

Inhibition of protein kinases A and C demonstrates dual modes of response in human eosinophils stimulated with platelet-activating factor

Takumi Takizawa, MD, PhD,^{a,*} Masahiko Kato, MD, PhD,^{a,*} Hirokazu Kimura, PhD,^b Masato Suzuki, MD,^a Atsushi Tachibana, MD,^a Hideru Obinata, MS,^c Takashi Izumi, MD, PhD,^c Kenichi Tokuyama, MD, PhD,^a and Akihiro Morikawa, MD, PhD^a Maebashi, Japan

Background: Platelet-activating factor (PAF) is a potent stimulator of human eosinophils involved in the pathogenesis of allergic diseases. However, intracellular signaling mechanisms in eosinophils involving the PAF receptor are incompletely understood.

Objective: We sought to determine the roles of protein kinase C (PKC) and cyclic AMP-dependent protein kinase (protein kinase A [PKA]) in signaling pathways of human eosinophils stimulated with PAF.

Methods: After pretreatment with a PKC inhibitor, bisindolylmaleimide I, or a PKA inhibitor, H89, we investigated PAF-evoked functions, such as CD11b expression, cellular adhesion, superoxide anion generation, and degranulation in human eosinophils.

Results: Preincubation of eosinophils with bisindolylmaleimide I resulted in enhancement of upregulated CD11b expression and adhesion induced by PAF. H89 pretreatment also enhanced PAF-induced cellular adhesion. Superoxide anion generation and degranulation were suppressed by means of inhibition of either PKC or PKA.

Conclusion: PKC and PKA negatively regulate PAF-induced CD11b upregulation and cellular adhesion but promote eosinophil effector functions, such as superoxide anion generation and degranulation. PKC and PKA modulate PAF-evoked intracellular signaling of the eosinophil function in distinct ways. (*J Allergy Clin Immunol* 2002;110:241-8.)

Key words: Eosinophils, protein kinase C, protein kinase A, CD11b, adhesion

Abbreviations used

Bis I:	Bisindolylmaleimide I (2-[1-(3-dimethylaminopropyl)-1 <i>H</i> -indol-3-yl]-3-[1 <i>H</i> -indol-3-yl]-maleimide)
cAMP:	Cyclic AMP
db-cAMP:	Debutyl-cAMP
EPX:	Eosinophil protein X
HSA:	Human serum albumin
MCLA:	Cypridina luciferin analogue (2-methyl-6-[<i>p</i> -methoxyphenyl]-3,7-dihydroimidazo-[1,2- <i>a</i>]pyrazin-3-one)
myr- ψ PKC:	Myristoylated PKC inhibitor peptide 20-28
O ₂ ⁻ :	Superoxide anion
PAF:	Platelet-activating factor
PIPES:	Piperazine-N, N'-bis(2-ethanesulfonic acid)
PKA:	Protein kinase A
PKC:	Protein kinase C
PTX:	Pertussis toxin

Eosinophils are involved in the pathogenesis of allergic diseases, such as pollinosis, atopic dermatitis, and bronchial asthma,¹ although a recent study of a humanized anti-IL-5 antibody in asthmatic patients has suggested that eosinophils might not be a prerequisite for the late asthmatic response and airway hyperresponsiveness.² In allergic inflammation, mediators released from epithelial cells or inflammatory cells induce migration of eosinophils from the blood into affected tissues. Once they have migrated, eosinophils are activated by appropriate stimuli, resulting in the release of inflammatory mediators, including arachidonic acid metabolites,³ superoxide anion (O₂⁻),⁴ and toxic cationic granule proteins.⁵ In the process of eosinophil activation, cellular adhesion has been shown to be one of the most important steps.^{6,7} For instance, we previously have demonstrated that treatment with an antibody against β 2 integrin can almost completely inhibit not only eosinophil adhesion but also degranulation induced by a lipid mediator

From ^athe Department of Pediatrics, Gunma University School of Medicine;

^bGunma Prefectural Institute of Public Health and Environmental Sciences; and ^cthe Department of Biochemistry, Gunma University School of Medicine, Maebashi.

*Drs Takizawa and Kato contributed equally to this work.

Received for publication November 28, 2001; revised April 29, 2002; accepted for publication April 30, 2002.

Reprint requests: Masahiko Kato, MD, PhD, Department of Pediatrics, Gunma University School of Medicine, 3-39-22 Showa-machi Maebashi, Gunma 371-8511, Japan.

© 2002 Mosby, Inc. All rights reserved.

0091-6749/2002 \$35.00 + 0 1/83/126303

doi:10.1067/mai.2002.126303

(platelet-activating factor [PAF]), a cytokine (IL-5), or an immunoglobulin (IgG).⁶⁻⁸ These findings suggest that cellular adhesion through $\beta 2$ integrins, especially $\alpha MB2$ (CD11b/CD18), which are induced by the above stimuli, is critical for the effector functions of eosinophils.⁸

PAF is a potent lipid mediator in allergic inflammation, participating in biologic activities, including airway constriction, microvascular leakage, and edema.^{9,10} PAF is known to bind to a G protein-coupled cell-surface receptor with 7-transmembrane portions.¹¹ PAF receptor deficiency in mice results in the absence of any acute airway allergic reaction to ovalbumin,¹² indicating that PAF plays a dominant role in acute allergic reactions. In eosinophils PAF has been shown to induce a variety of biologic functions, such as chemotaxis,¹³ CD11b upregulation,¹⁴ cellular adhesion,⁶ degranulation with release of toxic granule proteins,¹⁵ generation of O_2^- ,^{16,17} and release of arachidonic acid metabolites.¹⁸ Although PAF is a strong activator for eosinophils, intracellular signaling mechanisms in human eosinophils activated by PAF remain incompletely understood.

