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Protein kinase C activation inhibits eosinophil degranulation through stimulation of intracellular cAMP production

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1 The mechanism of inhibition of eosinophil degranulation by protein kinase C (PKC) was investigated in complement C5a (C5a)-stimulated degranulation of highly purified human eosinophils using the specific PKC activator – phorbol 12-myristate 13-acetate (PMA).

2 C5a-induced release of eosinophil peroxidase and eosinophil cationic protein was potently inhibited in a concentration-dependent manner by PMA (IC_{50}: 3 and 5 nM, respectively). The inhibition by PMA, but not histamine, was significantly reversed by the specific, but isoform nonselective, PKC inhibitor Ro 31-8220 (1 μM).

3 In the presence of phosphodiesterase inhibitor rolipram (5 μM), PMA stimulated a pronounced concentration-dependent increase in intracellular cAMP, with a potency 400 times that of histamine (EC_{50}: 55 nM vs 22.5 μM). The inactive PMA analogue, 4α-PMA, had no such effect.

4 The cAMP production by PMA, but not histamine, was significantly reversed by Ro 31-8220 (1 μM) and the selective inhibitor of the novel PKCδ, rottlerin (1–3 μM), but not the selective inhibitor of the classical PKC isoforms, Gö 6976 (0.01–0.1 μM).

5 Western blot analysis revealed the presence of six PKC isoforms (α, βI, βII, δ, ι and ζ) in isolated eosinophils.

6 Chelation of internal or external calcium had no effect on PMA-induced cAMP response, but abolished that induced by histamine.

7 There was a good correlation between increase in intracellular cAMP and inhibition of degranulation.

8 These results show, for the first time, that in human eosinophils, PMA, *via* activation of PKCδ isoform, can stimulate cAMP production, and that this may be the basis for its potent anti-degranulatory effect.

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Keywords: Protein kinase C; eosinophils; phorbol ester; cyclic adenosine; 3′,5′-cyclic monophosphate; degranulation

Abbreviations: AC, adenylyl cyclase; BAPTA, 1,2-bis(*O*-aminophenyl)-ethane-thane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; C5a, complement fragment 5a; CB, cytochalasin B; CI, confidence interval; DMSO, dimethyl sulphoxide; ECP, eosinophil cationic protein; EPO, eosinophil peroxidase; OPD, *O*-phenylenediamine; PAF, platelet-activating factor; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate

Introduction

Eosinophils are known to play prominent roles in the pathophysiology of allergic diseases, especially asthma (Gleich & Adolphson, 1999). Blood and sputum eosinophilia are common findings in asthma and the degree of eosinophil infiltration of the lung and their activation state correlate strongly with the severity of the disease (Motojima *et al*., 1998; Giembycz & Lindsay, 1999). Within the lungs, activated eosinophils release tissue-damaging granule proteins and oxygen-free radicals, which, in concert, orchestrate bronchial inflammation and cause severe damage to the lung epithelium (Robinson & Carver, 1998).

Eosinophils can be activated by a number of stimuli such as interlukin-5 (IL-5), C5a, leukotriene B_4, platelet-activating factor (PAF), complexed IgG and IgA, as well as phorbol 12-myristate 13-acetate (PMA) (van der Bruggen & Koenderman, 1996; Giembycz & Lindsay, 1999; Robinson, 1999), but the signal transduction pathways utilized by these stimuli have not been clearly defined. One signalling pathway that is increasingly being recognized as important in eosinophils is the phospholipase C (PLC) pathway, which involves the activation of protein kinase C (PKC) and the generation of intracellular Ca^{2+} liberator, inositol triphosphate (IP_3). The use of PMA, a well-known activator of PKC, as well as PKC inhibitors, has shown that a number of eosinophil functions such as superoxide ion release, degranulation and cell survival are mediated/modulated by PKC (Sedgwick *et al*., 1990; Ali *et al*., 1994; Bankers-Fulbright *et al*., 2001).

At least 12 isoforms of PKC are currently known to exist (Dekker & Parker, 1994). On the basis of molecular structure and biochemical properties, the isoforms have been divided into three families. The conventional PKCs (α, βI, βII and γ) are Ca^{2+}- and phospholipid-dependent. The novel PKCs (δ, ε, η, θ and μ) are Ca^{2+}-independent, but phospholipid-dependent, while the atypical PKCs (ι, λ and ζ) are both Ca^{2+}-

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and phospholipid-independent and lack PMA-binding sites (Nishizuka, 1992; 1995).

The difficulty in studying the signal transduction mechanisms involved in eosinophil activation stems not only from the diversity of the stimuli that activate the cells but also from the fact that different responses, involving apparently different signalling pathways, may result from a given stimulus. For example, PMA is a potent activator of eosinophil NADPH oxidase, leading to the release of superoxide anions (Sedgwick *et al*., 1990; Bankers-Fulbright *et al*., 2001), but the same stimulus will abolish eosinophil degranulation or leukotriene release (Ali *et al*., 1994; Kroegel *et al*., 1994). Similarly, the CD11b/CD18-induced superoxide release is PKC-dependent, whereas the induction of eosinophil adherence to surfaces, in response to the same stimulus, is not (Lynch *et al*., 1999). It has been suggested that the complex role of PKC in signal transduction may be dependent on both the type and level of the isoforms expressed in the relevant cell type, as well as the stimulus (Gusovsky & Gutkind, 1991; Lin & Chen, 1998).

In human eosinophils, few studies have investigated the expression of the different PKC isoforms and the association of individual isoforms with particular responses. Evans *et al*. (1999) have shown that human eosinophils express PKCα, βI, βII, δ, ε, ι, μ and ζ, and that only PKCζ expression was upregulated by allergen challenge. Other studies have implicated PKCδ as the isoform mediating human eosinophil NADPH oxidase activation and the consequent generation of superoxide ions (Bankers-Fulbright *et al*., 2001; Takizawa *et al*., 2003). So far, no isoform has been associated with PKC-mediated inhibition of eosinophil degranulation.

Given the role of eosinophil granule products in the pathophysiology of asthma and other allergic diseases, it has become imperative that an ideal antiallergic/anti-asthmatic drug must be able to prevent eosinophil degranulation. Hence, the profound inhibitory effect that PMA has on eosinophil degranulation (Kernen *et al*., 1991; Ali *et al*., 1994; Kroegel *et al*., 1994) has stimulated interest in PKC as an important target for the control of eosinophil degranulation.

