An atypical protein kinase C, PKC ζ, regulates human eosinophil effector functions

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Summary

Protein kinase (PK) C comprises a family of isoenzymes that play key roles in downstream signalling and cell functions. We studied PKC ζ participation in the effector functions of human eosinophils stimulated with platelet-activating factor (PAF) or complement (C) 5a. After pretreating eosinophils with a myristoylated specific PKC ζ inhibitor; bisindlolylmaleimide I (*Bis*I), an inhibitor of conventional and novel PKCs; or rottlerin, a PKC δ inhibitor, we examined PAF- and C5a-evoked functions. Induced PKC translocation was characterized by confocal laser scanning microscopy. The PKC ζ inhibitor blocked PAF- or C5a-induced eosinophil superoxide anion generation as effectively as *Bis*I or rottlerin. The PKC ζ inhibitor also attenuated PAF- or C5a-induced eosinophil degranulation and adhesion. In contrast, the PKC ζ inhibitor did not affect PAF- or C5a-induced CD11b expression. Finally, both eosinophil shape changes and the translocation of PKC ζ and $p47^{phox}$, a component of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, to the plasma membrane induced by PAF or C5a were completely inhibited by the PKC inhibitor. Thus, the atypical PKC ζ regulates human eosinophil adhesion and effector functions.

Keywords: eosinophils; protein kinase C; superoxide anion; degranulation; adhesion; platelet-activating factor; complement 5a

Introduction

Eosinophils are involved in the pathogenesis of allergic diseases such as bronchial asthma, pollinosis and atopic dermatitis, and in the inflammatory response to parasitic infections.[1] When eosinophils are activated by appropriate stimuli including immunoglobulins, cytokines and lipid mediators, these cells release inflammatory mediators, toxic cationic granule proteins and oxygen radicals, all resulting in tissue damage at inflammatory sites.[2]

Platelet-activating factor (PAF) and complement 5a (C5a) are among the most potent activators of eosinophil effector functions.[2,3] These effectors act as agonists at leucocyte surface seven-transmembrane receptors that are coupled to G proteins.[4,5] We have shown that cellular adhesion-mediated β2 integrins, especially αMβ2 (CD11b/CD18, Mac-1), are critical to the effector functions of eosinophils occurring in response to these receptor–ligand interactions.[6–8] This receptor-mediated stimulation induces a variety of functions, including αMβ2 expression on the cell surface and conformational changes of integrin molecules via a process termed 'inside-out' signalling.[9] Once clustering or multimerization of αMβ2 occurs at the site of focal adhesion, this stimulation of αMβ2 triggers activation of protein tyrosine kinases, phospholipase (PL) C and phosphatidylinostol 3-kinase (PI3K); calcium mobilization; and activation of protein kinase (PK) C, resulting in promotion of eosinophil effector functions. This phase is called 'outside-in' signalling.[8,9] In both signalling processes, PKC comprises a family of isoenzymes that play key roles in downstream signalling events and cell functions.[10–12] Activation of PKCs in intact cells

Abbreviations: C5a, complement 5a; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; EPX, eosinophil protein X; HSA, human serum albumin; NADPH, nicotinamide adenine dinucleotide phosphate; $p47^{phox}$, p47 phagocyte oxidase; PAF, platelet-activating factor; PI3K, phosphatidylinostol 3-kinase; PKC, protein kinase C; PTX, pertussis toxin.

generally is associated with translocation of the enzyme from the cytosol to the cell membrane.[13–15]

PKCs have been classified into three groups based on molecular structure and mode of activation: (1) 'conventional' PKCs (α, βI, βII and γ), which require phosphatidylserine (PS) and are activated by calcium and diacylglycerol (DAG); (2) 'novel' PKCs (δ, ε, μ, θ and η), which require PS and DAG but not calcium for activation; and (3) 'atypical' PKCs (ζ and τ/λ), which are calcium-independent and are not activated by DAG.[10–12] Among the atypical PKCs, PKC ζ can be stimulated by phospholipids including phosphatidic acid (PA), phosphatidylinositol triphosphates and ceramides.

A recent report demonstrated expression of at least eight PKC isoforms in human eosinophils (PKC α, βI, βII, ζ, δ, ε, ι and μ), and indicated that PKC ζ expression was induced after antigen challenge.[16] Another study indicated that PKC α, βI, βII, γ, δ and ζ are expressed in human eosinophils, and that PKC δ makes an important contribution to agonist-induced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity.[17] However, the role of atypical PKC ζ in mediating eosinophil effector functions has not been documented to date. In the present study, we sought to determine how PKC ζ modulates the effector functions of human eosinophils stimulated with PAF or C5a.

Materials and methods

Reagents

Platelet-activating factor (PAF) C-16 was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Complement (C) 5a was purchased from Novabiochem (San Diego, CA). Globulin-free human serum albumin (HSA, A3782), cytochrome *c*, superoxide dismutase (SOD) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Myristoylated peptide PKC ζ and peptide η inhibitors, a myristoylated peptide 20-28 PKC inhibitor, or bisindolylmaleimide I (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide; *Bis*I), and rottlerin were purchased from Calbiochem (San Diego, CA) and dissolved at 100 mM in distilled water or DMSO, respectively. The solutions were diluted in reaction medium immediately before use. A chemiluminescence probe for superoxide radicals, the *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. The concentration of MCLA was based on ε430 nm = 9600 $M^{-1}cm^{-1}$, as previously described.[18,19] The solutions were diluted in reaction medium immediately before use. Alexa Fluor 532 phalloidin, Alexa Fluor 488 rabbit anti-goat immunoglobulin G (IgG) (H + l), and 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Molecular Probes (Eugene, OR). $p47^{phox}$-specific or PKC ζ-specific goat polyclonal antibody and goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All of these reagents were diluted in each reaction medium at optimal concentration immediately before the experiments. Phycoerythrin (PE)-conjugated anti-CD11b mouse monoclonal antibody (mAb; ICRF44) and mouse IgG2a control immunoglobulin were purchased from Becton Dickinson (San Jose, CA).

