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The PGE_2-induced inhibition of the PLD activation pathway stimulated by fMLP in human neutrophils is mediated by PKA at the PI3-Kγ level

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ABSTRACT

Prostaglandin E_2 (PGE_2), an eicosanoid that modulates inflammation, inhibits several chemoattractant-elicited functions in neutrophils such as chemotaxis, production of superoxide anions, adhesion, secretion of cytotoxic enzymes and synthesis of leukotriene B_4. We previously reported that PGE_2 inhibits the fMLP signaling pathway that leads to PLD activation through suppression of PI3-Kγ activity and the decreased recruitment to membranes of PLD activation factors, PKC, Rho and Arf-GTPases. This effect is mediated via the EP_2 receptors known to raise cAMP in cells.

The inhibition of most fMLP-induced functional responses by PGE_2 via EP_2 receptors is mediated by PKA, except the chemotactic response. We have investigated the role of PKA in the EP_2-mediated inhibition of the PLD activation pathway. H-89, a selective PKA pharmacological inhibitor suppressed the inhibitory effects of PGE_2 at all stages of the PLD pathway activated by fMLP, i.e. PLD activity, translocation to membranes of PKCα, Rho and Arf-GTPases, calcium influx, tyrosine phosphorylation of proteins and finally translocation of p110γ catalytic subunit of PI3-K to membranes. However, neither PLD nor PI3-Kγ was substrate of PKA. These data provide evidence that PGE_2-stimulated PKA activity regulates the PLD pathway stimulated by fMLP at the level of PI3-Kγ and that the inhibition of PI3-Kγ activation by PKA is a complex mechanism that remains to be completely elucidated.

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Abbreviations: ADA, adenosine deaminase; AKAP, A-kinase anchoring protein; Arf, ADP-rybosilation factor; CB, cytochalasin B; DFP, diisopropylfluorophosphate; EP receptor, E prostaglandin receptor; fMLP, N-formyl-methionyl-leucyl-phenylalanine; GEF, guanine nucleotide exchange factor; PEt, phosphatidylethanol; PH, pleckstrin homology; PI3-K, phosphatidylinositol 3-kinase; PKA, cAMP-dependent protein kinase; PMN, polymorphonuclear neutrophil; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphophate; PLD, phospholipase D; PLC, phospholipase C

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1. Introduction

Prostaglandin E_2 (PGE_2) is an eicosanoid produced by cyclooxygenases (COX1/2) that is usually considered as a potent inflammatory mediator. However, PGE_2 has also the property to down-regulate the activation of leukocytes and plays an immuno-modulatory role at the level of the innate as well as the acquired immune responses [1,2]. Most particularly, PGE_2 inhibits fMLP-induced functional responses such as superoxide anions production [3–6], chemotaxis [7], release of cytotoxic enzymes [4,8], synthesis of leukotriene B_4 (LTB_4) [9] and adhesion to epithelial cells [10] in polymorphonuclear neutrophils (PMNs), important cell effectors of the innate immune response. These inhibitory effects on PMNs are all mediated via the E prostaglandin 2 (EP_2) receptors that trigger cAMP elevation in cells [7,11]. In a previous study on the inhibitory signaling mechanism of PGE_2, we reported that the main target of PGE_2 in the pathway that leads to activation of phospholipase D (PLD) by fMLP was phosphoinositol 3-kinase γ (PI3-Kγ), an important upstream regulator of PLD activity [12]. In this study, PGE_2 was shown to abolish the translocation of PI3-Kγ to membranes that allows the stimulation of the p110γ subunit enzymatic activity by binding to Gβγ subunits upon fMLP stimulation. The inhibition of the translocation of PI3-Kγ to Gβγ resulted in a decreased formation of PtdIns(3,4,5)P_3 and hence, in a reduced recruitment to membranes of the factors that activate PLD, i.e. Rho and Arf GTPases as well as PKCα.

Most of the EP_2 receptor-mediated inhibitory effects of PGE_2 on PMN functions activated by fMLP are paralleled by an increase in cAMP and hence have been attributed to an elevation of this second messenger. PKA, the main effector of cAMP in cells, has been shown to mediate the inhibition of fMLP-induced superoxide anion production by PGE_2 via EP_2 receptors [7]. However, the inhibition of chemotaxis by PGE_2 depends neither on a cAMP increase nor on PKA activation [7]. This latter observation suggests that EP_2 receptors may activate signaling pathways other than the cAMP/PKA pathway. Therefore, we have investigated the involvement of PKA in the EP_2 receptor-mediated inhibition of the different components of the PLD pathway induced by fMLP, i.e. PLD, Rho and Arf-GTPases, PKCα and PI3-Kγ, as well as calcium mobilization and tyrosine phosphorylation events.

2. Material and methods

2.1. Antibodies

The anti-PKCα (P16520) and anti-Cdc42 (C70820) monoclonal Abs were purchased from BD Transduction Laboratories (Mississauga, Ontario, Canada). Anti-RhoA (SC-179), anti-Rac2 (SC-96) polyclonal Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal anti-Akt (#9272) and anti-phospho-PKA substrate (#9621) Abs were obtained from Cell Signaling Technology (Beverly, MA). The polyclonal anti-Arf1 and anti-p110γ antisera, the polyclonal anti-CD32 (FcγRIIa) Ab were raised in rabbits as described previously [13–15]. The monoclonal anti-phosphotyrosine Ab (UBI 05-321, clone 4G10) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The rabbit polyclonal anti-Lyn Ab was obtained from Santa Cruz. Secondary anti-mouse (#NXA931) and anti-rabbit (#NA934V) Abs were obtained from Amersham Biosciences (Baie d'Urfé, Québec, Canada).

2.2. Reagents

Dextran T-500 was purchased from Pharmacia Biotech (Dorval, Québec, Canada) and Ficoll-Paque from Wisent (St-Bruno, Québec, Canada). Adenosine deaminase (ADA) was purchased from Roche Diagnostics (Laval, Québec, Canada), and di-isopropylfluorophosphate (DFP) from Serva (Heidelberg, Germany). fMet-Leu-Phe (fMLP) and cytochalasin B (CB) were obtained from Sigma–Aldrich Canada (Oakville, Ontario, Canada). PGE_2 and CAY10399 were purchased from Cayman Chemical (Ann Arbor, MI). H-89 and recombinant mouse PKA catalytic subunit were obtained from Calbiochem. Rp-8-Br-cAMPS was purchased from BIOLOG (Bremen, Germany). Fura-2/AM was obtained from Molecular Probes (Eugene, OR). Recombinant tagged PI3-K$\gamma^{His\text{-}GST}$ (p110γ^{His}/p101^{GST}) and p110γ^{His} catalytic subunits were purchased from Jena Bioscience (Jena, Germany). [γ-^{32}P] ATP (3000 Ci/mmol) (NEG 502A) was obtained from Perkin-Elmer Life Sciences (Woodridge, Ontario, Canada).