The biochemical pathway downstream of PKC activation, leading to the inhibition of granule extrusion in eosinophils, is currently unknown. Potentially, one such downstream pathway is the adenylyl cyclase (AC)–cAMP pathway. Intracellular cAMP is well established as an important second messenger regulating a wide range of cellular responses. In eosinophils, it is a powerful inhibitor of degranulation (Kita *et al*., 1991). Previous reports show that PKC activation can alter agonist-induced generation of intracellular cAMP by increasing or decreasing the activities of the various AC isoforms (Kawabe *et al*., 1994; Lai *et al*., 1997; Lin & Chen, 1998). Phorbol ester-activated PKC has been shown to increase the activities of mammalian ACI, II and V isoforms, leading to increased cAMP generation (Choi *et al*., 1993; Jacobowitz & Iyengar, 1994; Lin & Chen, 1998). We have, therefore, hypothesized that PKC-mediated down-regulation of eosinophil degranulation may be mediated by enhanced generation of cAMP.

The purpose of this study is, therefore, to investigate the involvement of PKC in PMA-induced inhibition of C5a-induced degranulation of human eosinophil and to determine the possible role of intracellular cAMP in the signalling pathway.

Methods

Isolation of blood eosinophils

Fresh blood was obtained from consenting individuals who were mildly atopic, but have no allergic disease nor taken any medication in the last 72 h. Granulocytes were isolated from sodium citrate-anticoagulated (13 mM final) blood by erythrocyte sedimentation, followed by percoll gradient centrifugation as described previously (Ezeamuzie & Philips, 1999). The granulocytes were washed twice in 'wash buffer' (Ca^{2+}- and Mg^{2+}-free HEPES-buffered Hank's balanced salt solution containing 0.25% bovine serum albumin, pH = 7.3) and re-suspended in the same buffer at 2×10^7 cells ml^{-1}. Eosinophils were finally purified by the immunomagnetic method (Hansel *et al*., 1991) using magnetic beads coated with anti-human CD16 monoclonal antibody to remove all neutrophils. The purified cells were recovered by centrifugation, washed twice and then re-suspended in 'reaction buffer' ('wash buffer' containing 2 mM Ca^{2+}, 1 mM Mg^{2+}) for experiments. The eosinophil purity was assessed by differential count of a Wright–Giemsa-stained cytosmear, and was routinely greater than 98%. Viability was determined by Trypan blue exclusion and always exceeded 98%.

Induction of degranulation/release of eosinophil peroxidase (EPO) and eosinophil cationic protein (ECP)

Purified eosinophils were used at a concentration of 5×10^5 cells ml^{-1}. A volume of 50 μl of pre-warmed cell suspension was dispensed into each well of a microplate. Then, 100 μl of the reaction buffer containing 10 μg ml^{-1} cytochalasin B (CB) and a two-fold concentration of the test drug was added. After a 10-min pre-incubation, the cells were stimulated with 50 μl of recombinant human C5a. The mixture was then further incubated for 30 min at 37°C. This time had been previously determined to be sufficient for the virtual completion of the degranulation process. Reaction was stopped by cooling on ice, and after centrifugation at $600 \times g$, for 10 min, 50 μl aliquots of the supernatant, as well as triton X-100-lysed cells (for total content determination), were taken for the determination of the released enzymes.

EPO activity was measured by the *O*-phenylenediamine (OPD) method as reported previously (Kroegel *et al*., 1989). Briefly, OPD substrate solution containing 0.4 mg ml^{-1} OPD and 0.4 mg ml^{-1} urea hydrogen peroxide in PBS-citrate buffer (pH 4.5) was prepared from SIGMA FAST® OPD tablets. In all, 100 μl of this substrate was added to 50 μl of the samples in a microplate and incubated for 30 min at 37°C. After incubation, the reaction was then stopped with 50 μl of 4 M H_2SO_4 and the plate read at 490 nm. Values were expressed as percentage of total content. The recovery of released EPO activity was usually above 80% at the end of 30 min incubation, but lower with more prolonged incubation.

ECP was assayed by the Pharmacia CAP ECP System (Pharmacia Biotech, Uppsala, Sweden) – an enzyme immunoassay-based method, using a commercially available kit.

Intracellular cyclic AMP determination

Purified eosinophils, re-suspended at a concentration of 10^7 cells ml^{-1} in BSA-free reaction buffer, were dispensed in

50 μl aliquots into each well of a 96-well plate. Then, 100 μl of reaction buffer containing rolipram (5 μM) and test drugs (2 × final concentrations), where indicated, was added. After incubating the mixture for 10 min at 37°C, the reaction was started by the addition of 50 μl of warmed stimuli or vehicle. After 3 min – a time found to be optimal for this response – the reaction was stopped by the direct addition of 22.2 μl of 1 N HCl. After a thorough mixing and a further incubation for 10 min, the plate was centrifuged at 1500 × *g* for 10 min and 200 μl of the supernatant was taken and stored at −43°C pending cAMP assay.

Cyclic AMP levels were measured, after acetylation, using a commercially available enzyme immunoassay kit, and following the manufacturer's instructions. The sensitivity of the assay was 0.01 pmol well^{-1}.

Eosinophil lysis and Western blotting

Purified eosinophils (10^7 cells ml^{-1}) were lysed with sodium dodecyl sulphate (SDS) sample buffer (2% SDS, 375 mM Tris–HCl, (pH 6.8), 4% β-mercaptoethanol, 0.1% bromophenol blue and 25 mM dithiothreitol)) and the lysate boiled for 10 min. Equivalents of 7.5×10^5 cells were then loaded per lane of a 10% polyacrylamide gel electrophoresis in the presence of 0.1% SDS (SDS–PAGE) and proteins were electroblotted unto nitrocellulose membranes (Amersham ECL grade). Blots were then blocked in blocking buffer (100 μM Tris–HCl, 150 mM NaCl and 0.1% Tween 20, pH 7.5) containing 5% nonfat dry milk powder for 30 min at room temperature and washed five times in blocking buffer. The separated proteins were then probed with commercially available antibodies to different PKC isoforms for 2 h. After five washes in PBS, the blots were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (diluted 1 : 200–1 : 1000) for 1 h at room temperature. Rat brain extract was used as positive controls for the detectability of the PKC isoforms (provided the antisera are sufficiently cross-reacting). Immunoreacting blots were then detected by the enhanced chemiluminescence detection (ECL) system (Amersham Pharmacia Biotech, Upsulla, Sweden) and recorded on a Kodak X-OMAIS-s film. The ECL reaction time was ≈60 min for PKCs α, βI, βII, ε and γ, but ⩽1 min for δ, ι and ζ isoforms.