Cell isolation

Eosinophils were purified by a previously described method with minor modifications, using a magnetic cell separation (MACS; Becton Dickinson, San Jose, CA) system.[19,20] Briefly, heparinized blood was obtained from healthy donors and diluted with an equal volume of phosphate-buffered saline (PBS). Diluted blood was layered over Histopaque solution (density, 1·083 g/ml; Sigma) and centrifuged at 1000 ***g*** for 30 min. Then the supernatant was removed and erythrocytes in pellets were subjected to two cycles of hypotonic water lysis. Isolated granulocytes were washed with piperazine-N,N′-bis (2-ethanesulfonic acid) (PIPES; Sigma) buffer containing 1% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), and an equal volume of anti-CD16 antibody-conjugated magnetic particles (Miltenyi Biotec GmbH, Bergish Gladbach, Germany) was added to the cell pellet. After 45 min on ice, cells were resuspended and loaded onto a separation column positioned in a strong magnetic field for MACS. Cells were eluted three times. The purity of eosinophils determined by counting Randolph's stain was more than 98%. Cell viability always exceeded 98% as determined by trypan blue exclusion and propidium iodide staining.

Superoxide anion (O_2^-) generation

O_2^- release by eosinophils was measured by MCLA-dependent chemiluminescence using a luminescence reader (BLR-102, 301; Aloka, Tokyo, Japan) as described previously.[18,19] Polyethylene test tubes (Aloka) were coated with 250 μl of 2·5% human serum albumin (HSA) dissolved in PBS at pH 7·40 and kept overnight at 4°. The next day, the tubes were washed three times with PBS and then used immediately. Eosinophils were washed with Hanks' balanced salt solution (HBSS) and resuspended in the same medium at 10^6 cells/ml. Stimulants were diluted in the same medium at the desired concentration. Assay mixtures contained $2{\cdot}5 \times 10^5$ cells, the various PKC inhibitors, 3 μM MCLA, PAF or C5a, and HBSS in a total volume of 2·0 ml. Briefly, cell suspensions (250 μl) and the various PKC inhibitors were added to the HSA-coated tubes, incubated for 10 min at 37°, and then prewarmed at 37° for 5 min with MCLA. After incubation, the reaction was then started by adding PAF or C5a as a

stimulant. The amount of O_2^- release (counts) was calculated as the area under the chemiluminescence curve (AUC) and corrected by subtracting control readings.

Eosinophil degranulation

Eosinophil degranulation was assessed by a slight modification of a previously described method.[20–22] Briefly, in HSA-coated, flat-bottomed, 96-well, flat-bottomed tissue culture plates, freshly isolated human eosinophils were suspended at 5×10^4 cells/well in RPMI 1640 with the addition of 25 mM HEPES. After a PKC inhibitor was added to each well and the plate was incubated for 15 min at 37°, the reactions were initiated by adding a stimulant (PAF or C5a at a final concentration of 1 μM or 100 ng/ml, respectively). After incubation for 4 hr, the supernatants were collected and stored at −20° until radioimmunoassay (RIA) for eosinophil protein X (EPX; Pharmacia-Upjohn, Tokyo, Japan) content to quantify eosinophil degranulation.[20–22] Total cellular EPX content was measured simultaneously in supernatants from cells lysed with 0·5% Nonidet P-40 (NP-40) detergent. All experiments were performed in duplicate.

Eosinophil adhesion assay

Numbers of adherent eosinophils were determined by measuring the content of EPX in adherent cells by RIA, as previously reported.[21,22] Briefly, in the HSA-coated wells of 96-well, flat-bottomed tissue culture plates, freshly isolated human eosinophils were suspended at 5×10^4 cells/well in RPMI 1640 including 25 mM HEPES. Then a PKC inhibitor was added to each well, and the plate was incubated at 37° for 15 min. After incubation, reactions were initiated by adding the stimulant, PAF or C5a, at respective concentrations of 1 μM or 100 ng/ml. After 60 min the supernatants were collected, and the plate was rinsed gently with warm RPMI 1640 to remove non-adherent cells. Adherent cells were then lysed with 0·5% NP-40 detergent, and EPX content in the lysate was measured by RIA. All experiments were performed in duplicate. Per cent adhesion was calculated as the ratio of EPX content in adherent eosinophils to total available EPX after incubation according to the following equation:

$$\text{Percent adhesion} = [\text{EPX in lysates of adherent cells after incubation}/(\text{total EPX in lysates of cells before incubation} - \text{EPX release into supernatants during incubation})] \times 100.$$

Expression of CD11b

Purified eosinophils were suspended in RPMI 1640 with 1% FBS at 10^6 cells/ml, preincubated with each PKC inhibitor for 15 min, and then stimulated with 1 μM PAF or 100 ng/ml C5a for 15 min at 37°. The reaction was then stopped on ice, and cells were resuspended in 50 μl of RPMI 1640 for incubation with an anti-CD11b mAb or isotype-matched immunoglobulin, mouse IgG2a, for 30 min at 4°. Cells were resuspended in PBS, and expression of CD11b was determined using a flow cytometer (Epics XLII; Beckman Coulter, Tokyo, Japan) and reported as mean fluorescence intensity (MFI).[21,22] The viability of cells was determined simultaneously by staining with propidium iodide (1 μg/ml).