2.3. Isolation of human neutrophils

Venous blood was collected from healthy adult volunteers in isocitrate anticoagulant solution. Neutrophils were separated as described previously [16]. Briefly, whole blood was centrifuged at 180 g for 10 min and the resulting platelet rich plasma was discarded. Leukocytes were obtained following 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+}.

2.4. 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 [16]. 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 pre-treated 5 min with 10 μM CB and 0.1 U/ml ADA to eliminate endogeneous adenosine and in the presence or the absence of the indicated concentrations of PGE_2 or EP_2 receptor agonist (CAY10399). Neutrophils were stimulated with 100 nM fMLP for 10 min in the presence of 1% ethanol. Incubations were stopped by adding 1.8 ml of chloroform/methanol/HCl (50:100:1, v/v/v) and unlabeled phosphatidylethanol (PEt) as a standard. Lipids were extracted and the levels of [^{3}H]PEt were quantified as described previously [16].

2.5. 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 pre-incubated 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 Me_2SO. 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 as described by Grynkievicz et al. [17]. Briefly, raw data were transformed using the following formula: $224((y - F_{min})(F_{max} - y))$. F_{max} was the level of fluorescence obtained with cells disrupted with 1% Triton X-100 and F_{min} was obtained by adding 5 mM of EGTA and 1N NaOH to cell suspensions.

2.6. 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 for an additional 5 min with 10 μM CB, 0.1 U/ml ADA and PGE_2 or EP_2 receptor agonist (CAY10399) or an equal volume of diluent (Me_2SO) as a control. Neutrophils were stimulated with 100 nM fMLP at 37 °C. Incubations were stopped by diluting the cells five-fold with ice-cold HBSS and total membrane proteins were collected as described previously [16]. Briefly, cell suspensions were centrifuged as indicated and resuspended at 1.6×10^7 cells/ml in ice-cold KCl–Hepes relaxation buffer (50 mM Hepes, 100 mM KCl, 5 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM PMSF and 1 mM orthovanadate, adjusted to pH 7.2). Cell suspensions were sonicated 20 sec and centrifuged 7 min at $1000 \times g$. Unbroken cells and nuclei were discarded and the supernatants ultracentrifuged at $180{,}000 \times g$ for 45 min in a Beckman TL-100 ultracentrifuge. Membrane pellets were washed once and resuspended in a small volume of solubilization buffer containing 0.25 M Na_2HPO_4, 0.3 M NaCl, 2.5% sodium dodecyl sulfate (SDS), 10 μg/ml aprotinin, 10 μg/ml leupeptin and 2 mM PMSF and samples were assayed for protein content with Pierce Coomassie Brilliant Blue Protein Assay. Protein samples (10–20 μg) were resolved on a 7.5–20% gradient SDS-PAGE and transferred to Immobilon PVDF membranes (Millipore Corporation, Bedford, MA, USA). Immunoblots were performed using anti-Arf1 (1/2500), anti-RhoA (1/1000), anti-PKCα (1/1000), anti-Rac2 (1/200), anti-Cdc42 (1/250), anti-p110γ (1/1000) Abs. The PVDF membranes were reprobed with an Ab against CD32, a marker of plasma membrane, to assure equal loading of proteins in all samples. Proteins were revealed with HRP-conjugated secondary anti-mouse or anti-rabbit Ab (1/20,000) and the Renaissance detection system (NEN, Perkin-Elmer Life Sciences).

2.7. Tyrosine and PKA phosphorylation patterns

Neutrophils (2×10^7 cells/ml) were pre-incubated at room temperature with 1 mM DFP, with or without the indicated concentrations of PGE_2 or EP_2 receptor agonists for 10 min at 37 °C prior to 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, 0.025% bromophenol blue) and boiled for 7 min. The samples were then subjected to 7.5–20% gradient SDS-PAGE and transferred to Immobilon PVDF membranes. Immunoblotting was performed using the monoclonal anti-phosphotyrosine Ab 4G10 (1/4000) or the polyclonal anti-phospho-PKA substrate Ab and revealed with HRP-conjugated secondary anti-mouse or anti-rabbit Ab (1/20,000) and the Renaissance detection system (NEN, Perkin-Elmer Life Sciences).

2.8. Phosphorylation of recombinant PI3-Kγ

Recombinant PI3-Kγ (700 ng $p110\gamma^{His}/p101^{GST}$ or 350 ng $p110\gamma^{His}$) was incubated in phosphorylation buffer (20 μl final volume; Tris–HCl, pH 7.5, $MgCl_2$ 5 mM, DTT 1 mM) with PKA catalytic subunit (50 U), 1 mM ATP and 2 μCi [γ-^{32}P]ATP at 30 °C for 30 min. When indicated, PI3-Kγ was pre-incubated with 100 nM wortmannin and PKA was pre-incubated with 1 μM H-89 for 10 min prior to the phosphorylation assay. In each experiment, histones were used as a substrate to monitor the PKA phosphorylation reaction. The reactions were stopped by addition of 10 μl SB 3× and 5 min boiling. The samples were resolved using 12% SDS-PAGE and the dried gels analyzed by autoradiography.

2.9. Statistical analysis

The data were analyzed using the unpaired Student's *t*-test. The levels of significance ($^{*}p < 0.05$ and $^{**}p < 0.01$) were determined between the samples treated with PGE_2 (or CAY10399) and the samples treated with both H-89 and PGE_2 (or CAY10399).

3. Results

3.1. Effects of fMLP and PGE_2 on PKA activation

Chemoattractants such as fMLP are known to elicit a rapid rise in intracellular cAMP that peaks at 15–20 s followed by a decrease to basal level by 2–5 min in PMNs [18–20]. EP_2 has been identified as the PGE_2 inhibitory receptor on PMNs [7,12,21] and has been shown to stimulate cAMP formation in cells [22]. In PMNs, PGE_2 (or PGE_1) is able to stimulate a modest increase in cAMP levels in the presence of phosphodiesterase inhibitors (IBMX, theophylline) only, but promotes a large and sustained enhancement of intracellular cAMP levels when PMNs are subsequently stimulated by fMLP [19,23,24]. PKA is the main effector of cAMP in cells and its activation is regulated by cAMP binding on its regulatory subunits. We have thus examined the effects of the cAMP elevation triggered by fMLP and PGE_2 on PKA activity in PMNs. To this purpose, we have analyzed whole cell lysates of fMLP-stimulated PMNs with a phospho-PKA

substrate Ab which specifically recognizes the phophorylated PKA consensus motif on PKA substrates. For all the experiments presented in this study, PMNs were pre-incubated in the presence of adenosine deaminase (ADA) in order to degrade the endogenous adenosine to inosine. We have previously shown that adenosine rapidly accumulates in PMN suspensions and activates the cAMP/PKA pathway via the A_{2a} inhibitory receptors [25,26]. Thus, this pre-incubation is necessary to isolate the effect of PGE_2 on the cAMP/PKA pathway.