Drugs and chemical reagents

The following drugs and reagents were obtained from Sigma-RBI, St Louis, MO, U.S.A.: percoll, HEPES buffer, bovine serum albumin, histamine dihydrochloride, recombinant human C5a, CB, dimethyl sulphoxide (DMSO), OPD, rolipram, Ro 31-8220, Gö 6976, Gö 6983, rottlerin and all inorganic salts. Mouse monoclonal anti-human CD16 antibody (clone FcR gran1) was obtained from CLB, Amsterdam, Netherlands, while the magnetic beads were supplied by Dynal AS, Oslo, Norway. The cAMP assay kit (direct method) was obtained from Assay Designs Inc, Ann Arbor, Michigan, U.S.A. All the anti-PKC antibodies, the HRP-labelled secondary antibodies and rat brain extract were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A. The anti-PKCs α, βI, βII, ε and δ antibodies were monoclonal, while the rest were polyclonal.

Stock solutions of all water-insoluble drugs were made in DMSO to concentrations in the range 100–400 mM and then diluted directly in buffer. The final concentration of the solvent in the highest drug concentrations did not exceed 0.05% – a concentration that had no significant effect on eosinophil responses.

Statistical analysis

Experimental data are presented as means ± s.e. Drug concentrations producing 50% of maximal increase in intracellular cAMP levels (EC_{50} values), or 50% inhibition of degranulation (IC_{50} values), were calculated from the concentration–effect curves by nonlinear regression analysis using GraphPad InPlot (GraphPad Software Inc., Philadelphia, U.S.A.). Statistical significance (*P*) was determined by the unpaired *t*-test or by one-sample *t*-test as appropriate (InStat, GraphPad, Software Inc., U.S.A.).

Results

PMA-induced inhibition of eosinophil degranulation

Pretreatment of eosinophils for 10 min with PMA resulted in a highly potent and concentration-dependent inhibition of C5a-induced eosinophil degranulation, measured as both the release of EPO and ECP (Figure 1). The concentrations producing 50% inhibition (IC_{50}) were 3.0 and 5.3 nM,

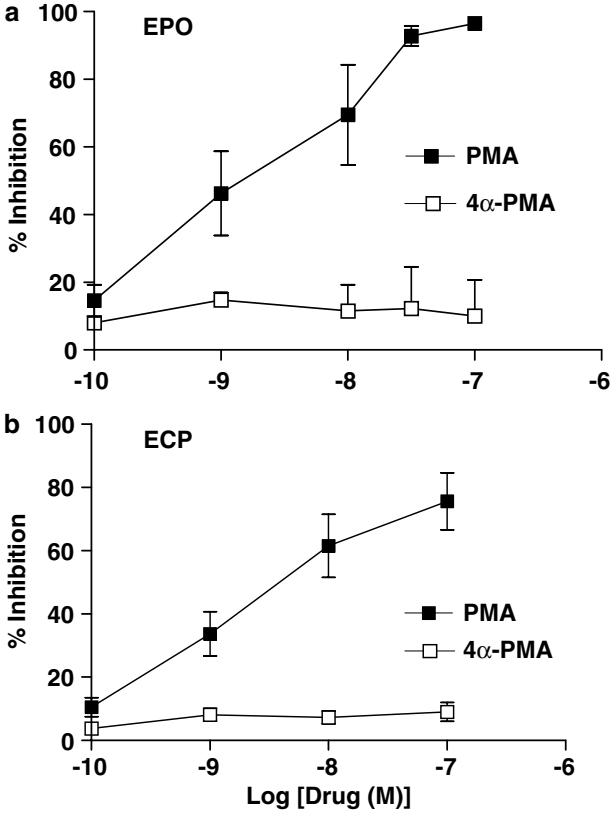

Figure 1 Effect of PMA and its inactive analogue 4α-PMA on EPO (a) and ECP (b) release from human eosinophils stimulated with C5a (30 nM) in the presence of CB (5 μg ml^{-1}). The uninhibited releases were 26.3 ± 4.1 and 29.8 ± 5.5% of the total contents of EPO and ECP, respectively. Results are means ± s.e. for six experiments.

respectively, for EPO and ECP releases. Maximal inhibitions achieved at 100 nM PMA were 100% for EPO ($n=6$) and 78 ± 5% for ECP ($n=4$). In contrast, 4α-PMA (an inactive analogue of PMA) at concentrations up to 100 nM had no effect on the release of both granule proteins.

To confirm the role of PKC in the observed PMA action and its specificity, the ability of Ro 31-8220 – a specific PKC inhibitor with no significant PKC isoform selectivity – to reverse the inhibitory effect of PMA was determined and compared with its ability to reverse the inhibitory effect of histamine. As shown in Figure 2a, pretreatment of the cells with Ro 31-8220 (1 μM) caused almost complete reversal of the PMA-induced inhibition of EPO release (92% inhibition with 30 nM PMA alone vs 27% inhibition in the presence of Ro 31-8220). In contrast, Ro 31-8220 (1 μM) had no effect on the inhibition of EPO release induced by 10 μM histamine (Figure 2b).

Induction of cAMP generation by PMA in comparison with histamine

As shown in Figure 3, in the presence of 5 μM rolipram, PMA induced a surprisingly strong generation of intracellular cAMP in eosinophils in the absence of any agonistic stimulus. With an estimated EC_{50} of 55 nM, the drug was about 400 times more potent than histamine ($EC_{50}=22.5$ μM) in producing this response. PMA was also more efficacious than histamine (apparent maximal effects: 6.2 ± 1.6 vs 3.8 ± 1.4 pmol cAMP per 10^6 cells, respectively). The inactive phorbol ester, 4α-PMA, had no such effect.

The specific involvement of PKC in this response was demonstrated by the ability of the PKC-specific, but PKC isoform nonselective, inhibitor, Ro 31-8220 (1 μM), to abolish PMA-induced stimulation of cAMP generation (Figure 4a), while having no such effect on a comparable response by histamine (Figure 4b).

The time-course of PMA-induced cAMP generation was rapid and similar to histamine, starting within 30 s of addition, peaking around 3 min, and declining to basal level by 30 min (Figure 5). The half-life ($t_{1/2}$) was approximately 1.7 min for PMA and 1.4 min for histamine.

The dependence of the PMA response on external and internal calcium was also studied and compared with histamine. As shown in Figure 6, PMA-induced cAMP generation was unaffected when experiments were performed in Ca^{2+}-free buffer containing 1 mM EGTA to exclude external Ca^{2+} (Figure 6a), or when cells were pre-incubated with the Ca^{2+} chelator BAPTA to remove internal Ca^{2+} (Figure 6b). In contrast, histamine-induced cAMP generation was dependent on the presence of both external and internal Ca^{2+}.