In guinea pig eosinophils protein kinase C (PKC) has been suggested to negatively regulate PAF-induced calcium influx and degranulation.¹⁹ In human eosinophils a previous study demonstrated that PKC negatively modulated PAF-induced arachidonic acid metabolite production²⁰ and augmented a sustained intracellular calcium increase and O_2^- generation induced by PAF.^{20,21} These results implicate PKC in the signaling pathway downstream from the PAF receptor in eosinophils. Furthermore, elevation of intracellular cyclic AMP (cAMP) concentrations by using phosphodiesterase inhibitors decreased PAF-induced CD11b expression on the eosinophil surface. Thus cAMP is also involved in the negative regulation of the signaling pathways evoked by PAF.¹⁴

In this study we used specific inhibitors to investigate the roles of PKC and cAMP-dependent protein kinase (protein kinase A [PKA]) in PAF-induced CD11b upregulation and cellular adhesion, which are critical steps for eosinophils to carry out their effector functions.⁶⁻⁸ We further examined the effect of changes in PKC and PKA on effector functions of human eosinophils, such as O_2^- generation and degranulation.

METHODS

Reagents

PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; PAF, C-16) obtained from Biomol Research Laboratories Inc (Plymouth Meeting, Pa) was dissolved in ethanol at 40 mmol/L and stored at -20°C . Bisindolylmaleimide I (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-[1H-indol-3-yl]-maleimide; Bis I), H89, staurosporine, pertussis toxin (PTX), herbimycin A, debutyl-cAMP (db-cAMP), and myristoylated PKC inhibitor peptide 20-28 (myr- ψ PKC) were from Calbiochem (La Jolla, Calif). Bis I and staurosporine were dissolved in dimethyl sulfoxide, and H89 was dissolved in 50% ethanol. Debutyl-cAMP and myr- ψ PKC were dissolved in doubly distilled water. All reagents were stored at -20°C until use. A chemiluminescence probe for superoxide radicals representing a *Cypridina* luciferin analogue (2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo-[1,2-a] pyrazin-3-one; MCLA) obtained from Tokyo Kasei

(Tokyo, Japan) was dissolved in doubly distilled water and stored at -80°C until use. The optimal concentration of the MCLA solution was determined as previously described.^{7,22} Phycoerythrin-conjugated anti-CD11b mouse mAb (ICRF44) and mouse IgG2a control immunoglobulin were purchased from Becton Dickinson (San Jose, Calif). Human serum albumin (HSA) was purchased from Sigma (St Louis, Mo; A3782), with the purity being certified by the manufacturer as containing no globulin.

Cell isolation

Eosinophil purification was performed by using a magnetic cell separation system (MACS; Becton Dickinson), as previously described, with minor modifications.²³ Briefly, granulocytes were isolated from heparinized peripheral blood obtained from normal healthy volunteers by using a Histopaque solution (density 1.083 g/mL; Sigma) and hypotonic lysis. The granulocytes were washed with piperazine-N, N'-bis(2-ethanesulfonic acid) (PIPES) buffer (25 mmol/L PIPES, 50 mmol/L NaCl, 5 mmol/L KCl, 25 mmol/L NaOH, and 5.4 mmol/L glucose at pH 7.4) with 1% FBS (JRH biosciences, Lenexa, Kan) and incubated with anti-CD16 mAb conjugated with magnetic particles (Miltenyi Biotec, Bergisch Gladbach, Germany). Then neutrophils were removed by using the MACS. Purified eosinophils were washed in PIPES buffer with 1% FBS and suspended in each reaction medium. The purity of eosinophils counted after Randolph staining was greater than 98%.

CD11b expression

Purified eosinophils were suspended in RPMI-1640 medium with 1% FBS at 10^6 cells/mL; preincubated with staurosporine, Bis I, H89, or db-cAMP at 37°C for 5, 15, 5, and 15 minutes, respectively; and then stimulated with $1\ \mu\text{mol/L}$ PAF for 15 minutes at 37°C . The reaction was stopped on ice. Then the cells were resuspended in $50\ \mu\text{L}$ of RPMI-1640 and incubated with an anti-CD11b mAb or isotype-matched immunoglobulin (mouse IgG2a) for 30 minutes at 4°C . The cells were resuspended in PBS, and expression of CD11b was determined by using a flow cytometer (Epics XL; Beckman Coulter, Tokyo, Japan) and expressed as mean fluorescence intensity. Viability of cells was simultaneously examined by staining for propidium iodide ($1\ \mu\text{g/mL}$).

Adhesion assay

Eosinophil adhesion was determined by measuring the eosinophil protein X (EPX) contents of adherent cells by means of RIA, as previously reported.⁸ Briefly, purified eosinophils (5×10^4) plated on HSA-coated plates were preincubated with Bis I or H89 for 30 minutes at 37°C and stimulated with PAF ($1\ \mu\text{mol/L}$) for 1 hour. Then the supernatants were collected, and the plates were gently rinsed with warm RPMI-1640 to remove nonadherent cells. Adherent cells were lysed, and the EPX content in the lysates and the supernatants was measured by means of RIA (Pharmacia-Upjohn, Tokyo, Japan). The percentage of adhesion was calculated as a ratio of EPX content in adherent eosinophils to total available EPX, according to the following equation:

$$\% \text{ Adhesion} = \frac{\text{EPX in lysates of adherent cells after incubation}}{\text{Total EPX in lysates of cells before incubation} - \text{EPX released into supernatant during incubation}} \times 100.$$

All assays were conducted in duplicate.