The data shown in Fig. 1 indicate that fMLP stimulated the phosphorylation of several proteins that are recognized by the anti-phospho-PKA substrate Ab (MW at approximately 47–50, 60–65, 95–97, 120–130 and 140 kDa). Phosphorylation peaked at 1 min and gradually decreased thereafter. In the presence of PGE_2, the levels of phosphorylation of PKA substrates stimulated by fMLP were globally increased, and the increased intensity of phosphorylation observed at 0.5 and 1 min indicated an earlier onset of the response when PMNs were pre-incubated with PGE_2 prior to fMLP stimulation. It is worthwhile to notice that pre-incubation of cells with PGE_2 alone increased the intensity of phosphorylation by PKA of some substrates at 50, 80–97 and around 120 kDa (fMLP versus fMLP + PGE_2, time 0). PMNs were also pre-incubated with H-89, a selective PKA inhibitor in order to ascertain the role of PKA in the phosphorylation pattern previously obtained. In the presence of H-89, the intensity of phosphorylation of the predominant PKA substrates in response to fMLP was attenuated (mainly substrates at 50, 60–65, 97 and 120 kDa) and the stimulating effect of PGE_2 on the PKA phosphorylation profile stimulated by fMLP was attenuated as well. Taken altogether, these data show that the PKA activity stimulated by fMLP is increased in the presence of PGE_2 but, although this eicosanoid has been shown to promote a long-lasting rise of cAMP in fMLP-stimulated PMNs, it does not seem to prolong the phosphorylation of proteins by PKA in these cells.

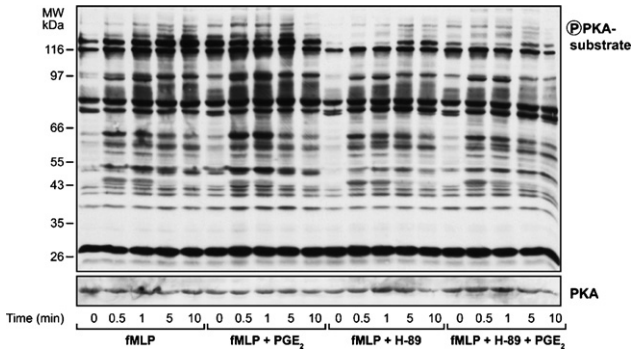

Fig. 1 – Activation of PKA by fMLP and PGE_2. PMNs were pre-incubated with 10^{-5} M PGE_2 at 37 °C for 10 min in the presence of 0.1 U/ml ADA and stimulated with 100 nM fMLP for the times indicated. Where indicated, PMNs were treated for 10 min with 10^{-5} M H-89 prior to incubation with PGE_2. Whole cell lysates were analyzed by immunoblotting with the anti-phospho-PKA substrate Ab as described in Section 2. As a control for equal loading, the PVDF membranes were reprobed with an anti-PKA mAb to monitor amounts of the kinase in each sample. The immunoblot shown is representative of at least three independent experiments.

3.2. *Role of PKA in the PGE_2-mediated inhibitory effect on fMLP-induced PLD activity*

We previously reported that PGE_2 inhibits PLD activity stimulated by fMLP via EP_2 receptors [12]. Since PGE_2 also activates PKA in fMLP-stimulated cells, we wondered whether PKA mediated the inhibitory effect of PGE_2 on PLD activity. To test this hypothesis, PMNs were pre-incubated in the presence of H-89 prior to addition of PGE_2 to cell suspensions and stimulation by fMLP. PMNs were primed with cytochalasin B 5 min prior to stimulation with fMLP in order to increase the amounts of phosphatidylethanol (PEt) produced by PLD. The data presented in Fig. 2A show that H-89 had no effect on PLD activity stimulated by fMLP but completely suppressed the inhibitory effect of PGE_2 on PLD activity. A distinct unrelated PKA specific inhibitor, Rp-8-Br-cAMPS, which inhibits preferentially type I PKA activity also significantly decreased the inhibitory effect of PGE_2 on fMLP-induced PLD activity but to a lesser extent than H-89 (Fig. 2B). These data clearly indicate that the PGE_2 inhibitory effect on PLD activity is mediated by PKA in fMLP-stimulated PMNs. Since Jang et al. have reported that PKA could phosphorylate PLD1 [27], the major PLD isoform expressed in PMNs, we verified the hypothesis of a direct phosphorylation of PLD1 in PMNs pre-incubated with PGE_2. PLD1 was immunoprecipitated from PMNs treated with PGE_2 and stimulated with fMLP. The PLD1 immunoprecipitates were analyzed by immunoblotting with the anti-phospho-PKA substrate Ab. Under these experimental conditions, we were not able to detect a phosphorylation of PLD1 in PMNs (data not shown).

3.3. *Role of PKA in the PGE_2-mediated inhibitory effect on Ca^{2+} influx induced by fMLP*

The rapid and transient rise in intracellular Ca^{2+} triggered by fMLP is an important regulator of PLD activity [28,29]. We have already reported that PGE_2 diminishes the late Ca^{2+} influx induced by fMLP via EP_2 receptors in PMNs [12]. We thus wondered whether PKA was implicated in the inhibition of Ca^{2+} influx by PGE_2. As shown in Fig. 3, the addition of PGE_2 reduced the late mobilization of cytoplasmic Ca^{2+} due to extracellular Ca^{2+} influx stimulated by fMLP. This effect of PGE_2 was abolished in the presence of H-89. The EP_2 selective agonist CAY10399 had a similar effect as PGE_2 on Ca^{2+} influx induced by fMLP and was similarly sensitive to H-89. These results are consistent with an inhibitory effect of PGE_2 and CAY10399 through the cAMP-mediated activation of PKA on the fMLP-induced Ca^{2+} influx.

3.4. *Role of PKA in the inhibition of PKCα and small GTPases translocation by PGE_2*

The activation of PLD is regulated by three cytosolic factors that need to translocate to membranes to be activated: classical PKC isoforms, and the small GTPases RhoA and Arf [30]. We previously reported that PGE_2, via EP_2 receptors, decreased the translocation to membranes of these PLD