Relationship between cAMP generation and inhibition of degranulation

To assess the causal relationship between PMA-induced inhibition of degranulation and induction of cAMP production, the concentration–response profiles of the two events were determined and compared. As shown in Figure 7, there was a fairly good agreement between the concentration–effect profile of PMA-induced inhibition of both EPO and ECP releases and that of the stimulation of cAMP. However, both the threshold concentration (0.1 nM) and the IC_{50} value for degranulation (3 and 5 nM for EPO and ECP, respectively)

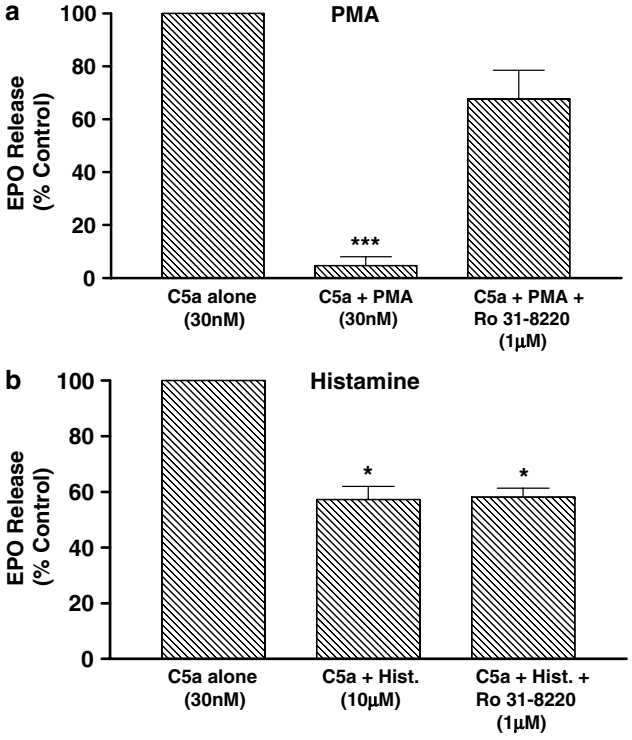

Figure 2 Reversal by the PKC inhibitor Ro 31-8220 (1 μM) of the inhibition of EPO release induced by 30 nM PMA (a) or 10 μM histamine (b). EPO release was stimulated with 30 nM C5a in the presence of CB (5 μg ml^{-1}). Eosinophils were pre-incubated with the inhibitor for 10 min before the addition of CB, followed 3 min later by PMA or histamine. After a further 5 min, cells were stimulated with C5a. Values are means ± s.e. for five experiments each. The net uninhibited release was 27.2 ± 6.0% of the total cell EPO content. At the concentration used, Ro 31-8220 had no significant effect of its own on C5a-induced EPO release. $^*P<0.05$; $^{***}P<0.001$ (compared with control).

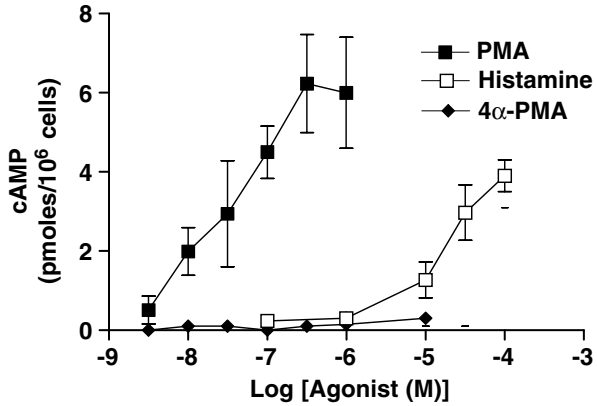

Figure 3 Stimulation of cAMP production in human eosinophils by PMA, its inactive analogue, 4α-PMA, and histamine. Cells were pre-incubated with rolipram (5 μM) for 5 min before being stimulated for another 3 min with the agonists. Values are means ± s.e. for 6–8 experiments. Basal releases (in the presence of rolipram alone) of 0.2–0.3 pmol 10^6 cells^{-1} have been subtracted from all values.

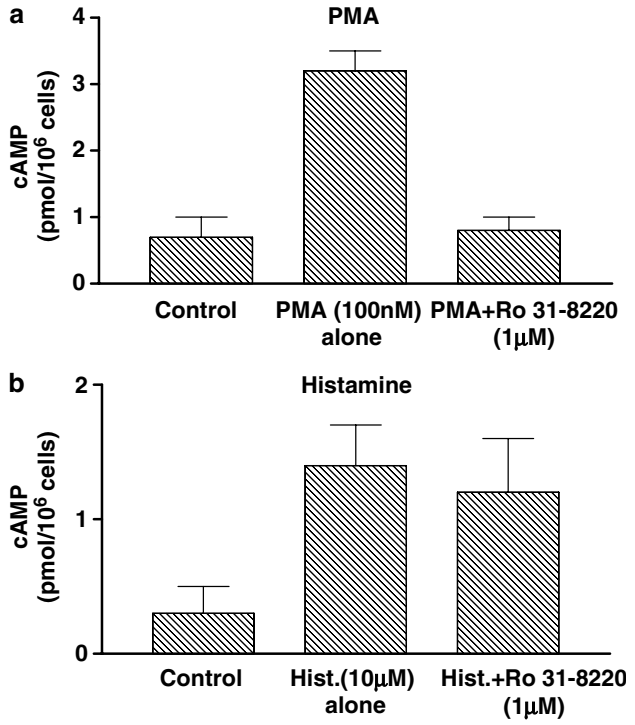

Figure 4 Inhibition by the PKC inhibitor Ro 31-8220 (1 μM) of the cAMP production stimulated by 100 nM PMA (a) or 10 μM histamine (b). Eosinophils were pre-incubated with the inhibitor for 10 min, followed by 5 min pre-incubation with rolipram (5 μM) before being stimulated with the agonists for an additional 3 min. Values are means $\pm$ s.e. for 5–6 experiments each.

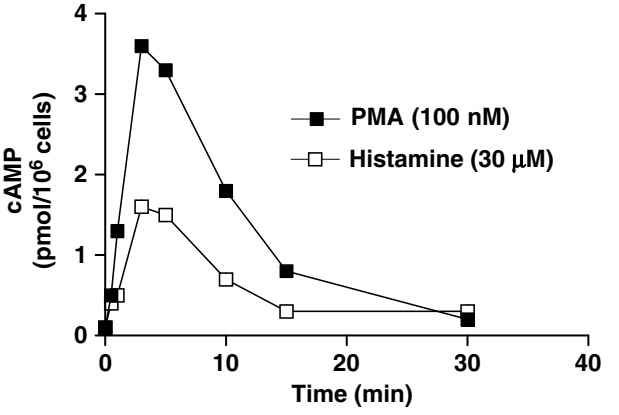

Figure 5 Time-course of cAMP production induced by PMA (100 nM) and histamine (30 μM). Values are means of duplicates of one experiment representing three others. Basal releases (in the presence of rolipram alone) of 0.2–0.4 pmol 10^6 cells^{-1} have been subtracted from all values.

were approximately one order of magnitude lower than the corresponding values for the stimulation of cAMP (threshold: 1 nM; EC_{50}: 55 nM).