O_2^- generation

Purified eosinophils (2.5×10^5 cells) were washed with HBSS and suspended in the assay mixture containing $3\ \mu\text{mol/L}$ MCLA and HBSS at a total volume of 2.0 mL. The cells were preincubated with

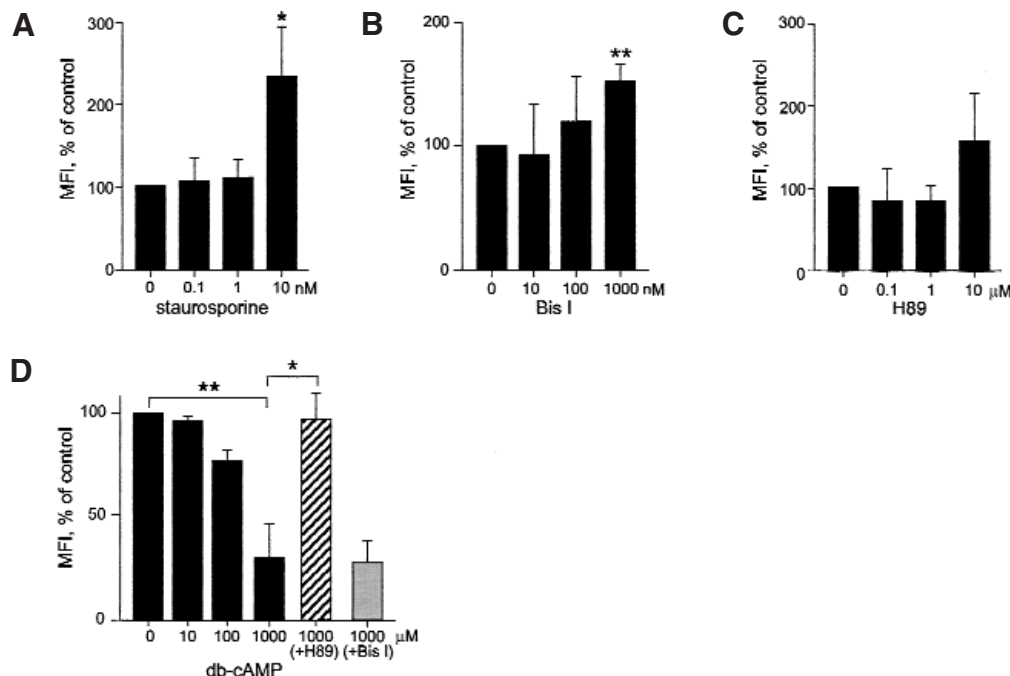


FIG 1. Effects of protein kinase inhibitors and a cAMP analogue on PAF-induced CD11b expression in human eosinophils. Purified eosinophils (2.5×10^5 cells/sample) were stimulated with or without $1 \mu\text{mol/L}$ PAF after pretreatment with staurosporine (A), Bis I (B), H89 (C), or db-cAMP (D; solid bar, db-cAMP alone; hatched bar, db-cAMP and H89; and shaded bar, db-cAMP and Bis I), or medium alone. CD11b expression was determined by means of flow cytometric analysis and described as mean fluorescence intensity (MFI). Mean fluorescence intensity of control cells with or without PAF stimulation was 28.3 ± 1.63 and 10.9 ± 0.78 , respectively (mean $\pm$ SEM, $n = 7$). * $P < .05$, ** $P < .01$.

Bis I, myr- ψ PKC, or H89 at 37°C for 15 minutes. The assay mixtures ($250 \mu\text{L}$) were moved to the polyethylene test tubes (Aloka, Tokyo, Japan) coated with 2.5% HSA dissolved in PBS and stimulated with PAF ($1 \mu\text{mol/L}$) at 37°C for 15 minutes. O_2^- generation was measured by means of MCLA-dependent chemiluminescence, as described previously,^{22,24} by using a luminescence reader (BLR-102; Aloka, Tokyo, Japan). The amount of O_2^- generation (counts) was calculated as the area under the chemiluminescence curve for 30 minutes. Amounts of O_2^- generation obtained for MCLA-dependent chemiluminescence were corrected by subtracting control readings. Luminescence was completely inhibited by $0.5 \mu\text{mol/L}$ superoxide dismutase (data not shown).

Eosinophil degranulation

Eosinophil degranulation was determined by measuring the contents of EPX in culture supernatants. Purified eosinophils (5×10^4) in RPMI-1640 medium were plated to tissue-culture plates coated with 2.5% HSA and pretreated with Bis I or H89 for 30 minutes at 37°C . Then the cells were stimulated with PAF for 4 hours. After stimulation, the concentrations of EPX in the supernatants were measured by means of RIA, as previously reported.⁸ Total cellular EPX contents were measured in parallel samples from cells lysed with 0.5% NP-40 detergent. All assays were conducted in duplicate.

Statistical analysis

All data were normalized to the increased values in response to PAF without the drugs as 100% and presented as means $\pm$ SEM of 3 to 7 experiments. The statistical significance of the differences from the increased values obtained without preincubation of the drugs in response to PAF was assessed by using paired or unpaired Student t tests.

RESULTS

Effects of protein kinase inhibitors on PAF-induced CD11b upregulation

Human eosinophils from peripheral blood constitutively express $\alpha\text{M}\beta 2$ integrin (CD11b/CD18) on their surfaces, which has been shown to be important for the cells to fully perform their effector functions⁶⁻⁸ and to be enhanced by stimulation with PAF.¹⁴ We examined whether PAF-induced upregulation of CD11b expression on eosinophils was modulated by the activity of PKC or PKA that had been manipulated by exposure to appropriate inhibitors.