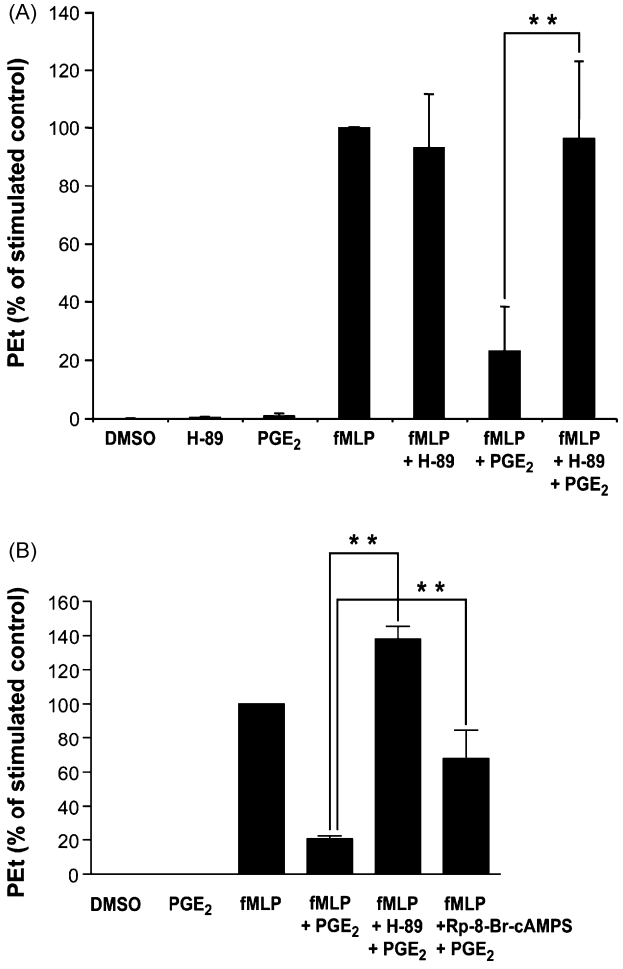

Fig. 2 – Effect of H-89 and Rp-8-Br-cAMPS on the PGE_2-induced inhibition of PLD activity stimulated by fMLP. (A) Neutrophils (10^7 cells/ml) were pre-incubated with 10^{-5} M H-89 for 5 min in the presence of 0.1 U/ml ADA and with 10^{-6} M PGE_2 for additional 5 min at 37 °C and 10 μM CB. Cells were stimulated with 100 nM fMLP in the presence of 1% ethanol for 10 min. (B) Neutrophils were pre-incubated with 0.1 U/ml ADA (5 min) and then with 250 μM Rp-8-Br-cAMPS for 45 min at 37 °C. Where indicated, 10^{-5} M H-89 was added for 10 min and 10^{-5} M PGE_2 for additional 5 min. 10 μM CB were added 5 min prior to stimulation of cells with 100 nM fMLP in the presence of 1% ethanol for 10 min. The amounts of [^{3}H]PEt formed were evaluated as described in Section 2. The levels of [^{3}H]PEt formed are expressed as the percentage of the fMLP-stimulated control. The data are from at least three different experiments performed in duplicates and are expressed as the means ± S.E.M.

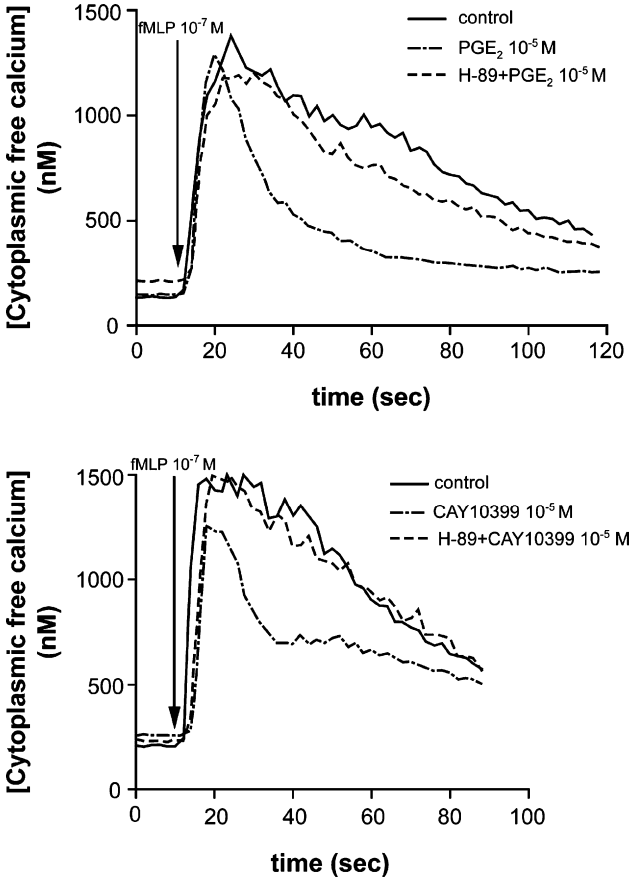

Fig. 3 – Effect of H-89 on the EP_2-mediated inhibition of calcium influx stimulated by fMLP. Neutrophils (5×10^6 cells/ml) were pre-incubated for 5 min at 37 °C with 10^{-5} M H-89 in the presence of 0.1 U/ml ADA and for additional 5 min with PGE_2 or CAY10399. Cells were stimulated with 100 nM fMLP at the time indicated by the arrow and cytosolic calcium concentrations were monitored as described in Section 2. The data shown are representative of three independent experiments.

activation cofactors stimulated by fMLP, as well as the translocation of two other Rho GTPases members: Rac2, which is a cytosolic component of the NADPH oxidase complex and Cdc42, an important regulator of actin polymerization [12]. To further investigate the role of PKA in the PGE_2 inhibitory mechanism mediated via EP_2 receptors, we tested the effect of H-89 on the fMLP-induced translocation of PKCα and Arf and Rho GTPases in the presence of PGE_2 or of the EP_2 receptor agonist CAY10399. PMNs were primed with cytochalasin B in order to increase the amounts of proteins recruited to membranes. The results illustrated in Fig. 4 show that PGE_2 and CAY10399 decreased the amounts of PKCα, Rho GTPases (RhoA, Rac2 and Cdc42) and Arf1 that translocate to membranes in response to fMLP. In PMNs pre-treated with H-89 and PGE_2 or CAY10399 prior to stimulation with fMLP, the amounts of these factors associated to membranes (lanes 9 and 10) are similar to those obtained from control fMLP-stimulated PMNs incubated in the presence (lane 8) or in the absence of H-89 (lane 5). These data show that H-89 almost completely (PKC, RhoA, Rac2) or completely (Arf, Cdc42) suppressed the inhibition of the translocation of these factors mediated via EP_2 receptors, and therefore indicate that PKA activated by the EP_2 pathway decreased, at least in part, the fMLP-induced recruitment of PKC and small GTPases to membranes.

3.5. Role of PKA in the PGE_2-mediated inhibitory effect on tyrosine phosphorylation

We have previously reported that PGE_2 decreases the fMLP-induced tyrosine phosphorylation of a subset of unidentified proteins at 116–120 kDa [12]. To assess whether PKA is involved in this inhibitory effect of PGE_2, PMNs were pre-incubated with H-89 prior to addition of PGE_2 to cell suspensions and then stimulated with fMLP. The levels of tyrosine phosphorylation were evaluated by analyses of whole cell lysates with an anti-phosphotyrosine Ab. As shown in Fig. 5, PGE_2 decreased the tyrosine phosphorylation of the proteins in the 116–120 kDa region stimulated by fMLP. Furthermore, H-89 slightly increased the fMLP-stimulated tyrosine phosphorylation of these proteins. The decreased tyrosine phosphorylation of the 116–120 kDa set of substrates caused by PGE_2 was no longer observed in the presence of H-89. These data indicate that (1) PKA modulates the tyrosine phosphorylation reactions triggered by fMLP and (2) the inhibition of tyrosine phosphorylations of the 116–120-kDa substrates by PGE_2 is mediated by PKA.