Visualization of PKCs

A suspension of $1–3 \times 10^5$ eosinophils in phenol red-free RPMI 1640 medium with 1 mM HEPES was incubated with or without 1 or 3 μM PKC ζ inhibitor for 15 min in 37° in an HSA-coated 35-mm glass-bottomed dish (Matsunami, Osaka, Japan). After incubation, PAF or C5a was added at a final concentration of 1 μM or 100 ng/ml, respectively. After 15 min at 37°, the mixture in each dish was gently aspirated, and 2 ml of 3·7% formaldehyde in PBS (v/v) was added carefully to each dish to fix attached cells for 5 min at room temperature. Then cells were washed gently with PBS and permeabilized with 2 ml of 0·1% Triton X-100 in PBS in each dish for 10 min. After permeabilization, cells were washed twice with PBS and blocked with BSA for 30 min at room temperature. Cells were then washed once, and incubated for 30 min at 4° with primary antibody ($p47^{phox}$-specific or PKC ζ-specific antibody, or goat IgG as a negative control; all dilutions 1 : 500). Then cells were washed three times and incubated for 30 min in darkness at room temperature with a mixture of Alexa Fluor 532 phalloidin (1 unit/dish) and secondary antibody (Alexa Fluor 488 rabbit anti-goat IgG; dilution 1 : 1000). Finally, after five washes with PBS, DAPI was mounted on the samples, and the fluorescent signal was observed with a confocal laser scanning microscope system (MRC-1024; Bio-Rad, Tokyo, Japan) as previously described with minor modifications.[22]

Statistical analysis

All data were normalized to the increased values in response to PAF or C5a without the drugs (considered 100%) and presented as standard errors of means (SEMs) of three to seven experiments. The statistical significance of the differences from the increased values obtained without preincubation of the drugs in response to PAF or C5a was assessed using paired or unpaired Student *t*-tests. Differences associated with a *P*-value of $< 0·05$ were considered significant.

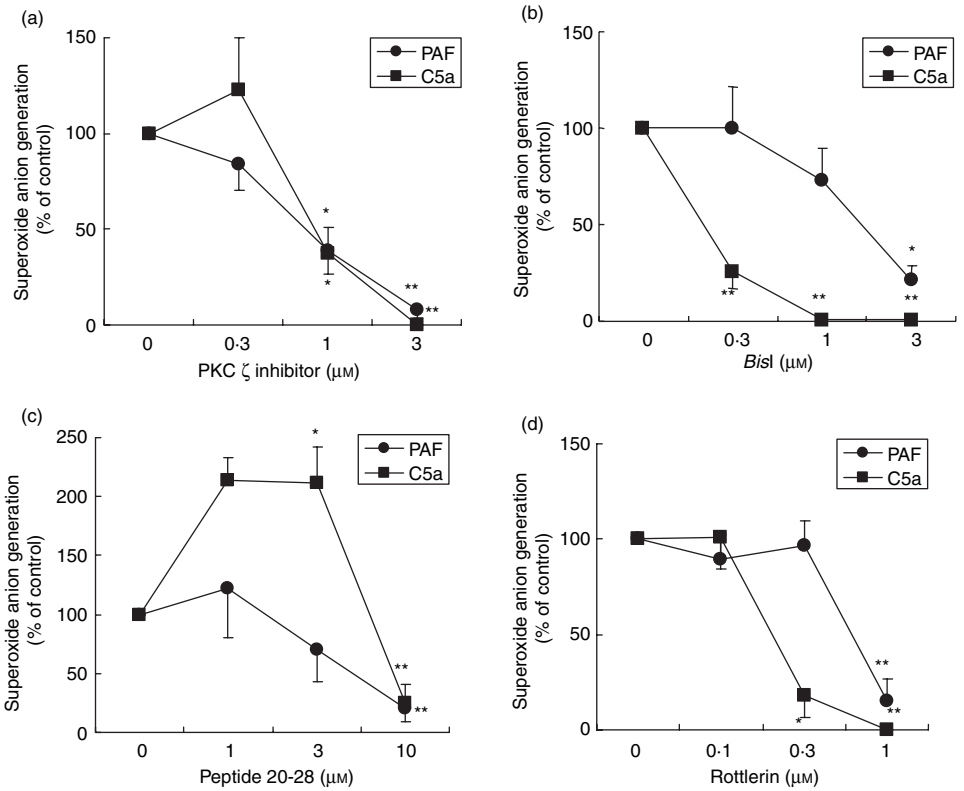

Figure 1. Effects of protein kinase C (PKC) inhibitors on superoxide anion (O_2^-) generation by human eosinophils. Purified human eosinophils in human serum albumin-coated wells ($2{\cdot}5 \times 10^5$ cells/tube) were stimulated with platelet-activating factor (PAF) (1 μM) or complement 5a (C5a) (100 ng/ml) after preincubation with each PKC inhibitor [(a) PKC ζ inhibitor; (b) bisindlolylmaleimide I (*Bis*I); (c) PKC inhibitor peptide 20-28; (d) rottlerin] for 10 min at 37° and then prewarmed at 37° for 5 min with 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo-[1,2-a] pyrazin-3-one (MCLA). The O_2^- generated for 30 min after stimulation by PAF or C5a is presented as the mean ± standard error of the mean (SEM) for four to 11 experiments. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; significant differences compared with cells stimulated with PAF or C5a in the absence of PKC inhibitors. O_2^- production in the absence of drug was as follows: PAF, 354·5 ± 41·1; C5a, 120·5 ± 23·9 ($\times 10^4$ counts; mean ± SEM; $n = 11$–14).