As shown in Fig 1, A, pretreatment with staurosporine, a potent PKC inhibitor that also inhibits other protein kinases, including PKA, was followed by PAF stimulation. CD11b expression was enhanced more than by PAF stimulation alone. We next examined whether this additional enhancement was dependent on inhibition of PKC or PKA by using specific protein kinase inhibitors. Preincubation of eosinophils with a PKC inhibitor, Bis I, also increased PAF-induced expression of CD11b in a dose-dependent manner (Fig 1, B). Pretreatment with a specific PKA inhibitor, H89, showed some degree of upregulation of PAF-induced CD11b expression, but the upregulation was not significant, even at a high dose ($10 \mu\text{mol/L}$; Fig 1, C). The effect of cAMP analogues on PAF-stimulated CD11b expression was then investigated. Preincubation with db-cAMP led to a significant decrease

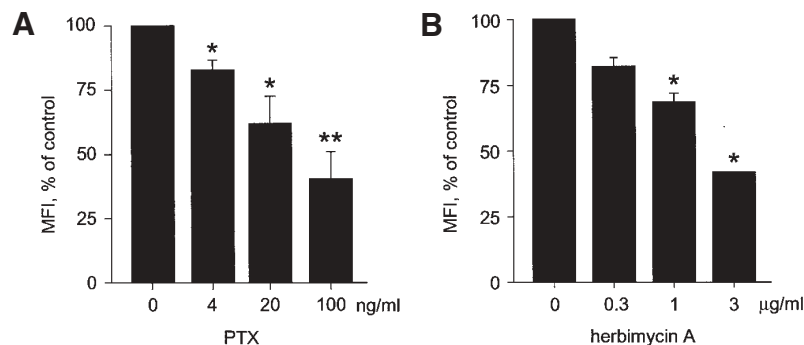


FIG 2. Effects of PTX and herbimycin A on PAF-induced CD11b expression. Eosinophils were stimulated with PAF (1 μ mol/L) after preincubation with PTX (**A**), herbimycin A (**B**), or medium alone. CD11b expression was determined as described in Fig 1. *MFI*, Mean fluorescence intensity. * $P < .05$, ** $P < .01$.

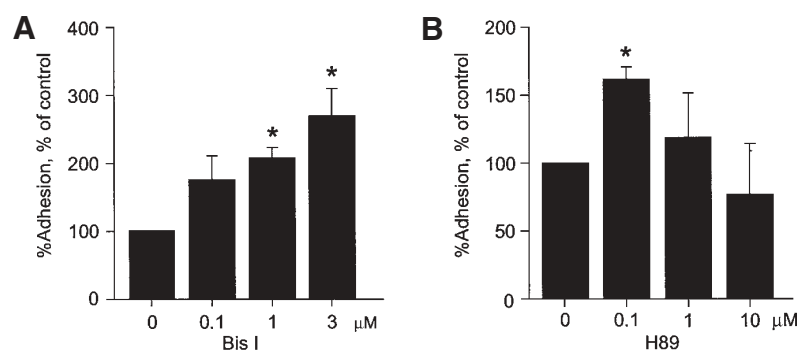


FIG 3. Role of PKC or PKA in PAF-induced cellular adhesion of human eosinophils. Eosinophils (5×10^4 /well) were incubated with or without 1 μ mol/L PAF after preincubation with Bis I (**A**), H89 (**B**), or medium alone. Percentage adhesion of control cells with or without PAF stimulation is $12.4 \pm 2.40\%$ and $6.58 \pm 1.23\%$, respectively (mean $\pm$ SEM, $n = 3$). * $P < .05$.

in CD11b expression on the cellular surface (Fig 1, *D*). Treatment with another cAMP analogue, 8-bromo-cAMP (1 mmol/L), also resulted in a decrease ($71.4 \pm 3.2\%$, $n = 2$). Furthermore, the suppression was completely recovered by pretreatment with H89. These results indicate that H89 potently inhibits PKA activity in eosinophils and that forced PKA activation by cAMP analogues suppresses CD11b expression induced by PAF. Although Bis I at high concentrations has been reported to inhibit PKA as well,²⁵ Bis I was not able to reverse the suppression by db-cAMP, even at the highest concentration studied (Fig 1, *D*). These results indicate that PKC, rather than PKA, inhibition enhanced PAF-induced CD11b expression. The cells were stained with propidium iodide to investigate the viability of eosinophils in the presence of inhibitors. No increase of apoptotic cells resulting from exposure to the drugs was observed.

Effects of Gi protein and protein tyrosine kinase inhibition on PAF-induced CD11b upregulation

Pretreatment with PTX significantly reduced the intensity of the staining with the anti-CD11b antibody (Fig 2, *A*). This suggests that a PTX-sensitive G protein

(Gi) is coupled to the PAF receptor, but other types of G proteins, such as Gq, also are likely to be involved in PAF-evoked signaling in human eosinophils. A tyrosine kinase inhibitor, herbimycin A, also caused partial suppression of PAF-induced CD11b expression (Fig 2, *B*). These results suggest that unlike PKA and PKC, activation of Gi protein or tyrosine kinases enhances PAF-induced CD11b expression in human eosinophils.

Effects of protein kinase inhibitors on cellular adhesion

Previously, we have reported that PAF-induced adhesion of eosinophils is largely dependent on α M β 2 integrin (CD11b/CD18).⁸ Because PKC inhibition upregulates PAF-induced expression of CD11b on eosinophils (Fig 1, *B*), it might result in enhancement of cellular adhesion. We therefore tested whether PKC inhibition increased the ability of PAF-stimulated eosinophils to adhere to plates coated with HSA, which is an in vitro ligand for CD11b/CD18.⁶ Indeed, pretreatment with a PKC inhibitor, Bis I, increased the number of PAF-stimulated eosinophils adhering to the plates (Fig 3, *A*).