Involvement of PKC isoforms in the stimulation of cAMP synthesis and inhibition of degranulation

To determine which PKC isoforms may be involved, we first determined the expression of the different isoforms in these cells. Western blot analysis of whole-cell lysates, using specific antibodies to eight PKC isoforms (α, βI, βII, γ, δ, ε, ζ and ι) revealed the expression of six isoforms: three classical (α, βI and βII), one novel (δ) and two atypical (ι and ζ) isoforms (Figure 8).

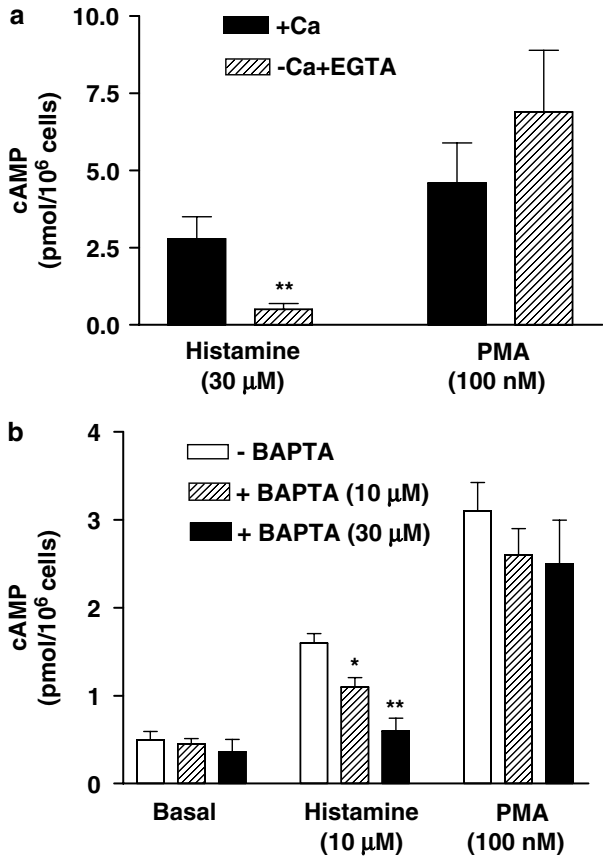

Figure 6 The requirement for external and internal Ca^{2+} in the stimulation of cAMP production by PMA and histamine. (a) Effect of removing external Ca^{2+} with EGTA (1 mM) in calcium-free buffer. (b) Effect of removing internal Ca^{2+} with the chelator BAPTA (10, 30 μM). Cells were incubated with BAPTA for 10 min before stimulation. Values are means $\pm$ s.e. for 4–6 experiments.

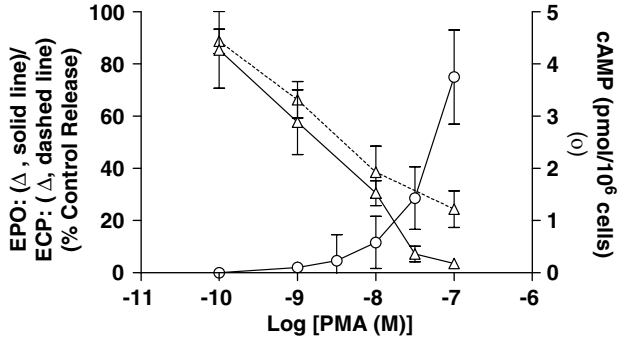

Figure 7 Correlation of PMA-stimulated cAMP generation with its inhibitory effect on degranulation (EPO and ECP release). Control (uninhibited) releases were 23.6 $\pm$ 3.8 and 27.2 $\pm$ 6.8% of total cell content for EPO and ECP, respectively. The cAMP values are net releases. Values are means $\pm$ s.e. for 4–6 experiments for each parameter.

The involvement of the individual isoforms in the PMA-stimulated cAMP production and the inhibition of degranulation were then studied using isoform-selective and nonselective PKC inhibitors. As shown in Figure 9a, treatment of eosinophils with the specific, but isoform nonselective, PKC inhibitor, Ro 31-8220 (0.01–1.0 μM), resulted in a profound concentration-dependent inhibition of PMA-induced cAMP production, with 1.0 μM achieving almost complete inhibition. This effect of Ro 31-8220 was mimicked by Gö 6983 (0.1 μM) – an inhibitor of all PKC isoforms, except PKCμ. A similar treatment with the selective inhibitor of the classical isoforms and PKCμ, Gö 6976 (0.01–0.1 μM), had no effect, whereas the PKCδ-selective inhibitor, rottlerin, significantly inhibited the response at 3 and 10 μM. The ability of these inhibitors to reverse PMA-inhibited EPO and ECP releases followed a similar trend (Figure 9b). Although the reversal by 3 μM rottlerin of PMA-inhibited EPO release did not reach statistical significance, PMA-inhibited ECP release was significantly reversed, $P<0.05$. Higher concentrations of this inhibitor could not be used in the EPO experiments because, at such concentrations, it had significant inhibitory effects of its own on eosinophil degranulation (data not shown).

Our attempt to determine if PMA caused the translocation of PKCδ as an index of its activation was technically unsuccessful, because we found that, in unstimulated eosinophils, this isoform was localized both in the cytoplasm and membrane.

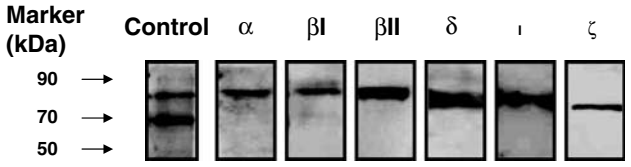

Figure 8 Western blot analysis of the expression of PKC isoforms in human peripheral blood eosinophils from a single patient, representative of six others. Rat brain extract was run as positive control and blotted with nonspecific PKC antibody. Exposure times for the ELC reaction were $\leqslant$1 min for PKCs δ and ζ and 60 min for all other isoforms.