Results

Effects of PKC inhibitors on superoxide anion (O_2^-) generation by human eosinophils

As shown in Fig. 1(a), myristoylated PKC ζ inhibitor peptide significantly suppressed PAF- or C5a-induced O_2^- generation in a dose-dependent manner. We compared this effect with those of other PKC inhibitors such as *Bis*I (a conventional and novel PKC inhibitor), PKC inhibitor peptide 20-28 (a PKC α, β inhibitor) and rottlerin (a PKC δ inhibitor). As shown in Figs 1(b) and (d), *Bis*I, PKC inhibitor peptide 20-28 and rottlerin blocked PAF- or C5a-induced O_2^- generation. However, a significant difference in the inhibitory effects of *Bis*I, peptide 20-28 and rottlerin was observed between PAF- and C5a-induced eosinophil superoxide generation. A summary of the inhibitory effects of PKC inhibitors, including IC_{50} values, is presented in Table 1; the IC_{50} of the specific PKC ζ inhibitor with respect to PAF- or C5a-induced O_2^- generation was very similar to that of rottlerin. The IC_{50} value for the effect of *Bis*I on C5a-induced O_2^- generation was much smaller than that on PAR-induced O_2^- generation. Preliminary studies demonstrated that concentrations of DMSO used in these experiments did not affect

Table 1. IC_{50} values of protein kinase C (PKC) inhibitors for superoxide anion generation induced by platelet-activating factor (PAF) or complement 5a (C5a)

	[IC_{50} (μM)]	
	PAF	C5a
PKC ζ inhibitor	0·8	0·9
*Bis*I	1·5	0·1
Peptide 20-28	5·1	7·5
Rottlerin	0·5	0·2

Purified human eosinophils in human serum albumin-coated wells (5×10^4 cells/well) were stimulated with PAF (1 μM) or C5a (100 ng/ml) after preincubation with each PKC inhibitor for 15 min. IC_{50} values of PKC inhibitors (μM) were calculated from the values of superoxide anion generation for 30 min after stimulation by PAF or C5a.
*Bis*I, bisindlolylmaleimide I.

eosinophil viability or stimulus-dependent O_2^- production, and each PKC inhibitor, by itself, did not affect spontaneous O_2^- generation and cell viability (data not shown).

Effects of PKC inhibitors on eosinophil degranulation

We next examined effects of PKC inhibitors on another major eosinophil effector function, degranulation, by quantifying release of a granule protein, EPX, into the supernatants by eosinophils. As shown in Fig. 2(a), the PKC ζ inhibitor suppressed PAF- or C5a-induced degranulation as well as *Bis*I (Fig. 2b). PKC ζ inhibitor or *Bis*I by itself did not affect the spontaneous degranulation response (data not shown).

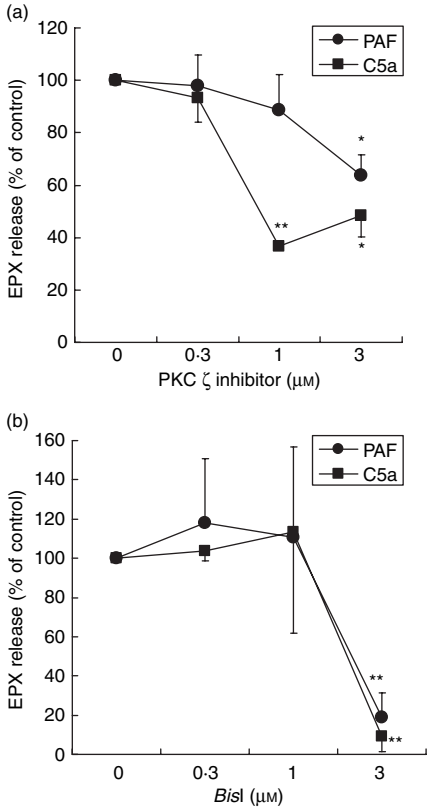

Figure 2. Effects of protein kinase C (PKC) inhibitors on eosinophil degranulation. Purified human eosinophils (5×10^4 cells/well) were stimulated with platelet-activating factor (PAF) (1 μM) or complement 5a (C5a) (100 ng/ml) in the presence or absence of PKC in a human serum albumin-coated plate at 37°. After 4 hr, concentrations of eosinophil protein X (EPX) in the cell-free supernatants were analysed by radioimmunoassay (RIA) as an indicator of eosinophil degranulation. Values are expressed as mean ± standard error of the mean (SEM) for four to seven independent experiments. EPX content in the absence of drug was as follows: medium alone, 92·9 ± 13·9; PAF, 512·9 ± 103·4; and C5a, 417·6 ± 91·9 ng/10^6 cells (mean ± SEM; $n = 8$–11). Total cellular EPX content was 2771·7 ± 332·76 ng/10^6 cells (mean ± SEM; $n = 11$). $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; significant differences compared with cells stimulated with PAF or C5a in the absence of inhibitor.

