα levels after okadaic acid treatment.

To test whether okadaic acid stimulates mast cell PKC activity, MC/9 cells were treated with 500 nM okadaic acid for 3 h and suspended in extraction buffer. Similar to *P. aeruginosa* treatment, okadaic acid treatment stimulated mast cell PKC activity using radioactive (Fig. 5c) and nonradioactive tests (Fig. 5d), suggesting that inhibition of PP2A increases PKC activity in mast cells.

**PKC Inhibitors Block Okadaic Acid-induced IL-6 Production by Mast Cells**—Increased PKCα phosphorylation by okadaic acid treatment suggests an effect on mast cell cytokine production. As shown in Fig. 6a, treatment of BMMC with okadaic acid for 24 h induced significant IL-6 production. To test whether PKC is involved in okadaic acid-induced cytokine production by mast cells, PKC inhibitor Ro 31-8220 (47) was used to treat BMMC during okadaic acid stimulation. Okadaic acid-induced IL-6 production by BMMC was inhibited by Ro 31-8220 in a dose-dependent manner (Fig. 6b). These data suggest a role of PKCα in okadaic acid-induced IL-6 production by mast cells and are consistent with a model that inhibition of PP2A increases PKCα activation and leads to IL-6 production by mast cells. This model helps our understanding of the role of PP2Ac-PKCα interaction in *P. aeruginosa*-induced IL-6 production by mast cells.

**Synergistic Effects of Okadaic Acid on *P. aeruginosa*- and PMA-induced IL-6 Production**—The physical and functional interaction between PP2Ac and PKCα in the regulation of IL-6 production suggests that modulation of these two enzymes will lead to an altered production of this cytokine by mast cells. When BMMC were treated with 50 nM okadaic acid together with 10 nM PMA for 24 h, okadaic acid demonstrated a synergistic effect on PMA-induced IL-6 production (IL-6 pg/ml: 9.8 ± 2.9, 395.8 ± 65.2, 209.1 ± 15.2, and 650.9 ± 55.8 by the treatment with medium, PMA alone, okadaic acid alone, and PMA + okadaic acid, respectively). Strikingly, a strong synergism on IL-6 production was observed when BMMC were treated with *P. aeruginosa* in the presence of okadaic acid (Fig. 7). These data together with the effects of *P. aeruginosa* treatment on the reduction of PP2Ac protein level and activation of PKC support a role of PP2Ac-PKCα interaction in *P. aeruginosa*-induced mast cell IL-6 production.
PP2A and PKC in *P. aeruginosa*-induced Mast Cell Activation

**DISCUSSION**

Previous studies have described the reciprocal regulation of PKCα and PP2A in *vitro* (33) and numerous overlapping effects between okadaic acid and PMA, suggesting an intimate interaction between these two enzymes. In the present study we have demonstrated that PKCα and PP2Ac are physically associated in mast cells during the resting state. Moreover, these two enzymes are functionally associated in the regulation of mast cell IL-6 production and are involved in *P. aeruginosa*-induced IL-6 production by mast cells.

Mast cells are abundant in the tissue area where they interface with external surfaces such as airway mucosa. Recently, several elegant studies have demonstrated that these cells are critical in the host defense against bacterial infection (6, 7, 48, 49); however, little is known about the signaling mechanisms involved. We have shown previously that two members of PKC family, PKCβ and δ, are involved in the internalization of *E. coli* by mast cells (17), suggesting that PKC plays a role in mast cell responses to bacterial pathogens. In this study, a role of PKCα and PP2Ac in *P. aeruginosa*-induced IL-6 production by mast cells is shown. Treatment of mast cells with cystic fibrosis-associated *P. aeruginosa* induced significant increases of PKCα phosphorylation and PKC activity. Moreover, PKC inhibitor Ro 31-8220 and PKC pseudosubstrate block *P. aeruginosa*-induced IL-6 production. These data suggest that PKCα activation is one of the mechanisms involved in *P. aeruginosa*-induced mast cell responses.

Interestingly, treatment of mast cells with *P. aeruginosa* induced a significant decrease of PP2Aα level. Treatment of mast cells with *P. aeruginosa* for 3 or 24 h did not affect the phosphorylation of several signaling proteins such as PKBα, CREB, STAT1, STAT5, Jak2, and RAF1 (data not shown), suggesting a specific effect on PKCα and PP2Ac. Given that PP2A in *vitro* has the capacity to down-regulate PKCα activation through dephosphorylation (33), we hypothesized that decreased PP2Aα is one of the mechanisms involved in *P. aeruginosa*-induced PKCα activation. This hypothesis prompted us to determine the possible interactions between PP2A and PKCα in mast cells. Although roles of PP2A and PKCα have been described individually in IgE-mediated signaling events, little is known about their interactions in regulating mast cell functions. In this study, the presence of PP2Aα in PKCα immunoprecipitates and the presence of PKCα in PP2Ac immunoprecipitates provided direct evidence of physical association between PKCα and PP2Ac. Confocal microscopy showed that these two enzymes are colocalized in cytosols. To our knowledge, this is the first direct evidence demonstrating the physical association between PKCα and PP2Ac in any system. The finding of a physical association of these two enzymes in mast cells could likely be applied to other cell types because coexistence of these two enzymes in the same cellular fraction was observed in COS cells (35).

In resting mast cells, association of PKCα and PP2Ac was observed in the cytosol. One of the dynamic features of PKCα upon activation is translocation from cytosol to membrane. Although the role of PP2A in PKCα translocation remains to be determined, it is likely that PP2Ac is translocated along with PKCα because of their physical association. This is supported by a recent study by Ludowyke et al. (9) that PP2A translocation to the mast cell membrane can be induced by PKC activator PMA. In COS cells, the presence of PP2Ac correlates with PKCα phosphatase activity in membrane fraction (35), suggesting the coexistence of PP2Ac and PKCα in the same cellular compartment. Dephosphorylation of PKCα was found in the membrane compartment (35). Thus, it is likely that PP2A, after translocation to the membrane along with PKCα, continues to play a role in the termination of PKCα activation by dephosphorylation.