3.6. Role of PKA in the inhibition of p110γ translocation by PGE_2

PI3-Kγ is the major PI3-K isoform activated in response to fMLP in PMNs [14] and is responsible for the rapid and massive accumulation of PtdIns(3,4,5)P_3 at the leading edge of the cell exposed to a gradient of chemotactic agent [14,31]. This accumulation allows the relocation of proteins containing PH domains, such as the exchange factors for small GTPases (GEFs), to PtdIns(3,4,5)P_3-enriched membrane regions. PI3-Kγ is a heterodimer 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 Gβγ units of heterotrimeric G protein. We have previously reported that PGE_2 inhibits the translocation of p110γ to membranes where its catalytic activity is stimulated by binding to the Gβγ units after stimulation by fMLP. This inhibition was mediated via EP_2 receptors and consequently inhibited PI3-Kγ activity and PtdIns(3,4,5)P_3 formation [12]. To assess the role of PKA in the inhibitory effect of PGE_2 on PI3-Kγ translocation, we have evaluated the amounts of membrane-associated p110γ in fMLP-stimulated PMNs in the presence of H-89 and PGE_2. The data presented in Fig. 6 show that a pre-incubation of cells with H-89 alone did not significantly modify the translocation of the p110γ catalytic subunit to membranes in response to fMLP (lane 8 versus lane 5) but, when added prior to PGE_2 or CAY10399, H-89 significantly blocked the inhibitory effect of PGE_2 or CAY10399 on p110γ translocation (lane 6 versus lane 9, $p = 0.0173$, and lane 7 versus lane 10, $p = 0.0412$, respectively). Together, these data show that H-89 blocks, at least in part, the EP_2-mediated inhibition of the translocation of PI3-Kγ induced by fMLP and indicate that PKA is involved in this PGE_2 inhibitory effect.

3.7. Phosphorylation of PI3-Kγ by PKA

Since PKA negatively regulates the translocation of p110γ induced by fMLP, we wondered whether this inhibitory effect could be mediated through a direct phosphorylation of the PI3-Kγ heterodimer (p110γ/p101) by PKA. First, we analyzed the protein sequences of p101 and p110γ by using the Scansite program (http://scansite.mit.edu) in order to identify putative motifs of phosphorylation by PKA (RxxT or RRxS) in these proteins. This theoretical analysis revealed and that human p101 contains two such potential sites: Thr-31 (RRST) and Ser-440 (RRDS) as well as two 14-3-3 protein binding groups that contain the consensus motif of phosphorylation by PKA: Thr-114(RFLTWP) and Thr-577(RSQTPP) and that human p110γ contains one potential site of phosphorylation by PKA: Ser-257 (KKKS).

To assess whether p110γ/p101 could be a substrate of PKA, we performed an in vitro PKA kinase assay using recombinant heterodimeric $p110\gamma^{His}/p101^{GST}$ or recombinant $p110\gamma^{His}$ as potential substrates. Phosphorylation of recombinant $p110\gamma^{His}/p101^{GST}$ and $p110\gamma^{His}$ was monitored using 50 U PKA catalytic subunit and [γ-^{32}P]ATP and was analyzed by autoradiography. The results of this assay, presented in Fig. 7A, show that heterodimeric $p110\gamma^{His}/p101^{GST}$ and $p110\gamma^{His}$ were phosphorylated whether or not they were incubated with PKA. The level of phosphorylation of $p110\gamma^{His}$ was much higher than that of the heterodimer $p110\gamma^{His}/p101^{GST}$. To further investigate the role of PKA in the in vitro phosphorylation of PI3-Kγ, we pre-incubated the PKA catalytic subunit with H-89 prior to the kinase assay. H-89 did not modify either the phosphorylation status of $p110\gamma^{His}/p101^{GST}$ or that of $p110\gamma^{His}$. Taken altogether, these data indicate that the observed phosphorylation of $p110\gamma^{His}/p101^{GST}$ and of $p110\gamma^{His}$ is not the result of PKA enzymatic activity but is rather attributable to the autophosphorylation of the catalytic subunit p110γ that has been already reported [32,33]. To verify the involvement of an autophosphorylation event on p110γ, recombinant $p110\gamma^{His}$ was incubated with wortmannin, a potent PI3-K inhibitor that covalently links the catalytic domain of p110 and then assayed for phosphorylation as described. As shown in Fig. 7B, $p110\gamma^{His}$ was phosphorylated whether or not it was incubated with PKA and wortmannin decreased the level of phosphorylation of $p110\gamma^{His}$. This result indicates that, under our experimental conditions, the observed p110γ phosphorylation is indeed due to autophosphorylation.

4. Discussion

In a previous study, we showed that PGE_2, via EP_2 receptors, inhibits the PLD pathway induced by fMLP at the level of PI3-Kγ, an important upstream regulator of PLD activity. But the inhibitory signaling mechanism of the EP_2 pathway itself has not been characterized yet. EP_2 receptors are linked to a Gαs protein known to stimulate adenylyl cyclase activity and hence the formation of cAMP in cells [22,34]. The main intracellular effector of cAMP is PKA, a well-known serine/threonine kinase activated by the binding of cAMP to its regulatory subunit that results in the release of two active catalytic subunits. However, the activation of EP_2 receptors may also signal via pathways independent of cAMP and of PKA. For example, the activation of EP_2 receptors stimulates the PI3-K-Akt pathway in dendritic cells [35–37] and inhibits fMLP-stimulated chemotaxis in PMNs in a cAMP/PKA inde-