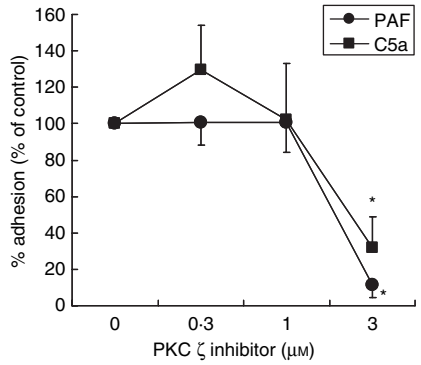

Figure 3. Effects of protein kinase C (PKC) inhibitors on eosinophil adhesion. Purified human eosinophils (5×10^4 cells/well) were stimulated with platelet-activating factor (PAF) (1 μM) or complement component 5a (C5a) (100 ng/ml) in the presence or absence of PKC inhibitor in a human serum albumin-coated plate at 37°. After 1 hr, percentages of eosinophils adherent to the wells of the plate were determined. Data are expressed as mean ± standard error of the mean (SEM) for four independent experiments. Percentage adhesion of control cells in the absence of drugs was as follows: medium alone, 4·3 ± 0·9%; PAF, 14·1 ± 1·7%; and C5a, 15·9 ± 1·9% (mean ± SEM; $n = 4$). Total cellular EPX content was 2817·7 ± 235·8 ng/10^6 cells (mean ± SEM; $n = 4$). $^*P < 0{\cdot}05$; significant differences compared with cells stimulated with PAF or C5a in the absence of inhibitor.

Effects of PKC inhibitors on eosinophil adhesion

Because cellular adhesion is considered critical to the effector functions of human eosinophils, we examined the effect of the PKC ζ inhibitor on adhesion of eosinophils to HSA-coated plates at 1 hr after stimulation using an adhesion assay. As shown in Fig. 3, the PKC ζ inhibitor suppressed PAF- or C5a-induced cellular adhesion. The PKC ζ inhibitor did not affect the spontaneous adhesion response (data not shown).

Effects of PKC inhibitors on CD11b expression in eosinophils

We tested the effect of PKC inhibitors on CD11b expression on the surfaces of eosinophils, an indicator of 'inside-out' signalling involving β2 integrin-mediated effector functions. As shown in Table 2 and Fig. 4, the PKC ζ inhibitors did not have as great an effect on spontaneous or PAF- or C5a-induced CD11b expression in eosinophils as other PKC inhibitors, while only *Bis*I enhanced PAF-induced CD11b expression, as previously reported.[21,34]

Effect of PKC ζ inhibitor on stimulus-induced translocation of $p47^{phox}$ or PKC ζ in eosinophils

Finally, to confirm the above results suggesting that PKC ζ modulates eosinophil effector functions, we examined

Table 2. Effects of protein kinase C (PKC) inhibitors on CD11b expression on eosinophils

	Mean fluorescence intensity (MFI)	
	PAF	C5a
PKC ζ inhibitor	91·6 ± 3·1	103·3 ± 4·4
*Bis*I	145·9 ± 7·4**	125·5 ± 12·3
Peptide 20-28	101·9 ± 9·6	118·0 ± 7·2
Rottlerin	88·1 ± 6·5	127·8 ± 16·4

Purified eosinophils ($2{\cdot}5 \times 10^5$ cells/sample) were stimulated with or without 1 μM platelet-activating factor (PAF) or 100 ng/ml complement 5a (C5a) after pretreatment with PKC inhibitor (3 μM PKC ζ inhibitor, 1 μM *Bis*I, 10 μM peptide 20-28 or 10 μM rottlerin) or medium alone for 15 min at the concentrations indicated. Expression of CD11b was determined by flow cytometric analysis as mean fluorescence intensity (MFI). The MFI in the absence of inhibitors was as follows: medium alone, 14·3 ± 1·1; PAF, 22·8 ± 1·7; and C5a, 24·2 ± 0·6 (mean ± standard error of the mean (SEM); $n = 5$–9). $^{**}P < 0{\cdot}01$; significant differences compared with the cells stimulated with PAF or C5a in the absence of inhibitors.
*Bis*I, bisindlolylmaleimide I.

the effect of the PKC ζ inhibitor on PAF- or C5a-induced translocation of $p47^{phox}$ or PKC ζ in eosinophils. Because these molecules translocate from the cytosol to just beneath the plasma membrane upon activation, we assessed the intracellular locarization of these molecules 15 min after stimulation by PAF or C5a. Immunofluorescent staining was used to mark the locarization of the molecules, as determined by confocal laser scanning microscopy. As shown in Fig. 5, $p47^{phox}$ (Figs 5c and i) or PKC ζ (Figs 5f and l), marked with green fluorescence, translocated from the cytosol to the plasma membrane upon stimulation by PAF (Figs 5b, c and f) or C5a (Figs 5c, i and l), but did not do so in medium alone (Figs 5a and b). Fluorescent phalloidin staining (F-actin), displayed in red, indicated that upon activation eosinophils changed shape dramatically, with extension of filopodia. Both the change in the cell shape and translocation of $p47^{phox}$ (Figs 5d, e, j and k) or PKC ζ (Figs 5g, h, m and n) were blocked by 1 or 3 μM PKC ζ inhibitor, while the effect of the pretreatment with 0·3 μM PKC ζ inhibitor was negligible (data not shown).