Next, the effects of PKA inhibition on PAF-induced adhesion of human eosinophils were examined (Fig 3, *B*). The results were surprising considering the limited

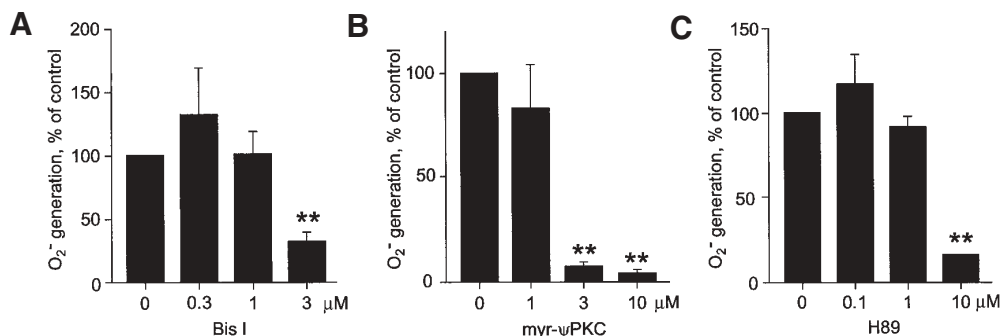


FIG 4. Reduction of O₂⁻ generation from PAF-stimulated human eosinophils by means of inhibition of PKC or PKA. Purified eosinophils (2.5×10^5 cells/sample) were incubated with or without 1 μmol/L PAF after preincubation with Bis I (**A**), myr-ψPKC (**B**), H89 (**C**), or medium alone. The O₂⁻ generation in control cells stimulated by PAF is $367.4 \pm 129.8 \times 10^4$ counts (mean $\pm$ SEM, $n = 6$). ** $P < .01$.

effect of inhibition on PAF-induced CD11b expression. Incubation with a low concentration (0.1 μmol/L) of a PKA inhibitor, H89, at which no significant enhancement of CD11b expression was observed, nonetheless enhanced adhesion induced by PAF. Thus negative modulation of PAF-induced eosinophil adhesion by PKA probably occurs at least in part independently of down-regulation of CD11b expression on the cell surface.

Effects of protein kinase inhibitors on PAF-induced O₂⁻ generation

O₂⁻ generation is one of the important effector functions of eosinophils. In allergic diseases O₂⁻ generated by activated eosinophils injures neighboring tissues and contributes to the development of eosinophilic inflammation.⁴ Several agents, such as phorbol ester,²⁰ opsonized particles,²⁶⁻²⁸ or N-formyl-methionyl-leucyl-phenylalanine,²⁹ have been demonstrated to potently induce generation of O₂⁻ by eosinophils. PAF, which is involved in allergic inflammation, has also been reported to trigger generation of O₂⁻^{16,17} but not as potently as the above-mentioned stimuli. We have previously demonstrated that PAF-induced O₂⁻ generation is abolished by inhibition of adhesion through CD11b/CD18. Because inhibition of PKC or PKA resulted in upregulation of CD11b expression or cellular adhesion, respectively, we investigated whether inhibition affects O₂⁻ generation by eosinophils. To detect O₂⁻ generation, we used a highly sensitive MCLA-dependent chemiluminescence system.^{22,24}

As shown in Fig 4, PAF stimulation after pretreatment with a 3 μmol/L concentration of a PKC inhibitor, Bis I, showed significant inhibition of PAF-induced O₂⁻ generation in eosinophils. Although Bis I at lower concentrations modestly enhanced this O₂⁻ generation, the enhancement was not significant. Additionally, when the cells were pretreated with another type of PKC inhibitor, myr-ψPKC, a pseudosubstrate for PKCα and PKCβ,³⁰ PAF-induced O₂⁻ generation was significantly inhibited (Fig 4, B). H89, a PKA inhibitor, displayed a similar dose-response curve to that of Bis I; it significantly

inhibited PAF-induced O₂⁻ generation at 10 μmol/L, while insignificantly enhancing O₂⁻ generation at lower concentrations that had been sufficient to significantly enhance PAF-induced adhesion (Fig 4, C). This minor enhancement of PAF-induced O₂⁻ generation by relatively low concentrations of Bis I or H89 might reflect enhanced adhesion.

Effects of protein kinase inhibitors on degranulation

Degranulation with release of toxic granule proteins is thought to be one of the most effective eosinophil functions as protection against parasites.¹ In allergic diseases, on the other hand, these cationic proteins are thought to exacerbate inflammation.⁵ PAF is a potent trigger for release of these proteins by eosinophils. We therefore determined whether PKC or PKA inhibition could alter PAF-induced EPX release in human eosinophils. EPX is a stable protein; determination of amounts of EPX released by eosinophils is an accurate way to assess degranulation activity of cells.

Fig 5 demonstrates that inhibition of PKC by Bis I at 3 μmol/L also led to reduction of PAF-induced EPX release from human eosinophils. The dose-response curve was similar to that for O₂⁻ generation, suggesting that PKC modulates degranulation by similar mechanisms. PKA inhibition also resulted in suppression of EPX release (ie, degranulation) induced by PAF (Fig 5, B). Furthermore, enhancement of PAF-induced degranulation at lower concentrations of Bis I and H89 might reflect enhancement of adhesion.

