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Prostaglandin E_2 Inhibits the Phospholipase D Pathway Stimulated by Formyl-methionyl-leucyl-phenylalanine in Human Neutrophils. Involvement of EP_2 Receptors and Phosphatidylinositol 3-kinase γ

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ABSTRACT

Prostaglandin E_2 (PGE_2), originally discovered as a pro-inflammatory mediator, also inhibits several chemoattractant-elicited neutrophil functions, including adhesion, secretion of cytotoxic enzymes, production of superoxide anions, and chemotaxis. In this study, we have examined the effects of PGE_2 and prostaglandin E (EP) receptor-selective agonists/antagonists on several steps of the formyl-methionyl-leucyl-phenylalanine (fMLP)-induced phospholipase D (PLD) activation pathway in human neutrophils to elucidate the PGE_2 inhibitory mechanism. PGE_2 and EP_2 receptor agonists inhibited the stimulation of the activity of PLD induced by fMLP in a concentration-dependent manner. The fMLP-stimulated translocation to membranes of protein kinase C α, Rho, and Arf GTPases was diminished in the presence of PGE_2 or EP_2 agonists. Moreover, PGE_2 and EP_2 agonists decreased the activation of phosphatidylinositol 3-kinase γ (PI3Kγ) and Tec kinases as well as the tyrosine phosphorylation of proteins stimulated by fMLP. These data provide strong evidence that 1) the inhibitory effects of PGE_2 on the fMLP-induced PLD activation pathway were mediated via EP_2 receptors and that 2) the suppression of PI3Kγ activity was the crucial step in the EP_2-mediated inhibition of the fMLP-induced signaling cascade.

Phospholipase D (PLD) catalyzes the hydrolysis of choline-containing lipids to produce the second messenger phosphatidic acid (PA). The PLD pathway is thought to play a critical role in regulating cell responses such as phagocytosis (Lennartz, 1999), secretion, and production of superoxide anions by the NADPH oxidase complex (Liscovitch et al., 2000) in human neutrophils. Two mammalian genes coding for PLD1 and PLD2 have been cloned (Frohman et al., 1999); however, only the PLD1 isoform has been detected in human neutrophils (Marcil et al., 1997). PLD1 requires phosphoinositides for activity and is regulated by direct interactions with conventional PKC isoforms and the small GTPases RhoA and ADP-ribosylation factor (Arf) (Exton, 1999). fMet-Leu-Phe (fMLP)-induced PLD activity is also modulated by variations in cytosolic calcium and its stimulation is controlled by phosphatidylinositol 3-kinase (PI3K) and tyrosine kinase-dependent events (Exton, 1999).

Besides their role in the regulation of PLD activity, the Rho GTPase family members (Rho, Rac, and Cdc42) also modulate multiple cellular processes by cycling between a GDP-bound inactive form and a GTP-bound form that directly activates several downstream targets. They are implicated in the regulation of the dynamics of the actin cytoskeleton, in changes in cell shape and motility (Hall, 1998), and they regulate various leukocyte functions including adhesion (Laudanna et al., 1996), phagocytosis (Chimini and Chavrier,

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ABBREVIATIONS: PLD, phospholipase D; PA, phosphatidic acid; PKC, protein kinase C; Arf, ADP-ribosylation factor; PI3K, phosphatidylinositol 3-kinase; PG, prostaglandin; EP receptor, E prostaglandin receptor; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; fMLP, formyl-methionyl-leucyl-phenylalanine; Ab, antibody; ADA, adenosine deaminase; DFP, di-isopropylfluorophosphate; PH, pleckstrin homology; CB, cytochalasin B; AM, acetoxymethyl ester; CAY 10399, 9-oxo-11α, 16*S*-dihydroxy-17-cyclobutyl-prosta-5*Z*,13*E*-dien-1-oic acid; AH23848, [1α(*Z*),2β,5α]-(±)-7-[5-[[(1,1′-biphenyl)-4-yl]methoxyl]-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid; HBSS, Hanks' balanced saline solution; PEt, phosphatidylethanol; PtdIns, phosphatidylinositol; GEF, guanine nucleotide exchange factors.

2000), and the migratory response to chemoattractants (Servant et al., 2000). In phagocytic cells, Rac also plays a major role in the production of superoxide anions by the NADPH oxidase complex (Babior, 1999). Rho GTPases are therefore critical regulators of the functional responsiveness in neutrophils.

Prostaglandins E (PGEs) are cyclooxygenase products that exert pleiotropic effects in a paracrine fashion. The physiological activities of PGEs are mediated via G-protein-linked seven transmembrane domain receptors that have been classified into four subtypes, EP_1 to EP_4, according to their structure and coupling to distinct signaling pathways (Breyer et al., 2001). EP_2 and EP_4 stimulate adenylyl-cyclase, whereas EP_1 triggers intracellular calcium release. The various EP_3 isoforms generated by alternative splicing are connected either to cAMP or to calcium metabolism. Pro-inflammatory functions have been attributed to PGEs by virtue of their vasodilator and nociceptive properties. However, PGEs also display inhibitory effects on most leukocyte functions and are considered modulators of inflammation rather than strictly pro-inflammatory mediators (Tilley et al., 2001). Most neutrophil functional responses, including adhesion to epithelial cells (Bloemen et al., 1997), motility and chemotaxis (Rivkin et al., 1975), production of superoxide anions (Fantone and Kinnes, 1983), and the release of cytotoxic enzymes and leukotriene B_4 (Ham et al., 1983; Hecker et al., 1990) are diminished by PGE_2 or PGE_1. Pharmacological studies have shown that the inhibitory effects of PGE_2 on fMLP-induced superoxide production, enzyme release, and chemotaxis are mediated via the EP_2 receptor (Armstrong, 1995; Talpain et al., 1995).

The effects of PGE_2 on signal transduction pathways induced by chemoattractants in neutrophils have only been incompletely characterized so far. PGE_2 has been shown to inhibit fMLP but not phorbol 12-myristate 13-acetate–induced PLD activation in human neutrophils (Agwu et al., 1991), but the mechanism involved has not been clarified. Therefore, we investigated further the effects of PGE_2 (and of specific EP receptor agonists) on the signaling cascade that leads to PLD activation upon fMLP stimulation. In this article, we report that PGE_2 inhibits the stimulation of the activity of PLD, the translocation of PKCα, Arf, and Rho GTPases and Tec kinases to membranes, the accumulation of PtdIns(3,4,5)P_3 and the activity of PI3Kγ induced by fMLP. These effects were mediated via EP_2 receptors. Our results suggest that suppression of fMLP-induced PI3Kγ activity by PGE_2 plays a prominent role in the inhibition of downstream signaling events such as the activation of small GTPases and of PKCα and Tec kinases, thereby resulting in decreased PLD activation.

Materials and Methods

Antibodies. The anti-PKCα (P16520) and anti-Cdc42 (C70820) monoclonal Abs were purchased from BD Transduction Laboratories (Mississauga, ON, Canada). Anti-RhoA (SC-179), anti-Rac2 (SC-96), anti-Tec (SC-1109), and anti-Btk (SC-1107) polyclonal Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal anti-EP_2 receptor Ab and the EP_2 receptor blocking peptide were obtained from Cayman Chemical (Ann Arbor, MI). The polyclonal anti-Arf1 and anti-p110γ antisera were raised in rabbits as described previously (Houle et al., 1999; Naccache et al., 2000). The anti-phosphotyrosine Ab (clone 4G10) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Secondary anti-mouse and anti-rabbit Abs were obtained from Amersham Biosciences (Baie d'Urfé, Québec, Canada) and the secondary anti-goat Ab from Santa Cruz Biotechnology.