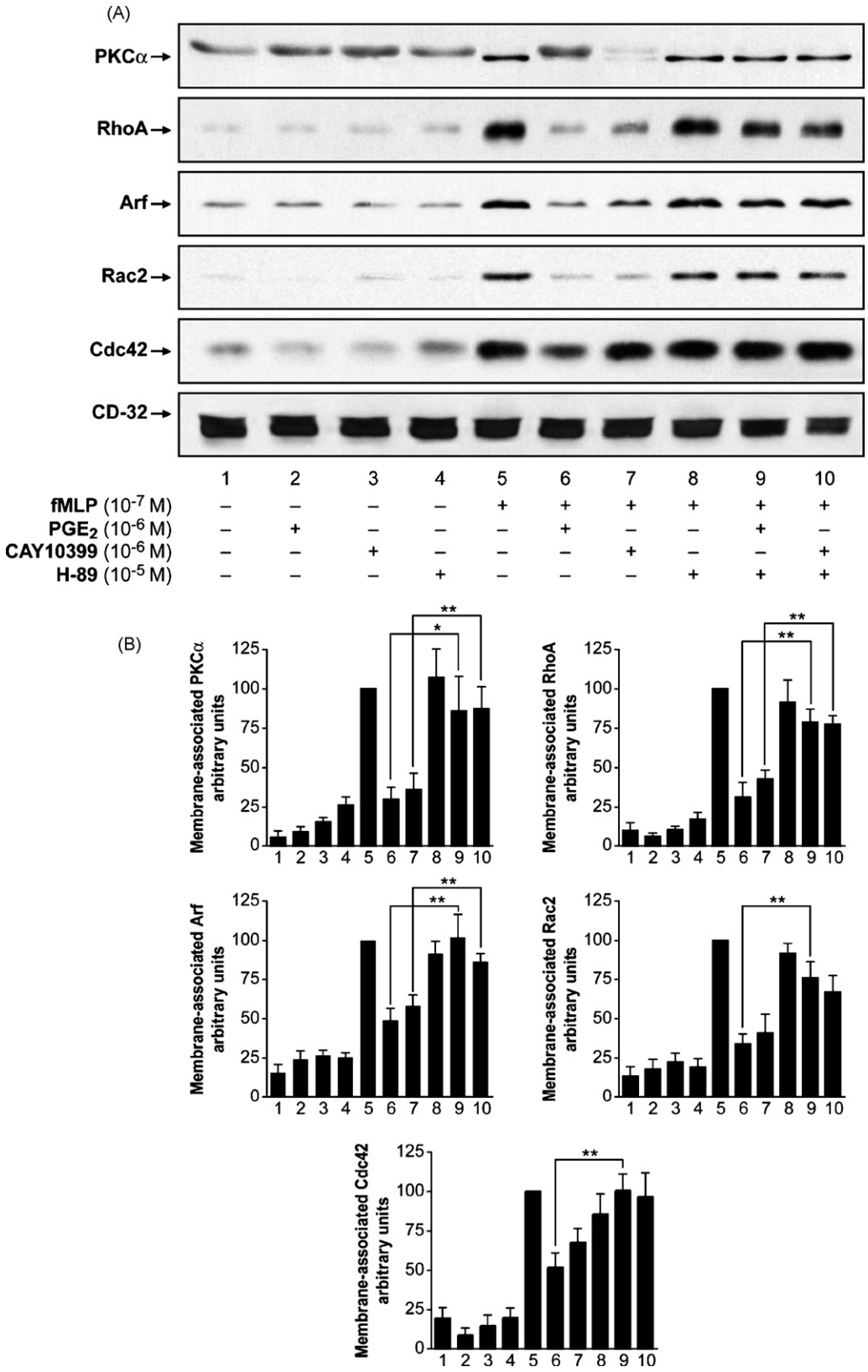

Fig. 4 – Effect of H-89 on the EP_2-mediated inhibition of PKCα, Arf and Rho-GTPases translocation stimulated by fMLP. (A) Neutrophils (10^7 cells/ml) were pre-incubated at 37 °C with 10^{-5} M H-89 for 5 min in the presence of 0.1 U/ml ADA and for additional 5 min with 10^{-6} M PGE_2 or CAY10399 and 10 μM CB. Cell suspensions were stimulated with 100 nM fMLP for 2 min. The incubations were stopped and the membrane fractions were prepared as described in Section 2. The samples were analyzed for PKCα, Arf1, RhoA, Rac2 and Cdc42 by immunoblotting. Each membrane was reprobed with an anti-CD32 Ab to assure equal protein loading in all samples. The immunoblots shown are representative of at least three independent

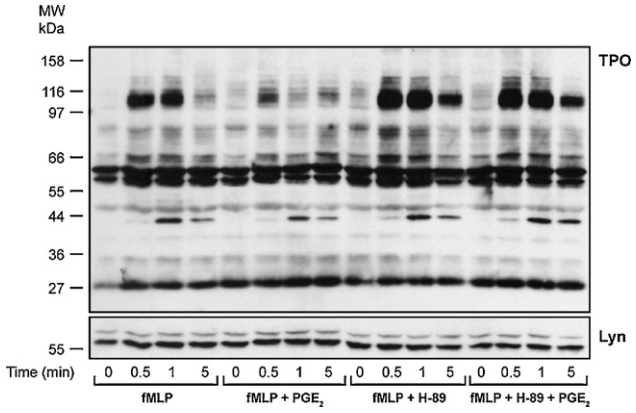

Fig. 5 – Effect of H-89 on the PGE_2-induced inhibition of tyrosine phosphorylation of proteins stimulated by fMLP. Neutrophils (2×10^7 ml^{-1}) were pre-incubated with 10^{-5} M H-89 for 10 min in the presence of 0.1 U/ml ADA and with 10^{-5} M PGE_2 at 37 °C for additional 10 min. The assay was performed as described in Section 2. The results shown are representative of at least three independent experiments Whole cell lysates were analyzed by immunoblotting with an anti-phosphotyrosine mAb as described in Section 2. As a control to assess equal loading, the PVDF membranes were reprobed with an anti-Lyn Ab.

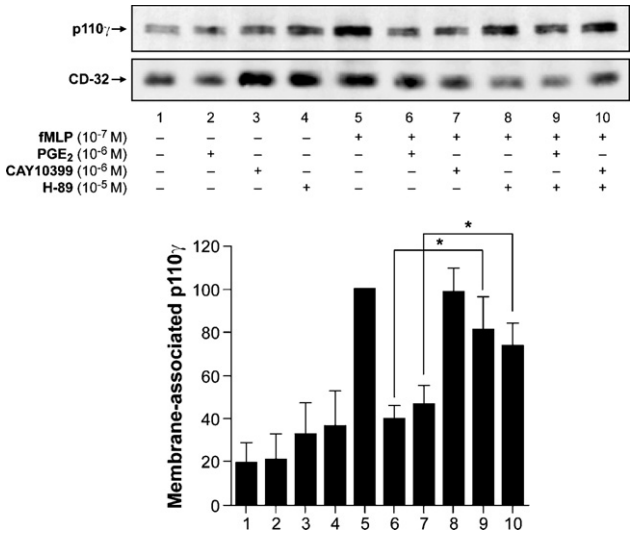

Fig. 6 – Effect of H-89 on the EP_2-mediated inhibition of p110γ translocation stimulated with fMLP. Neutrophils (10^7 cells/ml) were pre-incubated for 10 min with 10^{-5} M H-89 in the presence of 0.1 U/ml ADA and for additional 10 min with 10^{-5} M PGE_2 or CAY 10399 at 37 °C, 10 μM CB were added 5 min prior to stimulation with fMLP for 30 s. The reactions were stopped and the membrane fractions were prepared as described in Section 2. The samples were analyzed for p110γ by immunoblotting. Each membrane was reprobed with an anti-CD-32 Ab to assure equal protein loading in all samples. The immunoblot shown is representative of three independent experiments. Densitometric analyses are expressed as the percentage of fMLP-stimulated control and are the means ± S.D. from five independent experiments. The numbers identify the corresponding lanes of the immunoblots.

pendent manner [7]. These observations suggested that EP_2 receptors may stimulate multiple signaling mechanisms. Therefore, in the present study, we have investigated the role of PKA in the EP_2-mediated inhibition of the different components of the signaling cascade that leads to PLD activation in PMNs stimulated by fMLP.