DISCUSSION

We investigated the effects of PKC or PKA inhibition on PAF-induced functions of human eosinophils, finding that PKC inhibition caused by Bis I or PKA inhibition caused by H89 affected eosinophil responses to PAF. PAF-induced CD11b upregulation and cellular adhesion was augmented by Bis I treatment, whereas O₂⁻ genera-

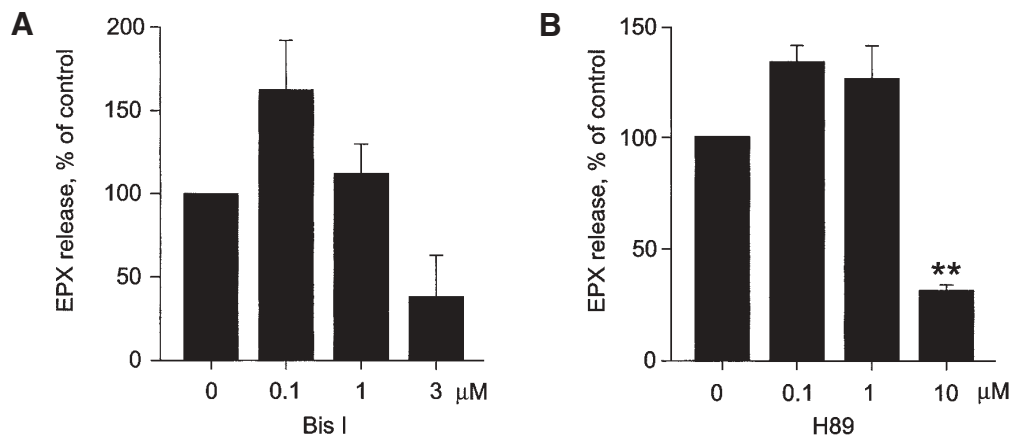


FIG 5. Suppression of PAF-induced degranulation from eosinophils by inhibiting PKA. Eosinophils (5×10^4 cells/well) were incubated for 4 hours with or without PAF ($1 \mu\text{mol/L}$) after preincubation with Bis I (**A**), H89 (**B**), or medium alone. Concentrations of EPX in the supernatants were determined. The amounts of EPX release in control cells with or without PAF stimulation and total cells are 335.5 ± 251.2 , 89.9 ± 59.9 , and 1798.5 ± 512.7 ng/ 10^6 cells, respectively (mean $\pm$ SEM, $n = 3$). ** $P < .01$.

tion was suppressed. PKA inhibition enhanced cellular adhesion induced by PAF but inhibited O_2^- generation and degranulation.

PKC is activated by G protein-coupled receptors, such as the PAF receptor,^{31,32} and is involved in a variety of cellular functions.^{33,34} In eosinophils PKC has been reported to negatively modulate PAF-evoked functions.^{19,20} For example, eicosanoid production and concentration of intracellular free calcium were increased by PKC inhibition in PAF-activated eosinophils, suggesting that PKC could suppress early events after PAF stimulation.^{20,21} In this study we showed that PKC negatively influences PAF-induced CD11b expression on the eosinophil surface, as well as adhesion by these cells. In contrast, PKC inhibition led to suppression of O_2^- generation and degranulation from eosinophils, indicating that PKC positively regulates these functions. These data imply that PKC differently modulates at least 2 distinct pathways in PAF-stimulated human eosinophils.

One explanation for such diverse effects of PKC inhibition is that PKC is likely to negatively modulate signaling directly from the PAF receptor, while it positively modulates signaling from integrins after adhesion. PAF and other stimulators of eosinophils, such as GM-CSF and IgG,^{6,7,35} which work through different receptors and different accessory signaling molecules, provide eosinophils with CD11b upregulation on the surface and an increasing avidity of integrins for their ligands. This process is called "inside-out signaling."³⁶ Once adhesion has occurred, clustered and bound integrins trigger intracellular signaling events, including activation of tyrosine kinases and phospholipase C, calcium mobilization, and activation of PKC,^{37,38} promoting effector functions of eosinophils (called "outside-in signaling"³⁸). In this study CD11b expression was dependent on inside-out signaling as opposed to outside-in signaling. PKC negatively modulates direct signaling from the PAF receptor

to suppress CD11b expression. Because PAF-evoked calcium mobilization has previously been reported to be increased by PKC inhibition,²¹ the effects of calcium depletion were investigated. Calcium depletion by BAPTA-AM ($1 \mu\text{mol/L}$) resulted in suppression of PAF-induced CD11b expression ($53.9\% \pm 8.6\%$, $n = 2$), suggesting calcium mobilization is also an important event for PAF-stimulated inside-out signaling and is likely to be involved in the process of CD11b upregulation induced by PKC inhibition. On the other hand, once eosinophils are primed by PAF and are adherent to their ligands, they are activated mainly through integrins by means of outside-in signaling. In human eosinophils we have previously demonstrated that clustering and binding of CD11b/CD18 triggers activation of tyrosine kinases and subsequent degranulation,³⁵ indicating that tyrosine kinases are involved in outside-in signaling in a positive regulatory manner. In the present study PKC also apparently acted as a positive regulator in outside-in signaling, including activation of O_2^- generation and degranulation in response to PAF. Enhancement of cellular adhesion of eosinophils has been considered to result in enhancement of effector functions. For instance, inhibition of tyrosine kinases suppressed not only PAF-induced cellular adhesion but also O_2^- generation and degranulation (Kato, unpublished data). In contrast to tyrosine kinases, with the PKC inhibitor studied here, the effects on CD11b expression and adhesion induced by PAF did not correlate with those on O_2^- generation and degranulation.