Reagents. Dextran T-500 was purchased from Pharmacia Biotech (Dorval, PQ, Canada) and Ficoll-Paque from Wisent (St-Bruno, PQ, Canada). Adenosine deaminase (ADA) was purchased from Roche Diagnostics (Laval, PQ, Canada), and di-isopropylfluorophosphate (DFP) from Serva (Heidelberg, Germany). fMLP and cytochalasin B (CB) were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada). 1-*O*-[^{3}H]alkyl-2-lyso-phosphatidylcholine was obtained from Amersham Biosciences (Baie d'Urfé, PQ, Canada). PGE_2, the butaprost acid-form CAY 10399, 11-deoxy PGE_1, sulprostone, and AH 23848 were purchased from Cayman Chemical (Ann Arbor, MI). [^{32}P]Orthophosphate (1000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). Fura-2/AM was obtained from Molecular Probes (Eugene, OR).

Isolation of Human Neutrophils. Venous blood was collected from healthy adult volunteers in isocitrate anticoagulant solution. Neutrophils were separated as described previously (Marcil et al., 1999). In brief, whole blood was centrifuged at 180*g* for 10 min and the resulting platelet rich plasma was discarded. Leukocytes were obtained after erythrocytes sedimentation in 2% Dextran T-500. Mononuclear cells were removed by centrifugation on Ficoll-Paque cushions and contaminating erythrocytes in the neutrophil pellets were removed by a 20-s hypotonic lysis in water. Neutrophils were resuspended in Hanks' balanced salt solution (HBSS), pH 7.4, containing 1.6 mM Ca^{2+} but no Mg^2. The cells were sterilely purified to avoid bacterial contamination of the neutrophil suspensions.

PLD Measurements. Neutrophils were labeled with 1-*O*-[^{3}H]alkyl-2-lyso-phosphatidylcholine (2 μCi/10^7 cells) for 90 min as described previously (Marcil et al., 1999). The cells were washed and resuspended at 10^7 cells/ml in HBSS. Cell suspensions (0.5 ml) were warmed at 37°C for 5 min and then pretreated for 5 min with 10 μM CB and 0.1 U/ml ADA to eliminate endogenous adenosine and in the presence or the absence of the indicated concentrations of agonists or antagonists. Neutrophils were stimulated with 100 nM fMLP for 10 min in the presence of 1% ethanol to monitor the formation of radiolabeled phosphatidylethanol (PEt) catalyzed by PLD. Incubations were stopped by adding 1.8 ml of chloroform/methanol/HCl (50:100:1, v/v/v) and unlabeled PEt as a standard. Lipids were extracted and the levels of [^{3}H]PEt were quantified as described previously (Marcil et al., 1999).

Measurement of Cytoplasmic Free Calcium Concentrations. Neutrophils (10^7 cells/ml) were incubated at 37°C for 30 min with 1 μM Fura-2/AM. The cells were washed and resuspended at 5×10^6 cells/ml in HBSS with 1.6 mM Ca^{2+}. Neutrophils were transferred to the thermostated cuvette compartment of a spectrofluorimeter (SLM 8000C) and preincubated 5 min at 37°C with 0.1 U/ml ADA in the presence of various EP receptor agonists/antagonists or the equal volume of dimethyl sulfoxide. Cells were stimulated with 100 nM fMLP at the time indicated by the arrow. The fluorescence of the cells was monitored at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. The internal calcium concentrations were calculated using the following formula: $224[(y - F_{min})(F_{max} - y)^{-1}]$ in which y represents the fluorescence of the samples. F_{max} was obtained by disrupting the cells with 1% Triton X-100 and F_{min} was obtained by adding 5 mM of both EGTA and NaOH.

Manganese Influx Measurement. Neutrophils were loaded with Fura-2/AM, washed and resuspended in HBSS without calcium and magnesium just prior incubation with ADA and PGE_2 or EP receptor agonists for 5 min at 37°C in a thermostated cuvette as described above. The wavelength used for these experiments were 360 and 505 nm for excitation and emission, respectively. After 10 s, 200 μM $MnCl_2$ was added to the cell suspensions that were then stimulated by 100 nM fMLP for 70 s.

Translocation Assays. Neutrophils (4×10^7 cells/ml) were treated with 1 mM DFP for 10 min at room temperature. The cell suspensions were centrifuged once and the cells were resuspended in HBSS at 10^7 cells/ml. The cells were warmed for 5 min at 37°C and treated 5 min with 10 μM CB, 0.1 U/ml ADA and PGE_2 or EP receptor agonists or an equal volume of diluent (dimethyl sulfoxide) as a control. Neutrophils were stimulated with 100 nM fMLP at 37°C. Incubations were stopped by diluting the cells 5-fold with ice-cold HBSS, and total membrane proteins were collected as described previously (Marcil et al., 1999). Protein samples (10–20 μg) were resolved on a 7.5 to 20% gradient SDS-polyacrylamide gel electrophoresis and transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA). Western blots were performed using anti-Arf1 (1/2500), anti-RhoA (1/1000), anti-PKCα (1/1000), anti-Rac2 (1/200) and anti-Cdc42 (1/250), anti-p110γ (1/1000), anti-Tec (1/1000) or anti-Btk (1/1000) Abs. Proteins were revealed with HRP-conjugated secondary anti-mouse or anti-rabbit Ab (1/20,000) or anti-goat Ab (1/15,000) and the Renaissance detection system (PerkinElmer Life and Analytical Sciences).

Phosphatidylinositol 3,4,5-trisphosphate Formation. Neutrophils (5×10^7 cells/ml) were incubated with 0.5 mCi/ml of [^{32}P] orthophosphate for 1 h at 37°C in a labeling buffer (137 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES, pH 7.4). Unincorporated radioactivity was discarded and the cells were washed twice in HBSS. The cells were resuspended in HBSS and preincubated with PGE_2/EP agonists or diluent in the presence of ADA 0.1 U/ml for 5 min at 37°C. The cells were stimulated with 100 nM fMLP for 30 sec and reactions were stopped by addition of 400 μl of chloroform-methanol (1:1, v/v). The samples were processed as described previously (Gilbert et al., 2003) and the final products visualized by exposure of X-ray films at −80°C or with a bio-imaging analyzer (Fujifilm BAS-1800).

Measurements of PI3Kγ Activity. Cell suspensions (4×10^7 cells/ml) were preincubated for 5 min at 37°C with 10^{-5}M PGE_2 (or an equal volume of dimethyl sulfoxide) and 0.1 U/ml ADA. Cells were stimulated with 100 nM fMLP and the reaction was stopped by addition of an equal volume ice-cold buffer I (phosphate-buffered saline containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 μM Na_3VO_4). Samples were processed as already described for the p110γ immunoprecipitation and the PI3K assays (Naccache et al., 2000).

Tyrosine Phosphorylation Patterns. Neutrophils (2×10^7 cells/ml) were preincubated at room temperature with 1 mM DFP, with or without the indicated concentrations of PGE_2 or EP agonists for 10 min at 37°C before stimulation with 100 nM fMLP for the indicated times. The reactions were stopped by transferring 100 μl of the cell suspensions to an equal volume of boiling 2× Laemmli sample buffer (SB) (1× is 62.5 mM Tris-HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol, 8.5% glycerol, 2.5 mM orthovanadate, 10 mM paranitro-phenylphosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.025% bromphenol blue) and boiled for 7 min. The samples were then subjected to 7.5 to 20% gradient SDS-polyacrylamide gel electrophoresis and transferred to Immobilon polyvinylidene difluoride membranes. Immunoblotting was performed using the monoclonal anti-phosphotyrosine Ab 4G10 (1/4000) and revealed as described previously (Gilbert et al., 2003).