We assessed first the effect of PGE_2 on the PKA phosphorylation pattern triggered by fMLP in PMNs. The data reported here show that PGE_2 increases the intensity of phosphorylation of PKA substrates, suggesting that PKA activity stimulated by fMLP is enhanced in the presence of PGE_2. We also noticed that PGE_2 alone was able to trigger a weak phosphorylation of PKA substrates. This latter observation is consistent with a study reported by Martin et al. that shows the capacity of PGE_2 to stimulate PKA activity in human PMNs [38].

The inhibitory effect of PGE_2 on fMLP-stimulated PLD activity was blocked by two distinct PKA selective inhibitors, H-89 and Rp-8-Br-cAMPS, indicating that the inhibition of PLD mediated via EP_2 receptors involves PKA activation. The fact that H-89 was more effective than Rp-8-Br-cAMPS in decreasing the inhibitory effect of PGE_2 on PLD activity could suggest that both type I and type II PKA are involved in this effect since Rp-8-Br-cAMPS preferentially inhibits type I PKA. Another possible explanation to the difference observed between the effects of both PKA inhibitors is that the cAMP analogs and H-89 inhibit PKA activity through different mechanisms. We verified that endogenous PLD1 in PMNs was not the direct target of PKA as reported by others in cells overexpressing this PLD isoform [27]. This result indicates that PKA regulation of PLD activity operates at an upper level than PLD itself. We next showed that the translocation of the PLD activation cofactors PKCα, RhoA and Arf1 was inhibited by PGE_2 in a PKA-dependent manner. Moreover, the fact that the translocation of all the Rho-GTPase family members (RhoA, Rac2 and Cdc42) as well as that of Arf1 and PKCα was inhibited by PGE_2 in a PKA-dependent manner suggested that PKA probably acts at a level upstream of the recruitment of these cofactors in the signaling cascade triggered by fMLP, possibly at the level of PI3-Kγ and/or at the level of tyrosine phosphorylation events since both PI3-K and tyrosine kinases are important regulators of small GTPases activation (see [12]). According to our results, the inhibition of fMLP-induced p110γ translocation by PGE_2 was, at least in part, dependent on PKA. The inhibition by PGE_2 of the tyrosine phosphorylation of the 116–120 kDa set of

experiments. (B) Densitometric analyses are expressed as the percentage of fMLP-stimulated control and are the means ± S.D. from five independent experiments ($n = 4$ for Rac2 and Cdc42). The numbers identify the corresponding lanes of the immunoblots shown in panel (A).

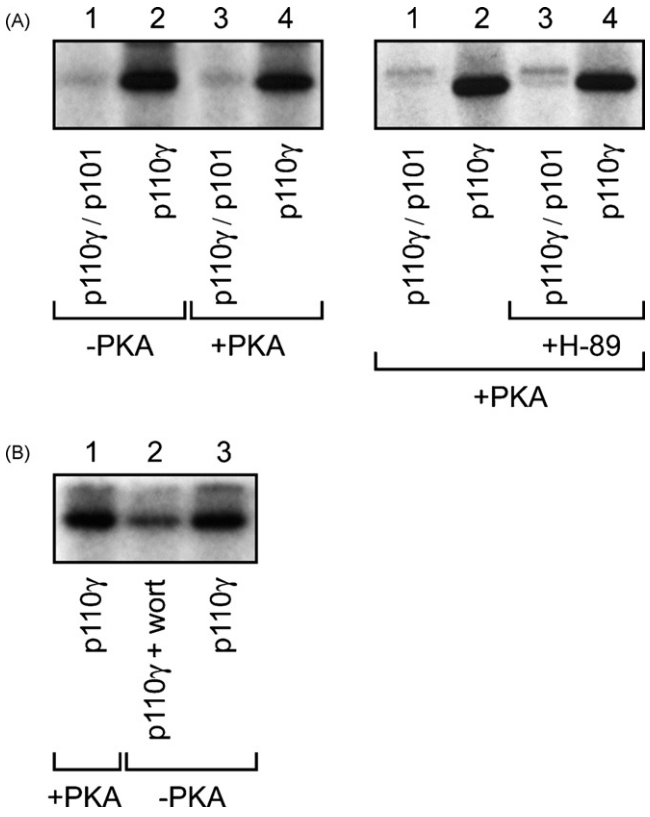

Fig. 7 – PKA phosphorylation assay. (A) Recombinant $p110\gamma^{His}/p101^{GST}$ (700 ng) and $p110\gamma^{His}$ (350 ng) were incubated or not with 50 U PKA catalytic subunit and a mix of 1 mM cold ATP and 2 μCi [γ-^{32}P]ATP at 30 °C for 30 min. When indicated, PKA catalytic subunit was pre-incubated with 1 μM H-89. The phosphorylation of proteins was analyzed by autoradiography. (B) Recombinant $p110\gamma^{His}$ was pre-incubated with 100 nM wortmannin 10 min prior to the onset of the phosphorylation reaction.

substrates stimulated by fMLP was also shown to depend on PKA. The fact that both the translocation of p110γ and the tyrosine phosphorylation of this particular set of substrates depend on PKA may suggest that the tyrosine-kinase(s) involved in this effect could be PI3-K-dependent and probably belong(s) to the Tec kinases family, one of the two tyrosine kinase families activated by fMLP [39]. Taken altogether, our results suggest that PGE_2 stimulates the activation of PKA via EP_2 receptors and that the inhibition of fMLP-stimulated PI3-Kγ activity by PKA regulates several downstream signaling events such as the translocation of Rho- and Arf-GTPase and PKCα and hence PLD activation. Finally, the fact that PKA has an impact on the mechanism of activation of PI3-Kγ induced by fMLP further confirms the model we have previously proposed to explain the inhibitory effect of PGE_2 on the PLD pathway [12].

We cannot exclude that PKA can also phosphorylate other substrates involved in the fMLP-induced PLD pathway. For example, RhoA has been shown to be phosphorylated on Ser-188 by PKA in cell-free systems [40] as well as in several cell types [27,41–46]. Among diverse effects, this RhoA phosphorylation inhibits cell motility [41], RhoA translocation promoted by integrins [47], adhesion of leukocytes induced by chemoattractants [42] as well as the interaction of RhoA with PLD1 [27,40]. It could therefore be possible that PKA activated by PGE_2 also regulates PLD activity via its direct inhibitory effect on RhoA. Another possible substrate for PKA is the Rac exchange factor P-Rex1, which is highly expressed in PMNs and plays a major role in Rac activation by fMLP [48]. The phosphorylation of P-Rex1 by PKA has been reported to inhibit the LPA-stimulated guanine nucleotide exchange activity of P-Rex1 on Rac in HEK293T cells [49]. However, in our experimental model, the direct inhibitory effect of PKA on RhoA and P-Rex1 would be additive to the effect mediated by PKA at the PI3-Kγ level which probably remains the major mechanism that explains the inhibition of small GTPases activation by PGE_2 since on one hand, Cdc42 is not a substrate for PKA [46] and PKA does not affect Arf1-mediated activation of PLD in cell-free system [40] and, on the other hand, Cdc42 and Arf1 activation is inhibited in a PKA-dependent fashion in the same way as RhoA. Taken together, our data suggest that PGE_2 inhibits fMLP-induced small GTPases translocation to PMN membranes through a common mechanism, possibly at the PI3-Kγ level.