Discussion

In the present study, a PKC ζ inhibitor blocked PAF- or C5a-induced eosinophil O_2^- generation, as did *Bis*I or rottlerin. This inhibitor also attenuated PAF- or C5a-induced degranulation and adhesion. In contrast, the PKC ζ inhibitor did not affect PAF- or C5a-induced CD11b expression. The PKC ζ inhibitor completely blocked PAF- or C5a-induced eosinophil shape changes as well as the translocation of PKC ζ and $p47^{phox}$ to the plasma membrane.

PKCs are being elucidated as an increasingly diverse family of enzymes involved in signal transduction in various cells, including eosinophils.[10–12] Studies using a phorbol ester, phorbol 12-myristate 13-acetate (PMA), that activates PKC suggest that this enzyme is involved in eosinophil functions such as cell adhesion,[23] degranulation,[6,24] and O_2^- generation.[6,25] Non-specific PKC inhibitors also modulate eosinophil functions stimulated with PAF or C5a that do not involve motility.[26–28] For example, staurosporine blocked a C5a- or PMA-induced respiratory burst,[26,27] and staurosporine or calphostin C inhibited PAF- or C5a-enhanced eosinophil cationic protein (ECP) release.[28] However, in contrast to eosinophil functions not involving motility, PAF-induced chemotaxis is independent of PKC activation,[29] while PKC negatively regulates PAF-induced aggregation.[30] In guinea pig eosinophils, PKC has been suggested to negatively regulate PAF-induced calcium influx and degranulation.[31] A previous study in human eosinophils demonstrated that PKC negatively modulated PAF-induced arachidonic acid metabolite production,[32] while augmenting sustained intracellular calcium increase and O_2^- generation induced

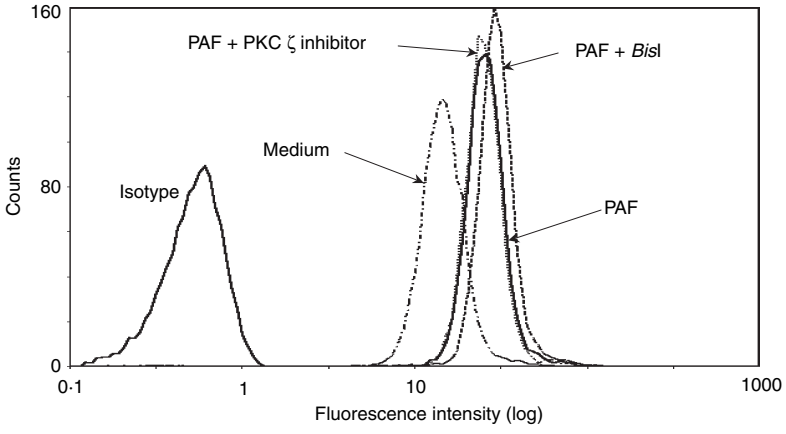

Figure 4. A typical result for CD11b expression on eosinophils. The histograms show a typical result for CD11b expression on eosinophils stimulated with platelet-activating factor (PAF). Purified eosinophils ($2{\cdot}5 \times 10^5$ cells/sample) were stimulated with or without 1 μM PAF after pretreatment with 3 μM protein kinase C (PKC) ζ inhibitor, 1 μM bisindlolylmaleimide I (*Bis*I), or medium alone for 15 min.