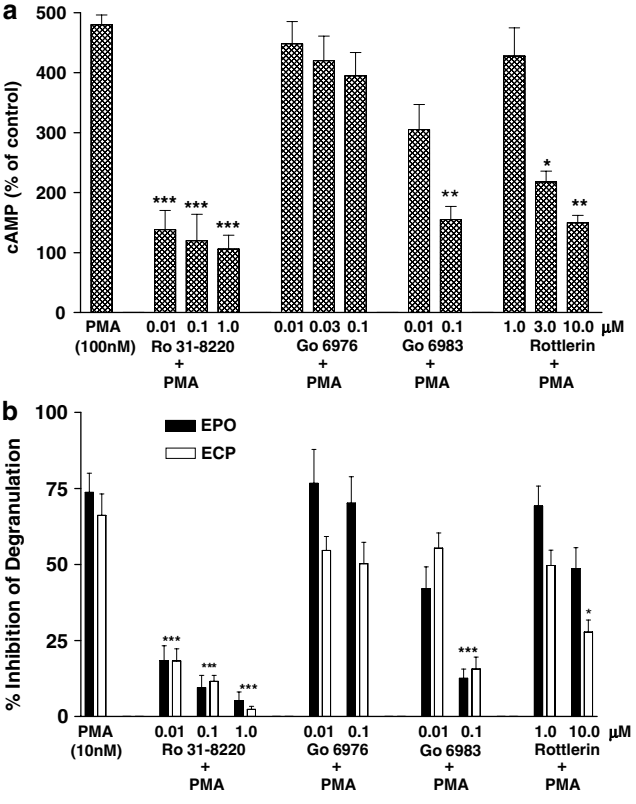

Figure 9 The ability of selective and nonselective PKC inhibitors to reverse the effects of PMA on human eosinophils. (a) Effect of the inhibitors on PMA-stimulated cAMP production. (b) Reversal of PMA-inhibited release of EPO and ECP. For the cAMP response, rolipram (5 μM) was added with the inhibitor, followed 10 min later by PMA. For degranulation, CB (5 μg ml^{-1}) was added 5 min before PMA and, after a further 5 min, C5a (30 nM) was added. At the concentrations used, the inhibitors had no significant effects of their own on the responses being measured. Values are means$\pm$s.e. for 4–6 experiments. ***$P<0.001$; **$P<0.01$; *$P<0.05$ (compared with PMA alone).

Discussion

This study investigated the PKC-dependent inhibition of C5a-induced degranulation of human peripheral blood eosinophils by PMA and the role of intracellular cAMP. In agreement with previous studies (Ali *et al*., 1994; Kroegel *et al*., 1994), we have shown here that PMA is an extremely potent inhibitor of human eosinophil degranulation. With an IC_{50} value of 3–5 nM, it is, perhaps, the most potent inhibitor of eosinophil degranulation known. Although PMA is also a potent inducer of superoxide ions (O_2^-), the observed inhibition of degranulation was not a consequence of the released EPO being consumed by the concurrently released O_2^- (and consequently H_2O_2). This is because eosinophils do not release O_2^- in the presence of microtubule depolymerizers such as CB. Furthermore, the release of another granule protein, ECP, which does not interact with O_2^-, was equally inhibited.

The inhibitory effect of PMA on degranulation appears to be PKC-mediated, since it was almost completely abolished by pretreatment of the cells with low concentrations of the specific PKC inhibitor Ro 31-8220. This is further supported by the fact that 4α-PMA – the non-PKC-activating analogue of PMA – had no such effect.

More importantly, the current study also revealed, for the first time, that PKC activation may bring about the down-regulation of eosinophil degranulation by stimulating the generation of intracellular cAMP. A number of studies have previously implicated PKC activation in the mediation of increased cAMP generation *via* stimulation of certain AC isoforms (Jacobowitz & Iyengar, 1994; Kawabe *et al*., 1994). However, in many whole-cell systems, such PKC activation often enhanced cAMP generation induced by other agonists/stimuli such as PGE_1, isoprenaline or forskolin (Tsu & Wong, 1996; Lin & Chen, 1998; Lin *et al*., 1999). Whether PKC activates AC directly by phosphorylating it or whether this is done indirectly *via* phosphorylation and inhibition of the inhibitory Gi protein (to release its brake on Gs) (Taussig *et al*., 1993) remains to be clarified. However, in the present study, PMA was clearly able to stimulate pronounced cAMP generation in the absence of any external agonist or additional stimulus, thus suggesting that direct AC activation by PKC was more likely. Furthermore, the possible involvement of

PKC-mediated inhibition of phosphodiesterases to raise cAMP levels can be ruled out because this response did not occur at all in the absence of phosphodiesterase inhibitors such as rolipram or isobutyl-methyl xanthine.

Studies characterizing PMA-stimulated generation of cAMP showed that the response was PKC-mediated, since it was abolished when the cells were pretreated with the specific PKC (but PKC isoform nonselective) inhibitor, Ro 31-8220 (0.1–1.0 μM). In contrast, such treatment had no effect on histamine-stimulated cAMP production. The PMA-stimulated cAMP production had a fast time-course ($t_{1/2} = 1.7$ min), which was similar to that of histamine. However, the response differed radically from that of histamine in being completely independent of both external and internal Ca^{2+}. This suggests that the PKC pathway utilized by PMA to activate AC is distinct from the Gs–AC pathway utilized by histamine. It may also suggest that the PKC isoforms involved in the PMA-stimulated cAMP response may be those that are Ca^{2+}-insensitive, essentially the novel PKC isoforms, since the classical isoforms are Ca^{2+}-sensitive and the atypical isoforms are not activated by PMA (Nishizuka, 1995). This is consistent with a previous report showing that the novel PKCs ε and μ were utilized by PMA to augment PGE_1-stimulated cAMP production in RAW 264.7 macrophages (Lin & Chen, 1998). The difference in Ca^{2+} requirement between PMA- and histamine-stimulated cAMP production may also lie in the AC isoforms activated by the two pathways, since some of the PKC-responsive AC isoforms, such as AC II, AC IV and perhaps AC VIII, are Ca^{2+}-insensitive (Cooper *et al*., 1995).

To determine which PKC isoforms may be involved in the PMA-stimulated cAMP generation, we first examined the expression of the different isoforms in whole-cell lysates of eosinophils by Western blotting. We detected the expression of three classical (α, βI and βII), one novel (δ) and two atypical (ι and ζ) isoforms. This is in general agreement with those recently reported by two other groups (Evans *et al*., 1999; Bankers-Fulbright *et al*., 2001), except that we did not detect the γ isoform as did Bankers-Fulbright *et al*. (2001), but not Evans *et al*. (1999), nor the ε isoform as did Evans *et al*. (1999), but not Bankers-Fulbright *et al*. (2001). These minor differences in the reported expression of PKC isoforms may reflect the quality/sensitivity of the specific antibodies used, or the activation status of the eosinophil populations used in the different studies.