The role of PKA in the regulation of PI3-Kγ activity has not yet been reported and the precise mechanism that underlies the PKA inhibitory effect at this level is still totally unknown. We report in this study that although PGE_2-induced PKA activity regulates the activation of PI3-Kγ triggered by fMLP, the regulatory p101 as well as the catalytic p110γ subunits of PI3-Kγ are not phosphorylated by PKA in vitro and hence, do not appear to be possible substrates of PKA in cells. PI3-Kγ activation is regulated by the binding of the heterodimer p101/p110γ to Gβγ subunits released upon GPCR activation by their ligands. The regulatory p101 subunit is thought to be involved in this binding and to play the role of an adaptor protein [50,51]. In fMLP-stimulated PMNs, Gβγ subunits bind to and directly activate other downstream effectors such as PLCβ2/3 or the Rac exchange factor P-Rex1. The fact that the Gβγ-mediated activation of these two effectors is inhibited as a result of their phosphorylation by PKA [49,52,53] suggests that Gβγ subunits are not likely targets of PKA. Moreover, PKA modulates the specificity of the coupling of several GPCRs to their Gβγ subunits through direct phosphorylation of the receptors, but the phosphorylation of the Gβγ subunits by PKA has never been described so far [54]. Moreover, Gβγ subunit protein sequences do not contain consensus motifs of phosphorylation by PKA. Nevertheless, the fMLP receptor itself is not phosphorylated by PKA [55]. Therefore, the targets of PKA in our model are not likely to be either Gβγ subunits since PKA usually inhibits the Gβγ-activated signaling pathway at the level of the effectors of Gβγ or the fMLP receptor. Intriguingly and contrary to PLCβ2/3, we observed that PKA activated by fMLP alone has no effect on PI3-Kγ translocation to membranes but that a pre-incubation step with PGE_2 is necessary to observe the inhibitory effect of PKA on PI3-Kγ. One possible explanation to this observation is that the recruitment of PI3-Kγ to membranes and its activation by Gβγ, a very early event in the fMLP signaling cascade, occurs before the elevation of cAMP and the subsequent activation of PKA triggered by fMLP. These temporal differences between PGE_2

and fMLP in the activation of PKA could explain that fMLP-activated PKA terminates the PLCβ2/3 signaling, whereas PGE_2-activated PKA prevents the mechanism of activation of PI3-Kγ triggered by fMLP.

Another possible explanation to the inhibitory effect of PKA activated by PGE_2 but not by fMLP on the PI3-Kγ activation mechanism could be a different compartmentalization of the cAMP/PKA signaling pathway induced by each agonist.

Taken altogether, these observations indicate that neither PI3-Kγ nor Gβγ subunits are PKA substrates and suggest that the inhibitory effect of PKA on PI3-Kγ activation is mediated through the phosphorylation of a yet unidentified protein that prevents the translocation of p101/p110γ to Gβγ subunits released upon fMLP stimulation by a mechanism that remains to be investigated. The fact that p101 contains 14-3-3 binding sites for which a function has not yet been ascribed to in the activation process of PI3-Kγ is intriguing. 14-3-3 proteins are small dimers that are able to bind a multitude of proteins, usually phosphorylated on ser/thr residues, to form signaling complexes that regulate many cellular processes [56]. Although the 14-3-3 binding site on p101 does not seem to be phosphorylated by PKA in our experimental model, 14-3-3 proteins could be involved in the inhibition of PI3-Kγ translocation mediated by PKA either by forming a molecular complex with the heterodimer p101/p110γ that would prevent its translocation to membranes or on the contrary, by preventing the formation of a complex that would allow the activation of PI3-Kγ. The hypothesis of the formation of an inhibitory molecular complex is strengthened by the fact that the effects of PKA in cells are often mediated by A-kinase anchoring proteins (AKAPs) which are scaffolding proteins that coordinate PKA signaling with other pathways by recruiting multiple enzymes and substrates. For example, some AKAPs such as AKAP-Lbc, a Rho-GEF, can be phosphorylated by PKA in a manner that promotes the binding of 14-3-3 proteins to AKAP signaling complex, thereby inhibiting the guanine nucleotide exchange activity of AKAP-Lbc [57,58]. This example shows that the PKA/AKAP and the 14-3-3 signaling pathways can intertwine and that both could possibly be involved in the PKA-mediated inhibitory mechanism on PI3-Kγ.

The inhibition of calcium influx by PGE_2 in fMLP-activated PMNs is also mediated by PKA since this inhibitory effect induced by EP_2 receptors was abolished in the presence of H-89. The metabolism of calcium and the formation of PtdIns(3,4,5)P_3 are totally independent signaling events in PMNs stimulated by fMLP [59], suggesting that PKA targets different substrates in both pathways. The calcium influx is thought to be mainly a capacitive calcium entry (SOCs) through plasma membrane non-selective cation channels [60,61]. Since PGE_2 does not affect the first rise of cytosolic calcium generated by the release of calcium from intracellular stores that brings about SOCs, the target of PKA could possibly be at the level of non-selective cation channels at the plasma membrane. Several types of ion channels have been shown to be PKA substrates and therefore the hypothesis of a direct inhibitory phosphorylation of the non-selective cation channels by PKA can be advanced. Other mechanisms such as the regulation of channel opening by the products of PI3-Kγ or PLD activities cannot be excluded.

In summary, the results reported in this study show that the inhibition of the fMLP-induced PLD activation pathway by PGE_2 via EP_2 receptors is mediated by the activation of PKA. Although some of the components of the PLD pathway can be PKA substrates, the main inhibitory effect of PKA that explains the inhibition of the whole PLD signaling cascade occurs more likely at the level of PI3-Kγ. The stimulation of PKA activity by PGE_2 via EP_2 receptors prevents fMLP-induced PI3-Kγ translocation to membranes but the p110γ/p101 heterodimer is not a substrate of PKA, at least in vitro. One of the various strategies that has been explored recently to treat inflammatory diseases consists of the selective inhibition by pharmacologic agents of the PI3-K isoforms involved in the migration of leukocytes, i.e. the γ and δ PI3-K isoforms [62–64]. Our study shows that the inhibition of PI3-Kγ by the cAMP/PKA pathway could be a valuable and alternative mean to achieve the inhibition of PI3-Kγ in PMNs.

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