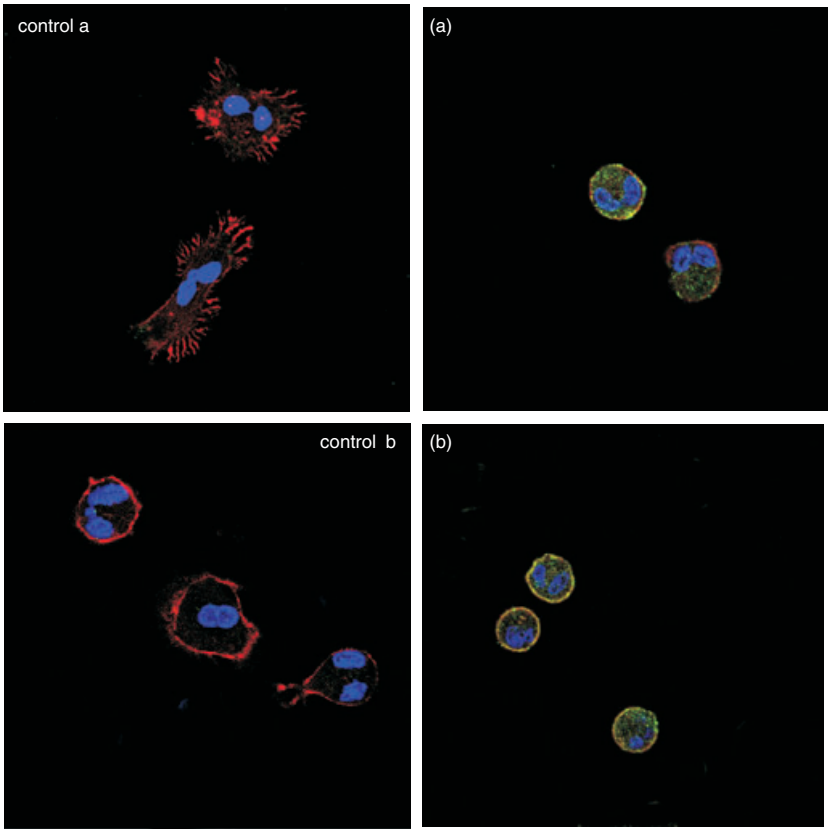

Figure 5. Effect of protein kinase C (PKC) ζ inhibitor on (b) platelet-activating factor (PAF)- or (c) complement 5a (C5a)-induced translocation of $p47^{phox}$ and PKC ζ in human eosinophils. Eosinophils were treated with medium alone (a and b), 1 μM PAF (c–h; control a) or 100 ng/ml C5a (i–n; control, b) in a human serum albumin-coated glass-bottomed dish without (a, b, c, f, i and l) or with 1 μM (d, g, j and m) and 3 μM (e, h, k and n) PKC ζ inhibitor. After adding PAF, C5a or medium, cells were fixed and stained using $p47^{phox}$-specific (a, c, d, e, i, j and k) and PKC ζ-specific (b, f, g, h, l, m and n) goat polyclonal antibody (primary antibody). Cells then were exposed to a staining cocktail containing Alexa Fluor 532 phalloidin and Alexa Fluor 488 rabbit anti-goat immunoglobulin G (IgG) as labelled second ligands. DAPI was mounted on each sample and fluorescence was observed with a confocal laser scanning microscope. The original magnification of all figures is the same (×600). The red colour shows fluorescence induced by Alexa Fluor 532 phalloidin (F-actin). The green colour shows fluorescence induced by Alexa Fluor 488 rabbit anti-goat IgG ($p47^{phox}$ or PKC ζ). The blue colour shows fluorescence induced by DAPI (nucleus). The negative control (goat IgG used instead of primary antibody) was shown as control a or b. The experiments were repeated three to five times with essentially identical results.

by PAF.[32,33] We also reported that PKC modulated PAF-activated eosinophil functions in two ways; PKC inhibition enhanced CD11b expression and adhesion, while suppressing O_2^- generation and degranulation.[21,34] Furthermore, in this study, a significant difference was observed in the effects of each PKC inhibitor on PAF- and C5a-induced eosinophil superoxide generation. Although the exact mechanism by which differences were produced between PAF and C5a in the IC_{50} values of each inhibitor is unknown, the involvement of the PKC isoform might differ between individual stimulant and eosinophil functions.

To date, 11 PKC isoforms have been identified: α, βI, βII, δ, ε, γ, η, ι, θ, μ and ζ. On the basis of molecular structure and biochemical properties, the PKC family can be divided into three groups: conventional PKCs (α, βI, βII and γ isoforms); the novel PKCs (δ, ε, η and θ isoforms); and the atypical PKCs (ι, μ and ζ isoforms).[10–12] At present, the biologic significance of heterogeneity as well as the function of individual isoenzymes largely remains unknown. Further, specific roles of PKC isozymes in eosinophil function remain to be investigated. In contrast to the many reports concerning other cells, such as neutrophils, little has been revealed about atypical, conventional or novel PKCs in eosinophils. A recent study demonstrated PKC α, βI, βII, γ, δ and ζ expression as well as important regulation by PKC δ of interleukin (IL)-5- or leukotriene (LT)B_4-evoked NADPH oxidase activity in human eosinophils.[17] We also showed that PKC δ is involved in O_2^- generation but not in CD11b expression in PAF-stimulated eosinophils.[33] Another report demonstrated expression of at least eight isoforms of PKC (PKC α, βI, βII, ζ, δ, ε, ι and μ) in human eosinophils, in which PKC ζ but not PKC