Statistical Analysis. The data were analyzed using the one-sample Student's t test for the PLD and translocation assays data or the Student paired t test (two-tailed) for the PI3K assay data. The levels of significance (*, $p < 0.05$; **, $p < 0.01$) were determined between the treated samples and the appropriate controls.

Results

fMLP-Induced PLD Activity Is Inhibited by PGE_2 via EP_2 Receptor. As is the case for other cAMP elevating substances (adenosine, forskolin, adrenergic β_2-agonists), PGE_2 has also been shown to inhibit fMLP-induced PLD activity in the presence of phosphodiesterase inhibitors (3-isobutyl 1-methylxanthine or theophylline) (Agwu et al., 1991). We have first investigated the effect of PGE_2 on PLD activity in our experimental conditions (i.e., in the absence of phosphodiesterase inhibitor and in the presence of ADA to eliminate the inhibitory effect of endogenous adenosine that could interfere with that of PGE_2) (Thibault et al., 2000). As shown in Fig. 1A, PGE_2 inhibited [^{3}H]PEt formation in a concentration-dependent manner. The amounts of [^{3}H]PEt formed were 25.89 ± 5.01% ($p = 0.0045$), 25.44 ± 5.74% ($p = 0.0059$), 35.27 ± 6.83% ($p = 0.011$), and 43.3 ± 5.21% ($p = 0.0083$) of the fMLP-stimulated control in the presence of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M PGE_2, respectively. To determine which EP receptor subtype mediated the inhibition of PLD activity by PGE_2, [^{3}H]PEt formation was assessed in the presence of EP receptor selective agonists and antagonists, all of which are PGE_2 analogs. We tested the effects of one EP_2 receptor-selective agonist (CAY 10399), one EP_2/EP_4 agonist (11-deoxy-PGE_1), one EP_1/EP_3 agonist, sulprostone, and one selective EP_4 receptor antagonist (AH 23848) on PLD activity. fMLP-induced [^{3}H]PEt formation was significantly reduced to 25.67 ± 2.37 ($p = 0.01$), 40.89 ± 5.8 ($p = 0.0095$), 65.3 ± 3.68 ($p = 0.011$), and 88.24 ± 2.15 ($p = 0.032$) of its level in untreated controls in the presence of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M 11-deoxy PGE_1, respectively. In the presence of 10^{-5}, 10^{-6}, and 10^{-7} M CAY 10399, [^{3}H]PEt formation was also reduced to 33.5 ± 4.25 ($p = 0.004$), 41.8 ± 3.9 ($p = 0.004$), and 58.75 ± 4,45 ($p = 0.011$) of its level in the untreated controls, respectively. The selective EP_1/EP_3 receptor agonist sulprostone had no effect on the fMLP-induced PLD activity (Fig. 1A). The selective EP_4 receptor antagonist

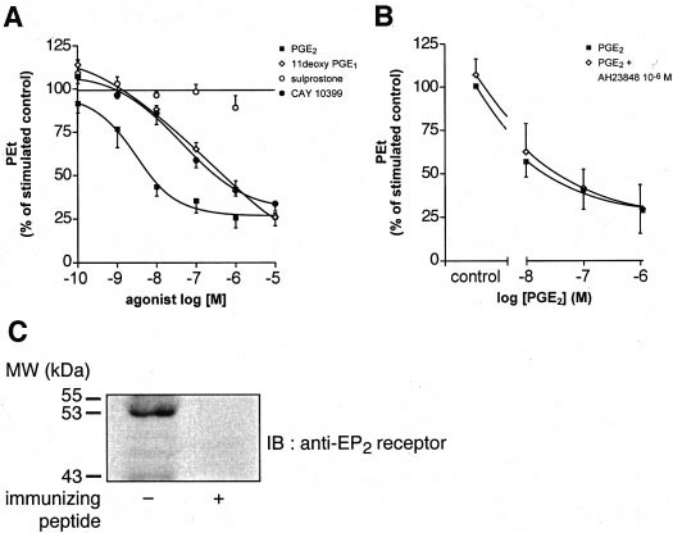

Fig. 1. Effect of PGE_2 and EP receptor agonists/antagonists on fMLP-induced PLD activity. A and B, neutrophils (10^7 cells/ml) were preincubated with the indicated concentrations of PGE_2 or/and the EP agonists/antagonists for 5 min at 37°C in the presence of 0.1 U/ml ADA and 10 μM CB. Cells were stimulated with fMLP in the presence of 1% ethanol for 10 min. The amounts of [^{3}H]PEt formed were evaluated as described under *Materials and Methods*. The levels of [^{3}H]PEt formed are expressed as the percentage of the fMLP-stimulated control. The data are from three different experiments performed in duplicate and are expressed as the means ± S.E.M. C, presence of EP_2 receptors on neutrophil membrane. Membranes were prepared as described under *Materials and Methods* and were analyzed by immunoblotting with the anti-EP_2 receptor Ab alone (lane 1) or preneutralized with the immunizing peptide (lane 2). The immunoblot shown is representative of three different experiments.

AH 23848 did not affect the inhibitory effect of PGE_2 on the stimulation of the activity of PLD by fMLP (Fig. 1B), indicating that PGE_2 did not exert its inhibitory effect via the EP_4 receptor. Because the EP_1/EP_3 agonist sulprostone did not affect fMLP-stimulated PLD activity and the EP_4 antagonist did not modify the PGE_2-mediated inhibition of PLD activity, these data suggested that EP_2 receptors mediated the inhibitory effects of PGE_2 .

The presence of EP_2 receptors on neutrophil membranes was assessed next by immunoblotting of the membrane fractions with an anti-EP_2 antibody. We observed a band at 53 kDa that was no longer detected when the antibody was preneutralized with the immunizing peptide (Fig. 1C).

PGE_2 Inhibition of Ca^{2+} Influx Is Mediated via EP_2 Receptor. fMLP induces a rapid increase in free cytoplasmic calcium resulting from the intracellular release of Ca^{2+} stores mediated by phospholipase Cβ-released inositol 3,4,5-trisphosphate followed by a sustained Ca^{2+} influx from the extracellular medium. fMLP-induced PLD activity has been shown to be dependent on this rise in Ca^{2+} (Exton, 1999) as well as on extracellular calcium (Pai et al., 1988). First, we examined the effect of PGE_2 on calcium mobilization in fMLP-stimulated human neutrophils. As already described, fMLP induced a rapid and transient increase in cytoplasmic free calcium as monitored using the fluorescent probe Fura-2. PGE_2 had no effect on the magnitude of the rapid rise in free cytoplasmic calcium but reduced the duration of the calcium spike suggesting that the contribution of calcium influx to the mobilization of calcium could be partially decreased by PGE_2 (Fig. 2A). We next assessed whether the effects of PGE_2 on calcium mobilization were also mediated via EP_2 receptors. The EP_2 receptor-selective agonist CAY 10399 reduced the duration of the calcium spike in a manner akin to PGE_2, (Fig. 2B), whereas the EP_4 receptor antagonist AH 23848 did not modify the reduction of the mobilization of cytosolic calcium induced by PGE_2 (Fig. 2C). Furthermore, the EP_1/EP_3 receptor agonist sulprostone influenced neither the magnitude nor the duration of the elevation of cytoplasmic calcium elicited by fMLP (Fig. 2D).