Another explanation for the differential responses to PKC inhibition involves differences between PKC isoforms implicated in PAF-evoked functions. Differences in specificity for substrates between PKC isoforms would contribute to differences in response to PKC inhibitors between eosinophil functions. An increasing number of PKC isoforms have been identified³⁴; in human eosinophils at least 8 isoforms have been report-

ed to be expressed (PKC α , β I, β II, ζ , δ , ϵ , ι , and μ).³⁹ Bis I has been shown to suppress conventional PKCs (PKC α , β I, β II, and γ), as well as novel PKCs (PKC δ , ϵ , η , and θ).²⁹ To ascertain which isoforms are involved in human eosinophil functions, we used the isoform-specific PKC inhibitors rotterlin (an inhibitor of PKC δ) and myr- ψ PKC (an inhibitor of PKC α and β). Pretreatment with rotterlin did not provide any significant change in PAF-induced CD11b upregulation, in contrast to Bis I. Furthermore, pretreatment with myr- ψ PKC led to inhibition of O₂⁻ generation but not CD11b expression (Fig 4, B, and data not shown). These data imply that the PAF-induced eosinophil functions are mediated through distinct PKC isoforms. Further study, however, is necessary with other specific inhibitors.

Intracellular cAMP elevation has been shown to inhibit PAF-induced CD11b upregulation in human eosinophils.¹⁴ Downregulation of CD11b induced by cAMP elevation was completely inhibited by pretreatment with H89 (Fig 1, D), suggesting that forced activation of PKA negatively regulates the PAF-evoked intracellular signaling pathway. In previous studies it has been reported that inhibition of PKA led to enhancement of PAF-induced eosinophil aggregation.⁴⁰ Our results showed that H89 treatment enhanced PAF-induced adhesion, indicating that PKA negatively modulates PAF-induced eosinophil adhesion in addition to PKC. Cellular adhesion through integrins includes several steps, such as mobility of integrin molecules, increase of affinity by conformational changes, clustering, and activation downstream of the integrins, with the latter steps being thought to be more important for cellular adhesion than upregulation of CD11b expression.^{38,41} H89 treatment enhanced adhesion of eosinophils to HSA-coated dishes at a concentration that did not enhance CD11b upregulation. This difference in effect of PKA inhibition between CD11b upregulation and adhesion indicates that although PKA does not affect CD11b upregulation, it negatively regulates PAF-induced adhesion by opposing the other steps necessary for cellular adhesion. As for the effector functions, such as O₂⁻ generation and degranulation, PKA inhibition, as well as PKC inhibition, resulted in suppression, indicating that PKA positively regulates signaling from activated integrins.

In summary, our study demonstrates for the first time that PAF-induced CD11b upregulation is augmented by PKC inhibition in human eosinophils. Thus PKC is involved in negative regulation of intracellular signaling from PAF receptors; as a result, PKC inhibition enhances PAF-induced eosinophil adhesion. PKA activation indeed inhibits PAF-induced CD11b expression, and PKA inhibition augments PAF-induced adhesion of eosinophils, presumably by favoring a change in steps other than CD11b expression. Effector functions of eosinophils, such as O₂⁻ generation and degranulation, involve signaling from integrins; either PKC or PKA inhibition results in suppression. These data imply dual modes of regulation by PKC and PKA of PAF-evoked signaling pathways in human eosinophils.

REFERENCES

- Butterfield JH, Leiferman KL, Gleich GJ. Eosinophil-associated diseases. In: Frank MM, Austen KF, Claman HN, Unanue ER, editors. *Samter's immunologic diseases*. Boston: Little, Brown and Co; 1995. p. 501-27.
- Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 2000;356:2144-8.
- Kita H, Adolphson C, Gleich GJ. Biology of eosinophils. In: Middleton E Jr, Reed CE, Ellis EF, Adkinson NF Jr, Yunginger JW, Busse WW, editors. *Allergy: principles and practice*. 5th ed. St Louis: Mosby-Year Book; 1998. p. 242-60.
- Sedgwick JB, Vrtis RF, Gourley MF, Busse WW. Stimulus-dependent differences in superoxide anion generation by normal human eosinophils and neutrophils. *J Allergy Clin Immunol* 1988;81:876-83.
- Abu-Ghazaleh RI, Fujisawa T, Mestecky J, Kyle RA, Gleich GJ. IgA-induced eosinophil degranulation. *J Immunol* 1989;142:2393-400.
- Horie S, Kita H. CD11b/CD18 (Mac-1) is required for degranulation of human eosinophils induced by human recombinant granulocyte-macrophage colony-stimulating factor and platelet-activating factor. *J Immunol* 1994;152:5457-67.
- Kaneko M, Horie S, Kato M, Gleich GJ, Kita H. A crucial role for β 2 integrin in the activation of eosinophils stimulated by IgG. *J Immunol* 1995;155:2631-41.
- Kato M, Abraham RT, Okada S, Kita H. Ligation of the β 2 integrin triggers activation and degranulation of human eosinophils. *Am J Respir Cell Mol Biol* 1998;18:675-86.
- Hanahan DJ. Platelet activating factor: a biologically active phosphoglyceride. *Annu Rev Biochem* 1986;55:483-509.
- Barnes PJ. Platelet activating factor and asthma. *Ann N Y Acad Sci* 1991;629:193-204.
- Izumi T, Shimizu T. Platelet-activating factor receptor: gene expression and signal transduction. *Biochim Biophys Acta* 1995;1259:317-33.
- Ishii S, Kuwaki T, Nagase T, Maki K, Tashiro F, Sunaga S, et al. Impaired anaphylactic responses with intact sensitivity to endotoxin in mice lacking a platelet-activating factor receptor. *J Exp Med* 1998;187:1779-88.
- Wardlaw AJ, Moqbel R, Cromwell O, Kay AB. Platelet-activating factor. A potent chemotactic and chemokinetic factor for human eosinophils. *J Clin Invest* 1986;78:1701-6.
- Momose T, Okubo Y, Horie S, Suzuki J, Isobe M, Sekiguchi M. Effects of intracellular cyclic AMP modulators on human eosinophil survival, degranulation and CD11b expression. *Int Arch Allergy Immunol* 1998;117:138-45.
- Kroegel C, Yukawa T, Dent G, Venge P, Chung KF, Barnes PJ. Stimulation of degranulation from human eosinophils by platelet-activating factor. *J Immunol* 1989;142:3518-26.
- Kroegel C, Yukawa T, Dent G, Chanez P, Chung KF, Barnes PJ. Platelet-activating factor induces eosinophil peroxidase release from purified human eosinophils. *Immunology* 1988;64:559-61.
- Zoratti EM, Sedgwick JB, Vrtis RR, Busse WW. The effect of platelet-activating factor on the generation of superoxide anion in human eosinophils and neutrophils. *J Allergy Clin Immunol* 1991;88:749-58.
- Bruijnzel PL, Kok PT, Hamelink ML, Kijne AM, Verhagen J. Platelet-activating factor induces leukotriene C4 synthesis by purified human eosinophils. *Prostaglandins* 1987;34:205-14.
- Kroegel C, Gienbycz MA, Matthys H, Westwick J, Barnes PJ. Modulatory role of protein kinase C on the signal transduction pathway utilized by platelet-activating factor in eosinophil activation. *Am J Respir Cell Mol Biol* 1994;11:593-9.
- Dent G, Munoz NM, Ruhlmann E, Zhu X, Leff AR, Magnussen H, et al. Protein kinase C inhibition enhances platelet-activating factor-induced eicosanoid production in human eosinophils. *Am J Respir Cell Mol Biol* 1998;18:136-44.
- Oshiro T, Kakuta Y, Shimura S, Nara M, Shirato K. Characterization of platelet-activating factor-induced cytosolic calcium mobilization in human eosinophils. *Clin Exp Allergy* 2000;30:699-705.
- Kato M, Tokuyama K, Morikawa A, Kuroume T, Barnes PJ. Platelet-activating factor-induced enhancement of superoxide anion generation in guinea-pigs. *Eur J Pharmacol* 1993;232:7-12.
- Ide M, Weiler D, Kita H, Gleich GJ. Ammonium chloride exposure inhibits cytokine-mediated eosinophil survival. *J Immunol Methods* 1994;168:187-96.