The physical association of PKCα and PP2Ac suggests a functional interaction between these two enzymes in mast cells. Treatment of mast cells with okadaic acid induced an increase of PKCα phosphorylation and PKC activity and stimulated IL-6 production. Moreover, okadaic acid-induced IL-6 production was blocked by PKC inhibitors. These data support the notion that in mast cells, PP2Ac physically binds to PKCα and regulates its activities. This interaction is involved in the regulation of IL-6 production by mast cells. The effects of okadaic acid or PP2A on cytokine production by mast cells have not been reported previously. The interaction of PKCα and PP2Ac in the regulation of IL-6 production in this study suggests that PP2A may have broader roles in the regulation of mast cell functions than thought previously. However, caution should be applied when making generalizations about this mechanism to other mast cell mediators such as histamine (degranulation) because mast cells possess different mechanisms in the regulation of different mediator secretion (50). Indeed, contrary to the stimulatory effects of okadaic acid on IL-6 production observed in this study, several studies demonstrated that okadaic acid inhibits mast cell degranulation in a time- and concentration-dependent manner (10–14).

Valuable information regarding the underlying signaling mechanisms mediated by PP2A has been obtained with the use of okadaic acid. *In vitro*, okadaic acid blocks both PP2A and PP1 activity at 0.1–10 nm concentrations, although it is 10-fold more effective against PP2A (36, 51–53). In intact cells, higher concentrations (up to 1 μM) are required to achieve an effect similar to that seen *in vitro* (53, 54). Okadaic acid has little or no effect on PP2B or PP2C. In mast cells, okadaic acid at the level of 1 μM inhibited PP2A activity but had very little or no effect on PP1 activity (9), suggesting that okadaic acid may have more selective effects on PP2A activity in mast cells than that seen in other cell types.

The demonstration of the physical and function interaction in mast cells between PP2Ac and PKCα and their roles in the regulation of IL-6 production provides a basis for the understanding of the mechanisms of *P. aeruginosa*-induced mast cell activation. Okadaic acid demonstrates a significant synergistic effect on *P. aeruginosa*-induced IL-6 production. These data together with the evidence of *P. aeruginosa*-induced PKCα activation and PP2Ac depletion are consistent with a model by
which down-regulation of PP2A by P. aeruginosa causes activation of PKCα and leads to IL-6 production by mast cells. This model provides a potential intracellular target for the therapeutic modulation of P. aeruginosa-induced inflammation.

The significant IL-6 production by mast cells after P. aeruginosa-stimulation suggests that mast cells may serve as a cellular source for this cytokine during P. aeruginosa infection. Early studies have demonstrated that mast cells secrete histamine and leukotriene C4 in response to P. aeruginosa stimulation (55, 56). Recently, the importance of mast cell-derived cytokines in the regulation of immune response has increasingly been recognized. IL-6 is a multipotent cytokine produced in the context of inflammation and infection and is critical to the development of the acute phase response during inflammation (57–59). We chose to examine the regulation of IL-6 production by mast cells in view of the wide range of biologic activities of IL-6 which are relevant to the initiation and progression of inflammation (59) and because production of IL-6 in P. aeruginosa-induced inflammation (62, 63), cytokine production by mast cells after P. aeruginosa stimulation has not been examined previously. Significant IL-6 production by mast cells induced by P. aeruginosa stimulation, together with the fact that mast cells are found in large numbers in the airway, suggests that mast cells may have a previously unrecognized role in P. aeruginosa-induced inflammation.

In summary, we have made the following novel observations in this study. First, PKCα and PP2Ac are found physically and functionally associated in mast cells and are involved in the regulation of IL-6 production by mast cells. Second, mast cells respond to P. aeruginosa to produce cytokine IL-6, suggesting a role of mast cells in P. aeruginosa-induced inflammation. Third, interaction between PKCα and PP2A is one of the mechanisms involved in P. aeruginosa-induced IL-6 production by mast cells.

REFERENCES
1. Nixon, L. S., Yung, B., Bell, S. C., Elborn, J. S., and Shale, D. J. (1998) Am. J. Respir. Crit. Care Med. 157, 1764–1769
2. Hodson, M. E. (1980) Thorax 35, 891–896
3. Doring, G., Albus, A., and Hoiby, N. (1988) Chest 94, 1096–1158
4. Osika, E., Cavaillon, J. M., Chadelat, K., Boule, M., Fitting, C., Tournier, G., Achong, M. K. (1998) Biochem. J. 334, 243–249
5. Lee, J. Y., Hannun, Y. A., and Obeid, L. M. (1996) J. Cell Biol. 135, 381–394
6. Hauswaard, J. F., and Beaven, M. A. (1993) Blood 72, 1188–1194
7. Riess, F., Konig, B., and Konig, W. (1992) Immunity 6, 86–94
8. Bergmann, U., Scheffer, J., Koller, M., Schonfeld, W., Erbs, G., Muller, F. E., and Konig, W. (1998) Infect. Immun. 66, 315–325
9. Bedard, M., McClure, C. D., Schiller, N. L., Konstan, M. W., and Blumberg, P. M. (2000) Am. J. Respir. Crit. Care Med. 161, 271–279
10. Tom, M., Stipes, G. J., and Stevenson, M. M. (1999) Am. J. Respir. Cell Mol. Biol. 20, 710–719