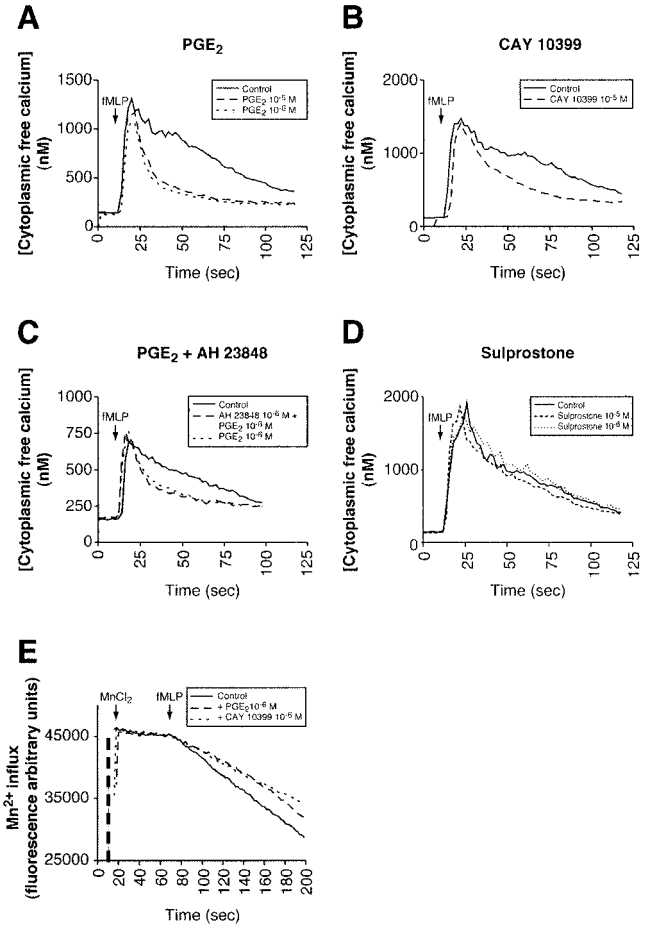

Fig. 2. Effects of PGE_2 and of EP agonists/antagonists on cytoplasmic free calcium concentration. A–D, neutrophils (5×10^6 cells/ml) were preincubated for 5 min at 37°C with PGE_2 or EP agonists/antagonists in the presence of 0.1 U/ml ADA. Cells were stimulated with fMLP at the time indicated by the arrow and cytosolic calcium concentrations were monitored as described under *Materials and Methods*. E, effect of PGE_2 and CAY 10399 on fMLP-stimulated Mn^{2+} influx. Neutrophils resuspended in HBSS with no Ca^{2+} were preincubated 5 min at 37°C with PGE_2 or EP agonists/antagonists in the presence of 0.1 U/ml ADA. 200 μM $MnCl_2$ and fMLP were added at the times indicated by the arrows. The results are representative of three separate experiments.

To confirm that PGE_2 and EP_2 agonists acted on the influx of calcium, we monitored the quenching of intracellular Fura-2 fluorescence that results from the entry of Mn^{2+} through divalent cation channels (Fig. 2E). This experiment showed that a pretreatment of neutrophils with PGE_2 or the EP_2 agonist CAY 10399 partially reduced the fMLP-induced rate of quenching of Fura-2, indicating that the stimulation of the EP_2 pathway exerted an inhibitory effect on the fMLP-stimulated calcium influx.

Effect of PGE_2 on Recruitment of PKCα, Arf, and Rho GTPases to Membranes. The cytosolic PLD activation factor PKCα and the small GTPases Arf1 and RhoA migrate to membranes, where they mediate the activation of PLD by fMLP (Houle et al., 1995). To investigate the mechanism implicated in the inhibition of fMLP-induced PLD activity by PGE_2, we assessed whether PGE_2 interfered with the translocation to membranes of the PLD1 activation factors. As shown in Fig. 3, 10^{-5} M PGE_2 alone had no noticeable effect on the basal membrane levels of PKCα, Arf1, and RhoA. In comparison, PGE_2 pretreatment before fMLP stimulation resulted in a significant and dose-dependent inhibition of the translocation of PKCα, Arf1, and RhoA to the membranes (compared with the fMLP-stimulated control). The recruitment to membranes of the two other Rho GTPase family members Rac2 and Cdc42 was also decreased by PGE_2 pretreatment.

PGE_2 Decreases PKCα, Arf, and Rho GTPases Recruitment to Membranes through EP_2 Receptor. We next assessed whether EP_2 agonists affected the membrane translocation of PKCα and small GTPases. As was the case with PGE_2, the EP_2-selective agonist CAY 10399 alone did not affect the basal level of PKCα, Arf, and Rho GTPases associated with membranes, but preincubation of cells with CAY 10399 before fMLP stimulation significantly decreased the amounts of membrane-translocated PKCα, Arf, and Rho GTPases in a concentration-dependent manner (Fig. 4). These data have been buttressed by the use of another selective EP_2 agonist: the 19(*R*)-hydroxy-PGE_2, which showed effects similar to those of CAY 10399 (data not shown), indicating that the inhibition of the translocation of PLD cofactors by PGE_2 was mediated via the EP_2 receptors.

Effect of PGE_2 on PtdIns(3,4,5)P_3 Formation and PI3Kγ Activity. PLD1 activation is also regulated by phos-

phoinositides and pharmacological inhibitors of PI3K strongly inhibit PLD activity induced by chemoattractants (Reinhold et al., 1990). Moreover, PI3K exerts a regulatory role over small GTPase activation, especially on Rac2 and Cdc42 (Benard et al., 1999). Thus, we tested the effects of PGE_2 on fMLP-induced PtdIns(3,4,5)P_3 formation in human neutrophils. As shown in Fig. 5A, PGE_2 (10^{-5} to 10^{-8} M) decreased the amounts of PtdIns(3,4,5)P_3 formed after fMLP stimulation. The amounts of PtdIns(3,4,5)P_3 formed in response to fMLP were also monitored in the presence of CAY 10399. As shown in Fig. 5B, CAY 10399 (10^{-5} and 10^{-6} M) also inhibited fMLP-stimulated PtdIns(3,4,5)P_3 formation, indicating that PGE_2 exerted its in-