24. Nakano M. Determination of superoxide radical and singlet oxygen based on chemiluminescence of luciferin analogs. *Methods Enzymol* 1990;186:585-91.
25. Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, et al. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* 1991;266:15771-81.
26. Blom M, Tool AT, Roos D, Verhoeven AJ. Priming of human eosinophils by platelet-activating factor enhances the number of cells able to bind and respond to opsonized particles. *J Immunol* 1992;149:3672-7.
27. Tool AT, Koenderman L, Kok PT, Blom M, Roos D, Verhoeven AJ. Release of platelet-activating factor is important for the respiratory burst induced in human eosinophils by opsonized particles. *Blood* 1992;79:2729-32.
28. van der Bruggen T, Kok PT, Blom M, Verhoeven AJ, Raaijmakers JA, Lammers JW, et al. Transient exposure of human eosinophils to the protein kinase C inhibitors CGP39-360, CGP41-251, and CGP44-800 leads to priming of the respiratory burst induced by opsonized particles. *J Leukoc Biol* 1993;54:552-7.
29. Lynch OT, Giembycz MA, Barnes PJ, Hellewell PG, Lindsay MA. 'Outside-in' signalling mechanisms underlying CD11b/CD18-mediated NADPH oxidase activation in human adherent blood eosinophils. *Br J Pharmacol* 1999;128:1149-58.
30. Eichholtz T, de Bont DB, de Widt J, Liskamp RM, Ploegh HL. A myristoylated pseudosubstrate peptide, a novel protein kinase C inhibitor. *J Biol Chem* 1993;268:1982-6.
31. Milligan G. Signal sorting by G-protein-linked receptors. *Adv Pharmacol* 1995;32:1-29.
32. Chao W, Olson MS. Platelet-activating factor: receptors and signal transduction. *Biochem J* 1993;292:617-29.
33. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 1992;258:607-14.
34. Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* 1995;9:484-96.
35. Kato M, Abraham RT, Kita H. Tyrosine phosphorylation is required for eosinophil degranulation induced by immobilized immunoglobulins. *J Immunol* 1995;155:357-66.
36. Lub M, van Kooyk Y, Figdor CG. Ins and outs of LFA-1. *Immunol Today* 1995;16:479-83.
37. Schwartz MA, Schaller MD, Ginsberg MH. Integrins: emerging paradigms of signal transduction. *Annu Rev Cell Dev Biol* 1995;11:549-99.
38. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 1992;69:11-25.
39. Evans DJ, Lindsay MA, Webb BL, Kankaanranta H, Giembycz MA, O'Connor BJ, et al. Expression and activation of protein kinase C-zeta in eosinophils after allergen challenge. *Am J Physiol* 1999;277:L233-9.
40. Teixeira MM, Giembycz MA, Lindsay MA, Hellewell PG. Pertussis toxin shows distinct early signalling events in platelet-activating factor-, leukotriene B₄-, and C5a-induced eosinophil homotypic aggregation in vitro and recruitment in vivo. *Blood* 1997;89:4566-73.
41. Bazzoni G, Hemler ME. Are changes in integrin affinity and conformation overemphasized? *Trends Biochem Sci* 1998;23:30-4.

Receive tables of contents by e-mail

To receive the tables of contents by e-mail, sign up through our Web site at

<http://www.mosby.com/jaci>

Choose *E-mail Notification*

Simply type your e-mail address in the box and click the *Subscribe* button

Alternatively, you may send an e-mail message to majordomo@mosby.com.

Leave the subject line blank and type the following as the body of your message:

subscribe jaci_toc

You will receive an e-mail message confirming that you have been added to the mailing list.

Note that TOC e-mails will be sent out when a new issue is posted to the Web site.