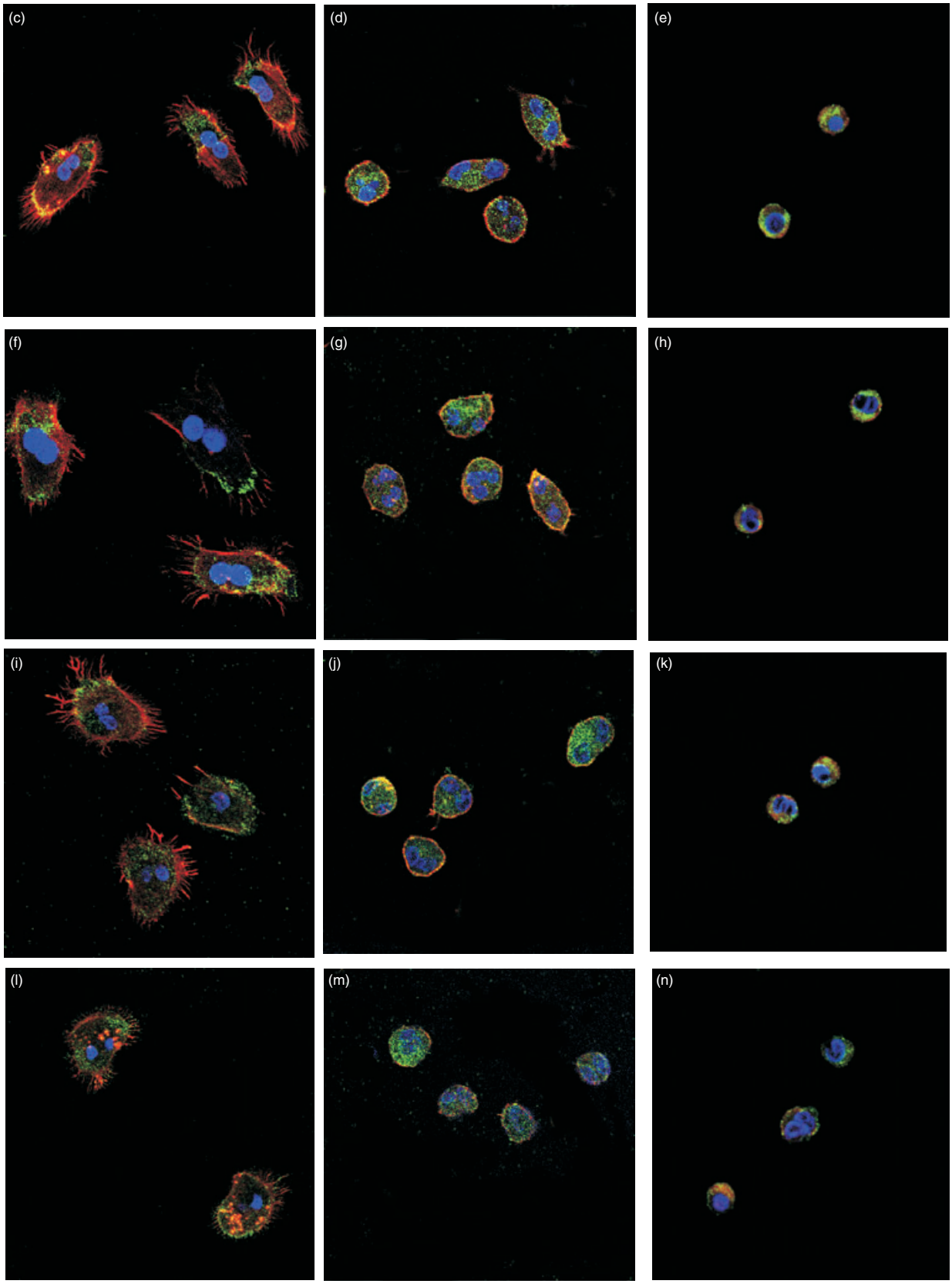

Figure 5. (*Continued*).

α, βI or βII showed increased translocation to the membrane *in vitro* 24 hr after antigen challenge in the asthmatic patients whose cells were sampled.[16] Very recently, we have shown that translocation of PKC βII, δ and ζ modulates eosinophil O_2^- generation and that an actin polymerization inhibitor, cytochalasin B, inhibits eosinophil shape change and the translocation of these PKCs and a NADPH oxidase component, $p47^{phox}$, suggesting that the actin cytoskeleton and the translocation of PKCs and $p47^{phox}$ play an important role in O_2^- generation in human adherent eosinophils.[22] In this study we further demonstrated that the PKC ζ inhibitor completely blocked both the eosinophil shape change and the translocation of $p47^{phox}$. This result is to a large extent comparable to that of Dang *et al*., who showed that $p47^{phox}$ is a substrate for PKC ζ and participates in the signalling cascade between the receptor and NADPH oxidase activation in human neutrophils stimulated with N-formyl-methionyl-leucyl-phenylalanine (FMLP).[35]

In the context of these observations, the present study is the first to show that PKC ζ modulates human eosinophil adhesion and effector functions *in vitro*. In human neutrophils, PKC ζ but no conventional PKC was found to participate in integrin-dependent adhesion.[36] This is comparable to our finding in eosinophils that at least PKC ζ modulated integrin-dependent adhesion and effector functions but did not affect CD11b expression. With respect to integrin activation, cellular events preceding adhesion are referred to as 'inside-out' signalling, a view supported by observation of CD11b expression before adhesion occurred. In contrast, most effector functions of eosinophils, such as O_2^- generation, require adhesion through integrins ('outside-in' signalling). In this study, treatment with a myristoylated PKC inhibitor (20-28 peptide), rottlerin, and the PKC ζ inhibitor did not affect PAF- or C5a-induced CD11b expression, while only *Bis*I enhanced PAF-induced CD11b expression. This indicates that PKC isoforms other than PKC α, β, δ and ζ are involved in PAF-induced CD11b expression, representing inside-out signalling. In contrast, at high concentrations, *Bis*I up-regulated PAF- or C5a-induced eosinophil adhesion, while it decreased PAF- or C5a-induced superoxide generation and degranulation (reference 21 and data not shown). Furthermore, rottlerin or peptide 20-28 inhibited the adhesion response stimulated by PAF or C5a (data not shown). These results suggest that conventional and novel PKCs are diversely involved in both cell adhesion and effector functions of eosinophils, representing 'outside-in' signalling.

Recently, we have found that PAF activates two distinct effector pathways leading to O_2^- generation: one is pertussis toxin (PTX)-sensitive with immediate and transient activation, and the other is PTX-resistant with late and extended activation. We have discovered that the latter pathway evokes substantial O_2^- production and is mediated by PI3K.[20] In addition, previous reports have shown that PI3K is essential for PKC δ[37] and PKC ζ activation.[38] Taken together, these observations and our present results suggest that substantial stimulus-induced O_2^- generation, one of the functions mediated by outside-in signalling, is modulated by PKC ζ and δ, which might be activated by PI3K. In marked contrast, inhibition of PI3K did not notably reduce PAF-induced CD11b expression (Y. Motegi, M. Kato *et al*., unpublished data), which suggests that CD11b expression is mediated by a signalling pathway distinct from O_2^- generation, and independent of PI3K activation and PKC ζ and δ.

In conclusion, we have demonstrated that PKC ζ modulates eosinophil effector functions. Although much about the mechanism of PKC ζ participation in eosinophil functions requires further elucidation, our results provide evidence that an atypical PKC, PKC ζ, as well as a novel PKC, PKC δ, might be therapeutic targets for decreasing eosinophilic inflammation in clinical states such as asthma.

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