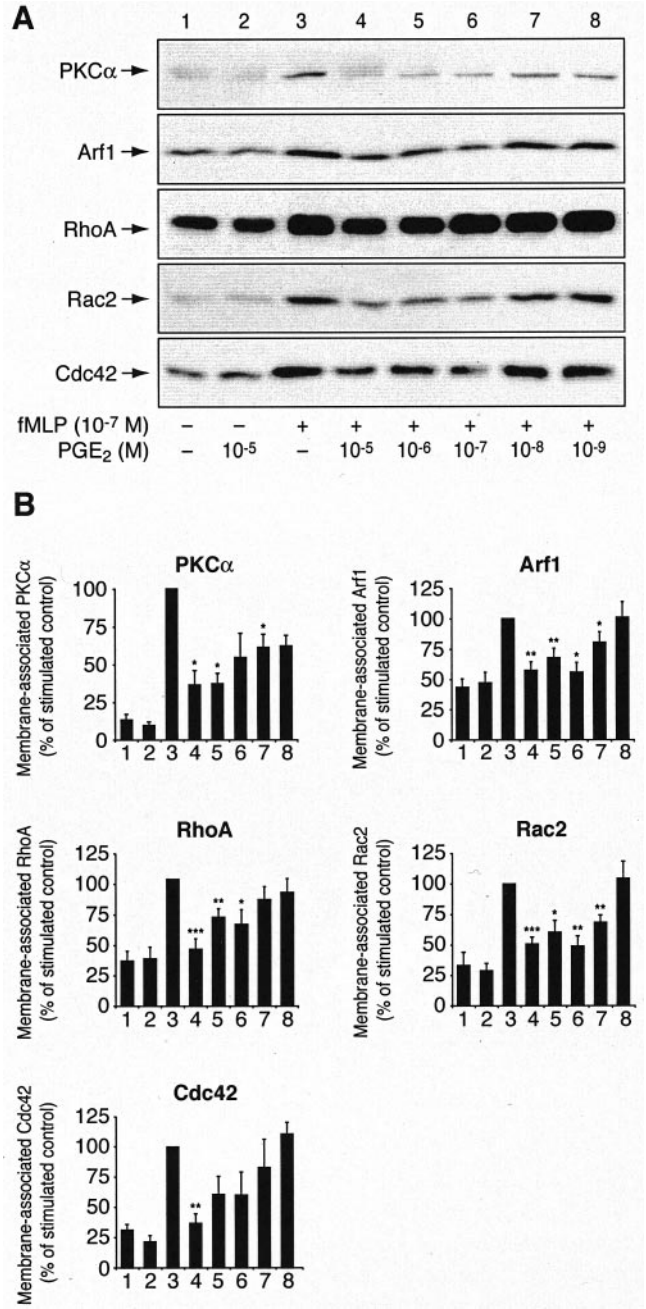

Fig. 3. Effect of PGE_2 on the fMLP-induced translocation of PKCα, Arf1, and Rho GTPases to the membranes. Neutrophils (10^7 cells/ml) were preincubated with the indicated concentrations of PGE_2 for 5 min at 37°C, in the presence of 0.1 U/ml ADA and 10 μM CB. Cell suspension were stimulated with fMLP for 2 min and the incubations were stopped as described under *Materials and Methods*. The samples were analyzed for PKCα, Arf1, RhoA, Rac2, and Cdc42 by immunoblotting. A, the immunoblots shown are representative of at least three independent experiments. B, the data of densitometric analyses are expressed as the percentage of fMLP-stimulated control and are the means ± S.D. from at least three experiments. The numbers identify the corresponding lanes of the immunoblots.

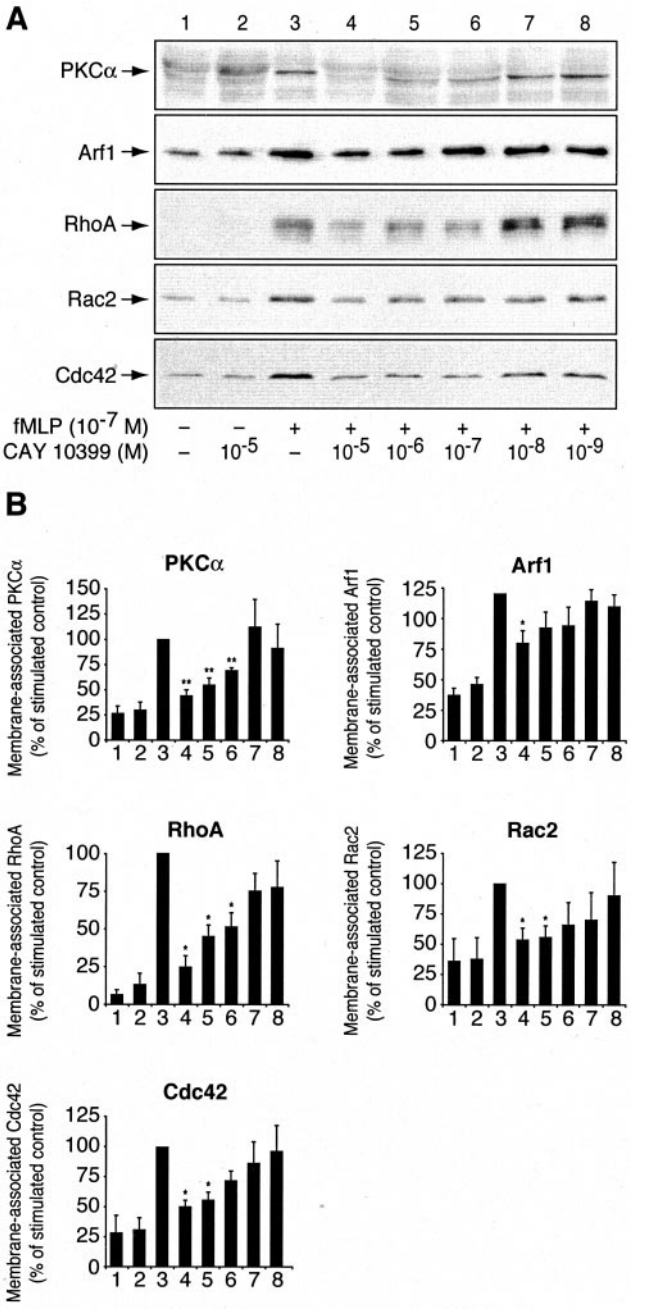

Fig. 4. Effect of CAY 10399, an EP_2 agonist, on the fMLP-induced translocation of PKCα, Arf1 and Rho GTPases to the membranes. Neutrophils (10^7 cells/ml) were preincubated with the indicated concentrations of CAY 10399 for 5 min at 37°C, in the presence of 0.1 U/ml ADA and 10 μM CB. Cell suspensions were stimulated with fMLP 100 nM for 2 min, and the reactions were stopped as described under *Materials and Methods*. The samples were analyzed for PKCα, Arf1, RhoA, Rac2, and Cdc42 by immunoblotting. A, the immunoblots shown are representative of at least three independent experiments. B, the data of densitometric analyses are expressed as the percentage of fMLP-stimulated control and are the means ± S.D. from at least three experiments. The numbers identify the corresponding lanes of the immunoblots.

hibitory effect on PtdIns(3,4,5)P_3 formation via the EP_2 receptors. Decreased accumulation of PtdIns(3,4,5)P_3 could be caused by decreased transformation of phosphatidylinositol 4,5-bisphosphate into PtdIns(3,4,5)P_3 or to accelerated dephosphorylation of PtdIns(3,4,5)P_3. To distinguish between these possibilities, we monitored the activity of p110γ induced by fMLP in the presence of PGE_2, for p110γ is the major PI3K isoform activated by chemoattractants in neutrophils (Hirsch et al., 2000; Naccache et al., 2000). In brief, PI3Kγ was immunoprecipitated using an anti-p110γ Ab at different times after fMLP stimulation, and PI3Kγ activity was tested using an exogenous substrate (PtdIns). The results of these experiments (Fig. 6A) indicated that fMLP-induced p110γ activity was maximal at 15 to 30 s, and this peak of p110γ activity was totally abolished by PGE_2 ($p = 0.0264$ at 15 s and $p = 0.0089$ at 30 s). The activation of p110γ by chemotactic factors is characterized by its rapid recruitment to membranes, and we have therefore examined the effects of PGE_2 and EP_2 agonists on this translocation step. PGE_2 and CAY 10399 alone had no effect on the amounts of membrane-associated p110γ in unstimulated cells but reduced the levels of membrane-associated p110γ to that of the unstimulated controls in fMLP-stimulated neutrophils (Fig. 6B). Thus, the inhibition of the fMLP-stimulated accumulation PtdIns(3,4,5)P_3 by PGE_2 seems to be the direct consequence of the inhibition of p110γ activity.