Subsequently, isoform-selective and nonselective PKC inhibitors were employed to investigate the involvement of the individual PKC isoforms in the observed PMA effects. Particular attention was paid to the choice of inhibitors and their concentrations to avoid nonspecific effects common to enzyme inhibitors. At $\leqslant 1\,\mu$M, Ro 31-8220 is considered to be a general PKC-specific, but PKC isoform nonselective, inhibitor, though less activity against atypical PKCs has been reported (Yeo & Exton, 1995). In the concentration range 1–100 nM, Gö 6976 is selective for the classical PKC isoforms, with some activity against PKCμ as well (Martiny-Baron *et al*., 1993; Gschwendt *et al*., 1996), while its analogue Gö 6983 appears to inhibit all isoforms, except PKCμ (Gschwendt *et al*., 1996). Rottlerin is considered a selective inhibitor of PKCδ with an IC_{50} value of 3–6 μM (Gschwendt *et al*., 1994).

Results obtained using the above PKC inhibitors suggest that PKCδ was involved in mediating the effect of PMA on cAMP production. Several pieces of evidence led to this conclusion. Firstly, the effect of PMA was blocked by the isoform nonselective PKC inhibitor Ro 31-8220 and the PKCμ-sparing PKC inhibitor Gö 6983, but not by Gö 6976, which is a selective inhibitor of the classical isoforms (α, βI, βII and γ) and PKCμ (Martiny-Baron *et al*., 1993; Gschwendt *et al*., 1996). This suggests that the classical PKCs, as well as PKCμ, do not play a significant role in the cAMP response. Even though we detected two atypical PKCs (ι and ζ) in our cells, their involvement in this response can be ruled out since PMA does not activate atypical PKCs (Nishizuka, 1995). This leaves the novel PKCs ε, δ, η and θ as the only isoforms likely to be involved, but the only novel isoform we detected was PKCδ. Secondly, rottlerin, which is a selective inhibitor of PKCδ, significantly inhibited the ability of PMA to stimulate cAMP production. Thirdly, the PMA-stimulated cAMP response was completely independent of external and internal Ca^{2+}. It is well known that among the PMA-sensitive PKC isoforms only the novel PKCs are Ca^{2+}-insensitive (Nishizuka, 1992; 1995). In a previous report, Lin & Chen (1998) showed that PMA-induced potentiation of PGE_1-stimulated cAMP generation in RAW 264.7 macrophages was mediated by novel PKCs. However, they implicated the μ or ε isoforms rather than the δ isoform. The difference between their result and ours is likely to be cell-specific.

Given the above pieces of evidence, we propose that the PKCδ-mediated stimulation of AC to generate cAMP may be the hitherto unknown mechanism by which PMA down-regulates eosinophil degranulation. Although a direct cause and effect relationship between inhibition of degranulation and elevation of cAMP is difficult to prove, several pieces of evidence suggest that this is likely to be the case. Firstly, intracellular cAMP is a well-known second messenger with powerful inhibitory effect on eosinophil degranulation (Kita *et al*., 1991). Secondly, there is a good correlation between PMA-induced increase in intracellular levels of cAMP and inhibition of degranulation. Although the IC_{50} for inhibition of degranulation, 3–5 nM, appeared to be 10–18 times less than the EC_{50} for the induction of cAMP (55 nM) generation, this can be accounted for by signal amplification that usually occurs in signal transduction mechanisms. Thirdly, the rapid time-course of PMA-stimulated cAMP generation ($t_{1/2} = 1.7$ min) places it well ahead of degranulation ($t_{1/2} \approx 15$ min). Temporally, this is consistent with a role for a cAMP-sensitive step in the events leading to degranulation. Finally, PKC inhibitors that blocked PMA-induced cAMP generation also significantly reversed the PMA-mediated inhibition of degranulation.

Activation of PKC is a common event in the signalling mechanisms of many cells. Perhaps, the key significance of PKC-stimulated cAMP generation is that it may act as an important braking system by which cells limit or terminate responses to cellular activation. In the case of eosinophils where PKC also mediates O_2^- production (Bankers-Fulbright *et al*., 2001; Takizawa *et al*., 2003), it may be a crucial innate mechanism that prevents the concurrent release of O_2^- and EPO – two products whose interaction leads to the generation of highly toxic hypohalous acids.

In summary, this study has confirmed that PMA, *via* PKC activation, potently inhibits C5a-induced degranulation of human eosinophils. It also shows, for the first time, that PMA is able to stimulate pronounced cAMP generation in human eosinophil in the absence of any external agonist or additional

stimulus and that this effect appears to be mediated *via* activation of the novel PKCδ isoform. We, therefore, propose that PKCδ-dependent activation of AC and the consequent increase in the intracellular cAMP levels may be the hitherto unknown mechanism by which PMA inhibits human eosinophil degranulation. Given the central role of PKC in signal transduction mechanisms, PKC-mediated generation of cAMP may have a wider significance as a counter-regulatory system that checks excessive response (or toxic consequences) to cellular activation.

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References

ALI, A., FORD-HUTCHINSON, A.W. & NICHOLSON, D.W. (1994). Activation of protein kinase C down-regulates leukotriene synthase activity and attenuates cysteinyl leukotriene production in an eosinophilic sub-strain of HL-60 cells. *J. Immunol.,* **153,** 776–788.

BANKERS-FULBRIGHT, J.L., KITA, H., GLEICH, G.J. & O'GRADY, S.M. (2001). Regulation of human eosinophil NADPH oxidase activity: a central role for PKCδ. *J. Cell. Physiol.,* **189,** 306–315.

CHOI, E.J., WONG, S.T., DITTMAN, A.H. & STORM, D.R. (1993). Phorbol ester stimulation of the type I and type II adenylyl cyclases in whole cells. *Biochemistry,* **32,** 1891–1894.

COOPER, D.M.F., MONS, N. & KARPEN, J.W. (1995). Adenylyl cyclases and the interaction between calcium and cAMP signaling. *Nature,* **374,** 421–424.

DEKKER, L.V. & PARKER, P.J. (1994). Protein kinase c – a question of specificity. *Trends Biochem. Sci.,* **19,** 73–77.

EVANS, D.J., LINDSAY, M.A., WEBB, B.L.J., KANKAANRANTA, H., GIEMBYCZ, M.A., O'CONNOR, B.J. & BARNES, P.J. (1999). Expression and activation of protein kinase C-ς in eosinophils after allergen challenge. *Am. J. Physiol.,* **277,** L233–L239.

EZEAMUZIE, C.I. & PHILIPS, E. (1999). Adenosine A_3 receptors on human eosinophils mediate inhibition of degranulation and superoxide anion release. *Br. J. Pharmacol.,* **127,** 188–194.