Effect of PGE_2 on the fMLP-Induced Pattern of Tyrosine Phosphorylation. Tyrosine kinases are well known regulators of PLD activity induced by various agonists and have been shown to act upstream of fMLP-induced recruitment of cytosolic PLD cofactors to membranes (Houle et al., 1999). We have therefore sought evidence for an effect of PGE_2 on the tyrosine phosphorylation of proteins stimulated by fMLP. The pattern of tyrosine phosphorylation induced by fMLP was examined in the presence or absence of PGE_2 or the EP_2 agonist CAY 10399. As shown in Fig. 7A, incubation of neutrophils with 10^{-5} M PGE_2 or CAY 10399 before fMLP stimulation decreased the intensity and the duration of phosphorylation of the band at 120 kDa. For a few donors, the tyrosine phosphorylation of the 42- to 44-kDa bands corresponding to the mitogen-activated protein kinases as evidenced by reprobing the membranes with an anti-phospho extracellular signal-regulated kinase 1/2 Ab (data not shown) was also reduced by PGE_2 (middle) but not by CAY 10399 (right). Taken together, these data indicate that PGE_2 reduced the activation of some still unidentified tyrosine kinase(s) via EP_2 receptor.

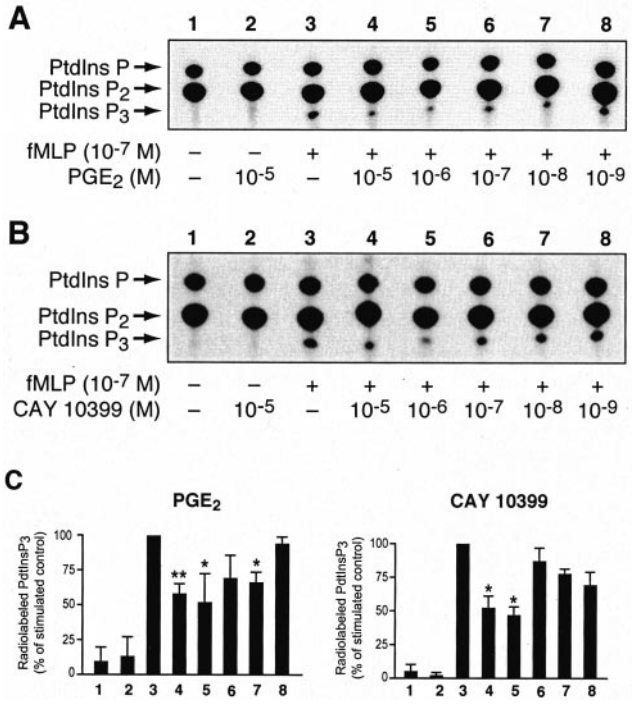

Fig. 5. Effect of PGE_2 and CAY 10399 on the fMLP-induced accumulation of PtdIns(3,4,5)P_3. [^{32}P]-labeled neutrophils (5×10^7 cells/ml) were preincubated 5 min at 37°C with the indicated concentrations of PGE_2 (A) or CAY 10399 (B) in the presence of 0.1 U/ml ADA. Cells were stimulated with fMLP for 30 s at 37°C and the amounts of PtdIns(3,4,5)P_3 were measured as described under *Materials and Methods*. The autoradiograms shown are representative of three independent experiments. C, the data of the densitometric analyses are expressed as the percentage of the fMLP-stimulated control and are the means ± S.D. from three experiments. The numbers identify the corresponding lanes of the autoradiograms.

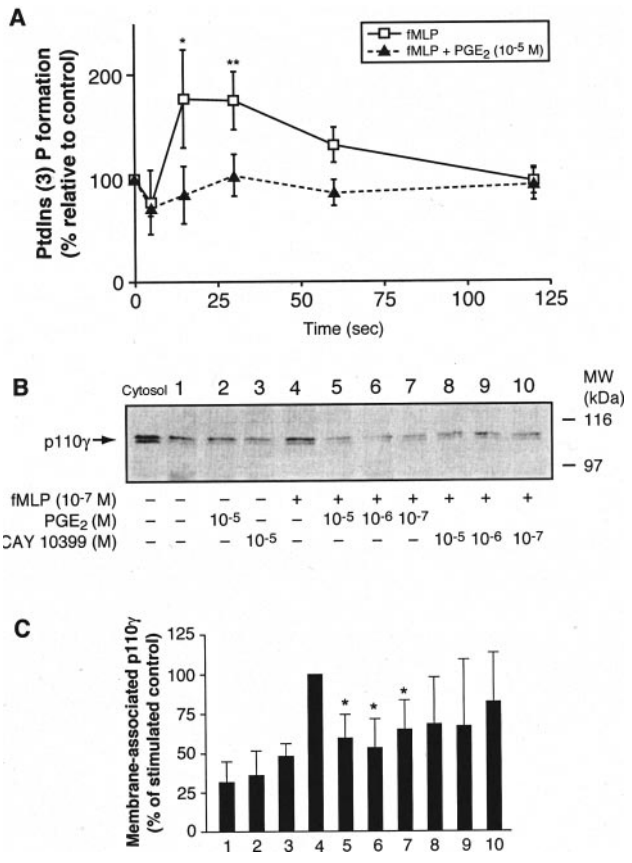

Fig. 6. Effect of PGE_2 on fMLP-induced p110γ activity and translocation to membranes. A, neutrophils (10^7 cells/ml) were preincubated 5 min at 37°C with 10^{-5} M PGE_2 in the presence of 0.1 U/ml ADA. Cells were stimulated with fMLP for the indicated times, lysed, and the immunoprecipitated p110γ was assayed for lipid kinase phosphorylation as described under *Materials and Methods*. The results are from four independent experiments and are expressed as percentage of the unstimulated sample at t = 0. B, neutrophils (10^7 cells/ml) were preincubated with PGE_2 or CAY 10399 at the indicated concentrations for 5 min at 37°C, in the presence of 0.1 U/ml ADA and 10 μM CB. Cell suspensions were stimulated with fMLP for 30 s and the reactions were stopped as described under *Materials and Methods*. The samples were analyzed for p110γ by immunoblotting. The immunoblot shown is representative of three independent experiments. C, the data of densitometric analyses are expressed as the percentage of fMLP-stimulated control and are the means ± S.D. from at least three experiments. The numbers identify the corresponding lanes of the immunoblots.

Several members of the Tec family kinases (Tec, Btk, and Bmx) have been shown to be involved in response to chemoattractants in human neutrophils (Lachance et al., 2002). The activation of Tec kinases is dependent on PI3K and characterized by a rapid translocation to the PtdIns(3,4,5)P_3-enriched membranes. We thus examined the effect of PGE_2 and EP_2 agonist on this index of activation of Tec kinases. The data shown in Fig. 7B indicate that PGE_2 as well as CAY 10399 (10^{-5}-10^{-7} M) decreased the amounts of Tec and Btk translocated to membranes in fMLP-stimulated cells.

Discussion

In this study, we have investigated the mechanisms underlying the inhibition of PLD activity by PGE_2. We demonstrated that PGE_2 inhibited several critical events stimulated by fMLP, including the influx of calcium, translocation of PKCα and small GTPases, the activation of PI3Kγ and tyrosine kinases, and the stimulation of PLD activity as well. Using various agonists and antagonists of the EP receptors, we demonstrated that EP_2 but not EP_1, EP_3, or EP_4 receptors mediate the inhibitory effect of PGE_2 on the fMLP-induced PLD activation pathway. Taken together, our results are consistent with previous pharmacological studies attributing a major role for EP_2 receptors in PGE_2-induced inhibition of neutrophil functional responses (Armstrong, 1995; Talpain et al., 1995).

The mechanism underlying the PGE_2-induced inhibition of the stimulation of PLD activity by fMLP has not yet been clarified. The fact that PGE_2 inhibited fMLP- but not phorbol 12-myristate 13-acetate–induced PLD activity suggested that PGE_2 did not exert its inhibitory effect directly on PLD but rather at a site nearer to the fMLP receptors (Agwu et al., 1991). This hypothesis led us to examine the effect of PGE_2 on the signaling steps located upstream of PLD in the fMLP-stimulated PLD activation pathway. We confirmed that PGE_2 did not influence the mobilization of Ca^{2+} from intracellular stores but decreased the influx of Ca^{2+} from the extracellular medium (Ahmed et al., 1995). The inhibitory mechanism of PGE_2 on influx has not yet been elucidated, but several hypotheses can be advanced. An up-regulating role for PI3K in Ca^{2+} influx has been reported in chemokine-stimulated cells (Kansra et al., 2001), but this does not seem to be the case in our model, because we and others (Ahmed et al., 1995) have found that wortmannin, a potent PI3K inhibitor, does not decrease fMLP-stimulated calcium influx in neutrophils; on the contrary, it slightly augments this influx (P. H. Naccache, unpublished results). Therefore, the inhibition of PI3K by PGE_2 cannot explain the reduced calcium influx observed in our experimental model. Because PGE_2 has no impact on the release of Ca^{2+} from intracellular stores, the capacitive calcium entry is probably not affected by PGE_2, and a relevant hypothesis is that PGE_2 rather inhibits the opening of a receptor-operated calcium channel (Ahmed et al., 1995). Another possibility is that, as shown previously for adenosine and other cAMP elevating agents, PGE_2 could promote an accelerated clearance of cytosolic calcium by up-regulating the *endo*-membrane Ca^{2+}-ATPase that recaptures cytosolic calcium (Theron et al., 2002). However, this hypothesis would account in part for the decreased mobilization of calcium but not for the inhibition of Mn^{2+} influx observed in the presence of PGE_2. Because the influx of Ca^{2+} has been shown to increase PLD activity stimulated by fMLP (Pai et al., 1988), the reduction of this influx of Ca^{2+} by PGE_2 could possibly down-regulate fMLP-induced PLD activity, although this

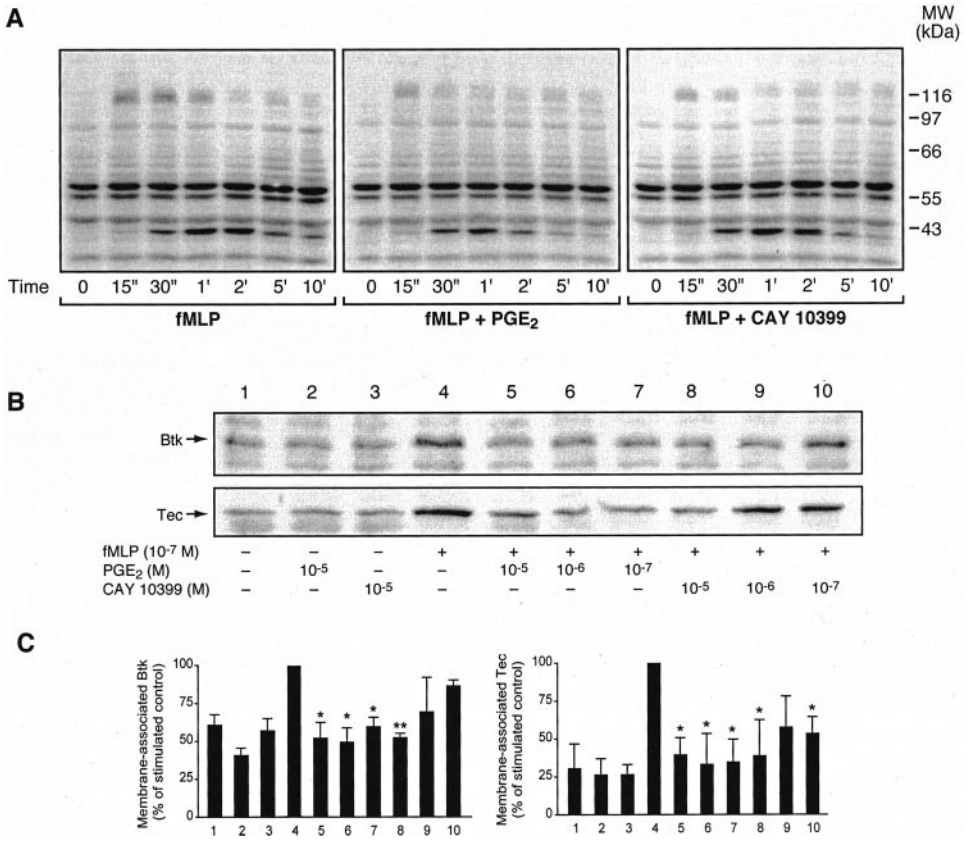

Fig. 7. Effect of PGE_2 and CAY 10399 on the global profile of tyrosine phosphorylation and on the translocation of Tec kinases induced by fMLP. A, neutrophils (2×10^7/ml) were preincubated with 10^{-5} M PGE_2 or 10^{-5} M CAY 10399 in the presence of 0.1 U/ml ADA at 37°C for 10 min. The assay was performed as described under *Materials and Methods*. The results shown are representative of at least three independent experiments. B, neutrophils (10^7 cells/ml) were preincubated with the indicated concentrations of PGE_2 or CAY 10399 for 5 min at 37°C in the presence of 0.1 U/ml ADA and 10 μM CB. Cell suspensions were stimulated with fMLP for 30 sec and the reactions were stopped as described under *Materials and Methods*. The samples were analyzed for Btk and Tec by immunoblotting. The immunoblots shown are representative of three independent experiments. C, the data of densitometric analyses are expressed as the percentage of fMLP-stimulated control and are the means ± S.D. from at least three experiments. The numbers identify the corresponding lanes of the immunoblots.

effect of PGE_2 is probably of minor influence on PLD activation because other factors than calcium directly regulate PLD.

We observed that PGE_2 and EP_2 agonists significantly decreased the translocation of PKCα, Arf1 and RhoA to fMLP-stimulated neutrophil membranes. These PLD activation cofactors act synergistically to enhance PLD activity (Exton, 1999) and their simultaneous inhibition by PGE_2 provides a mechanistic explanation for the inhibitory effect of the EP_2 signaling pathway on fMLP-induced PLD activity. These results are in accordance with and further support a previous study from our laboratory indicating that adenosine inhibited the fMLP-induced PLD pathway via the A_{2A} receptor by diminishing membrane recruitment of PKCα, Arf1, and RhoA (Thibault et al., 2000). The translocation of two other Rho GTPase family members, Rac2 and Cdc42, are involved in several functions inhibited by PGE_2 is also reduced by PGE_2 via the EP_2 receptors.

The concomitant inhibition of Rho and Arf GTPases suggested that PGE_2 exerts an inhibitory effect upstream of both small GTPase families. Although the intermediary signaling steps linking membrane bound receptors to small GTPase activation are not yet fully understood, PI3K and tyrosine kinases seem to play key roles in membrane recruitment and activation of small GTPases (Benard et al., 1999).