GIEMBYCZ, M.A. & LINDSAY, M.A. (1999). Pharmacology of eosinophils. *Pharmacol. Rev.,* **51,** 213–340.

GLEICH, G.J. & ADOLPHSON, C.R. (1999). The eosinophil and bronchial asthma: evidence for a critical role of eosinophils in pathophysiology. In: *Interlukin-5 from Molecule to Drug Target for Asthma,* ed. Lenfant C. pp. 1–37. New York: Marcel-Dekker.

GSCHWENDT, M., DIETERICH, B., RENNECKE, J., KITTSTEIN, W., MULLER, H.J. & JOHANNES, F.J. (1996). Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes. *FEBS Lett.,* **392,** 77–80.

GSCHWENDT, M., MULLER, H.J., KIELBASSA, K., ZANG, R., KITTSTEIN, W., RINCKE, G. & MARKS, F. (1994). Rottlerin, a novel protein kinase inhibitor. *Biochem. Biophys. Res. Commun.,* **199,** 93–98.

GUSOVSKY, F. & GUTKIND, J.S. (1991). Selective effects of the activation of PKC isozymes on camp accumulation. *Mol. Pharmacol.,* **39,** 124–129.

HANSEL, T.T., De VRIES, I.J.M., IFF, T., RIS, S., WANDZILAK, M., BETZ, S., BLASER, K. & WALKER, C. (1991). An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J. Immunol. Methods,* **145,** 105–110.

JACOBOWITZ, O. & IYENGAR, R. (1994). Phorbol ester-induced stimulation and phosphorylation of adenylyl cyclase 2. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 10630–10634.

KAWABE, J., IWAMI, G., EBINA, T., OHNO, S., KATADA, T., UEDA, Y., HOMEY, C.J. & ISHIKAWA, Y. (1994). Differential activation of adenylyl cyclase by protein kinase C iso-enzymes. *J. Biol. Chem.,* **269,** 16554–16558.

KERNEN, P., WYMANN, M.P., VON TSCHARNER, V., DERANLEAU, D.A., TAI, P.C., SPRY, C.J., DAHINDEN, C.A. & BAGGIOLINI, M. (1991). Shape change, exocytosis and cytosolic calcium changes in stimulated human eosinophils. *J. Clin. Invest.,* **87,** 2012–2017.

KITA, H., ABU-GHAZALEH, R.I., GLEICH, G.J. & ABRAHAM, R.T. (1991). Regulation of Ig-induced eosinophil degranulation by adenosine 3′,5′-cyclic monophosphate. *J. Immunol.,* **146,** 2712–2718.

KROEGEL, C., GIEMBYCZ, M.A., MATTHYS, H., WESTWICK, J. & BARNES, P.J. (1994). 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.,* **11,** 593–599.

KROEGEL, C., YUKAWA, T., DENT, G., VENGE, P., CHUNG, K.F. & BARNES, P.J. (1989). Stimulation of degranulation from eosinophils by platelet activating factor. *J. Immunol.,* **142,** 3518–3526.

LAI, H.L., YANG, T.H., MESSING, R.O., CHING, Y.H., LIN, S.C. & CHERN, Y. (1997). Protein kinase c inhibits adenylyl cyclase type VI activity during desensitization of the A2a-adenosine receptor-mediated camp response. *J. Biol. Chem.,* **272,** 4970–4977.

LIN, W.-W., CHANG, S.-H. & WANG, S.-M. (1999). Roles of atypical protein kinase C in lysophosphatidic acid-induced type II adenylyl cyclase activation in RAW 264.7 macrophages. *Br. J. Pharmacol.,* **128,** 1189–1198.

LIN, W.-W. & CHEN, B.C. (1998). Distinct isoforms mediate the activation of cPLA2 and adenylyl cyclase by phorbol ester in RAW264.7 macrophages. *Br. J. Pharmacol.,* **125,** 1601–1609.

LYNCH, O.T., GIEMBYCZ, M.A., BARNES, P.J., HELLEWELL, P.G. & LINDSAY, M.A. (1999). 'Outside-in' signaling mechanisms underlying CD11b/CD18-mediated NADPH oxidase activation in human adherent blood eosinophils. *Br. J. Pharmocol.,* **128,** 1149–1158.

MARTINY-BARON, G., KAZANIETZ, M.G., MISCHAR, H., BLUMBERG, F.M., KOCHS, G., HUG, H., MARME, D. & SCHACHTELE, C. (1993). Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. *J. Biol. Chem.,* **268,** 9194–9197.

MOTOJIMA, S., FAKUDA, T. & MAKINO, S. (1998). Eosinophil activation in asthma. *Lung Biol. Health Dis.,* **117,** 179–204.

NISHIZUKA, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science,* **258,** 607–613.

NISHIZUKA, Y. (1995). Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.,* **9,** 484–496.

ROBINSON, C. & CARVER, J.E. (1998). Interactions between eosinophils and the airway epithelium. *Lung Biol. Health Dis.,* **117,** 263–286.

ROBINSON, D.S. (1999). Interlukin-5 and the interleukin 5 receptor in asthma. *Lung Biol. Health Dis.,* **125,** 51–68.

SEDGWICK, J.B., GEIGER, K.M. & BUSSE, W.W. (1990). Superoxide generation by hypodense eosinophils from patients with asthma. *Am. Rev. Respir. Dis.,* **142,** 120–125.

TAKIZAWA, T., KATO, M., SUZUKI, M., TACHIBANA, A., MOTEGI, Y., FUJIU, T., KIMURA, H., ARAKAWA, H., MOCHIZUKI, H., TOKUYAMA, K. & MORIKAWA, A. (2003). Distinct isoforms of PKC are involved in human eosinophil functions induced by platelet activating factor. *Int. Arch. Allergy Immunol.,* **131** (Suppl 1), 15–19.

TAUSSIG, R., INIGUEZ-LLUHI, J.A. & GILMAN, A.G. (1993). Inhibition of adenylyl cyclase by Gi alpha. *Science,* **261,** 218–221.

TSU, R.C. & WONG, Y.H. (1996). Gi-mediated stimulation of type II adenylyl cyclase is augmented by Gq-coupled receptor activation and phorbol ester treatment. *J. Neurosci.,* **16,** 1317–1323.

VAN DER BRUGGEN, T. & KOENDERMAN, L. (1996). Signal transduction in eosinophils. *Clin. Exp. Allergy,* **26,** 880–891.

YEO, E.J. & EXTON, J.H. (1995). Stimulation of phospholipase D by epidermal growth factor requires protein kinase C activation in Swiss 3T3 cells. *J. Biol. Chem.,* **270,** 3980–3988.

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