We report here that PGE_2 significantly reduced the amounts of PtdIns(3,4,5)P_3 formed in response to fMLP via EP_2 receptor. Moreover, we observed an almost complete inhibition of p110γ kinase recruitment and activation by PGE_2. These results strongly suggest that PGE_2 inhibited the chemoattractant-induced activation of p110γ rather than stimulating an accelerated rate of dephosphorylation of PtdIns(3,4,5)P_3. It is therefore unlikely that the phosphoinositide-specific phosphatases PTEN or SHIP are involved in the inhibitory effect of PGE_2 on PtdIns(3,4,5)P_3 accumulation. This inhibitory effect of PGE_2 on p110γ adds a new element to the mechanism involved in EP_2-mediated inhibition of the fMLP-induced PLD signaling pathway.

The activation of small GTPases is tightly controlled by the opposite actions of guanine nucleotide exchange factors (GEFs) and guanine nucleotide activating proteins that stimulate GTP hydrolysis. GEFs for the Rho and Arf family GTPases contain a PH domain known to bind phosphoinositol lipids, thereby targeting GEF to PtdIns(3,4,5)P_3-enriched membrane regions. Increasing evidence indicates that Rho GTPases are indirectly regulated by PI3K via specific GEFs (Welch et al., 2003). Of particular relevance to our work is the observation that the Rho GEF Vav stimulated by the fMLP receptor mediates the activation of Rac by PI3Kγ. The relationship between Arf GEFs and PI3K in vivo is more controversial, and reports differ according to the GEF and the cellular type studied. Nevertheless, the PH domain of cytohesin-1, the main Arf-GEF expressed in human neutrophils, binds to PtdIns(3,4,5)P_3 (Corvera and Czech, 1998), and we observed that Arf translocation to membranes is down-regulated by wortmannin (N. Thibault, unpublished results). These lines of experimental evidence linking PI3K to GEF activation in various cellular systems support our hypothesis that the inhibition of p110γ by PGE_2 decreases the translocation of Arf and Rho GTPases.

The inhibition of PKCα translocation by PGE_2 may at first seem intriguing because PGE_2 neither alters the first peak of cytosolic calcium released from intracellular pools by fMLP-induced phospholipase C activation nor effectively inhibits diacylglycerol formation in fMLP-stimulated neutrophils (Takenawa et al., 1986). The reduced calcium influx could account in part for the inhibitory effect of PGE_2 on PKCα activation, but PKCα inhibition is more likely to be attributable to the decreased activity of PI3Kγ because PKC has been shown to be located downstream of PI3K in the fMLP signaling pathway (Vlahos et al., 1995).

PLD and small GTPase activation is also regulated by tyrosine phosphorylation events. There is no evidence for a direct tyrosine phosphorylation of PLD after fMLP stimulation in neutrophils (Marcil et al., 1999), but the translocation of PLD cofactors (PKCα, Arf, and RhoA) as well as Rac2 and Cdc42 activation has been shown to occur downstream of tyrosine phosphorylation events in fMLP-stimulated HL-60 cells, providing a functional link between tyrosine kinases and PLD activation (Benard et al., 1999; Houle et al., 1999). PGE_2 (or CAY 10399) reduced the fMLP-stimulated tyrosine phosphorylation of a set of substrates around 120 kDa by as-yet-unidentified kinases. This suggests that tyrosine kinase(s) could be another target for PGE_2. Because the profile of tyrosine phosphorylation cannot provide precise information on the putative kinases involved, we further investigated the effect of PGE_2 on Tec kinases that have been shown to be activated by fMLP (Lachance et al., 2002). PGE_2 significantly decreased Tec and Btk translocation to membranes, an effect that can be viewed as a consequence of PI3Kγ inhibition because the recruitment and the activation of these PH-domain containing kinases are dependent on PI3K. This latter result is to be related to a previous study that established a functional link between Btk (and possibly other Tec kinases) and the activation of Rho, Arf GTPases and PLD in human neutrophils (Gilbert et al., 2003) and further support the involvement of PI3Kγ as a crucial target of the EP_2 inhibitory pathway.

The mechanism underlying the inhibition of PI3Kγ by PGE_2 remains to be further investigated. PI3Kγ is composed of a p110γ catalytic subunit tightly bound to a p101 regulatory unit, and upon fMLP-receptor stimulation, this cytosolic complex is activated by binding to the $G_{\beta\gamma}$ units of heterotrimeric G protein. The noncatalytic p101 subunit is thought to act as an adaptor that mediates the recruitment of the enzymatic complex to the membrane where $G_{\beta\gamma}$ directly activates the catalytic p110γ subunit (Brock et al., 2003). Because PGE_2 inhibits the translocation of the p110γ, we can suggest that PGE_2 prevents the binding of the enzymatic complex to $G_{\beta\gamma}$ by targeting a site either on the p101/p110 complex or on $G_{\beta\gamma}$ and that the decreased amount of p101/p110γ recruited explains the suppression of the stimulation of p110γ activity by the EP_2 pathway.

The signaling events linking the EP_2 receptor to its fMLP-stimulated targets (possibly PI3Kγ) also remain to be characterized. The EP_2 receptor is coupled to a $G_{\alpha s}$ protein that increases cAMP levels by stimulating adenylate cyclase (Breyer et al., 2001). Although several suppressive effects of PGE_2 on neutrophil functions have been paralleled by an increase in cAMP (Rivkin et al., 1975; Talpain et al., 1995; Bloemen et al., 1997), others (e.g., chemotaxis and aggregation triggered by fMLP) have not (Armstrong, 1995), suggesting that EP_2 could transmit signals through an alternative and still unknown pathway. Nevertheless, the fact that superoxide production stimulated by fMLP was inhibited by PGE_2 in a cAMP/cAMP-dependent protein kinase-dependent manner (Armstrong, 1995) would be in favor for a role of this pathway in the inhibition of PLD activity. A more detailed study of the involvement of cAMP-dependent protein kinase in the inhibition of the fMLP

signaling cascades, more particularly in the inhibition of PI3K, is presently underway.

The present study also provides new insights into the signaling mechanisms underlying the EP_2 mediated inhibition of the respiratory burst, chemotaxis, and secretion elicited by chemoattractants in human neutrophils. The assembly and activation of the NADPH complex is tightly regulated at multiple levels and has been shown to require 1) the phosphorylation of several components by PKC, 2) the presence of PLD-derived PA implicated in phosphorylation processes, 3) the translocation of Rac2 and, finally, 4) the activation of PI3K, mainly p110γ (Vlahos et al., 1995; Babior, 1999; Hirsch et al., 2000). The EP_2-mediated inhibitory effects on fMLP-induced PI3K and PLD activities as well as on the translocation of Rac2 and PKC can account for the strong reduction of the respiratory burst by PGE_2. PI3Kγ and Rho GTPases also play essential roles in the polarization of the cell that initiates the chemotactic response (Servant et al., 2000), and their inhibition by the EP_2 pathway explains, at least in part, the impairment of chemotaxis by PGE_2. Exocytosis and degranulation processes are regulated by Arf and PLD in neutrophils (Cockcroft, 2001), and their inhibition by the EP_2 pathway might also account for the inhibition of the fMLP-stimulated release of cytotoxic enzymes by PGE_2.

In conclusion, the present study shows that PGE_2 inhibits the chemoattractant-stimulated PLD pathway by binding to the EP_2 receptor in human neutrophils. Moreover, we provide evidence that this inhibitory pathway decreases p110γ activation by fMLP and, as a result activation of Tec kinases, small GTPases and PLD. The results of this investigation provide new insights into the signaling mechanisms implicated in the down-regulation by PGE_2 of the inflammatory response to chemoattractants.

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