Biosynthesis of paf-acether

Activators of protein kinase C stimulate cultured mast cell acetyltransferase without stimulating paf-acether synthesis

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Antigen stimulation of cultured bone-marrow-derived mast cells sensitized with specific monoclonal IgE induced cell degranulation and paf-acether (paf; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) biosynthesis via the deacylation/acetylation (remodelling) pathway. Phorbol myristate acetate (PMA; 20–100 ng/ml) triggered only acetyltransferase activation, without concomitant lyso-paf (1-*O*-alkyl-*sn*-glycero-3-phosphocholine) and paf formation. A low concentration of PMA (5 ng/ml) potentiated antigen-induced degranulation, acetyltransferase activation and paf formation by about 30%, but did not change the level of lyso-paf formation. Stimulation of mast cells with antigen increased intracellular Ca^{2+} from 61 to 269 nM, whereas no modification of Ca^{2+} influx was observed when cells were pretreated with PMA (5 ng/ml) before antigen challenge. Gas chromatography coupled to electron capture detection revealed that the composition of paf formed by cells stimulated by antigen alone was similar to that of paf formed by PMA-primed antigen-stimulated cells; $84 \pm 8\%$ and $79 \pm 2\%$ (means $\pm$ S.E.M., $n = 3$) of molecules respectively bore the $C_{16:0}$ alkyl chain moiety, with the remainder bearing essentially $C_{18:0}$ molecules. Overnight treatment of mast cells with PMA (200 ng/ml) caused disappearance of protein kinase C (PKC) from both cytosol and membranes. When such cells were stimulated further with antigen, they failed to degranulate, and acetyltransferase activation, paf production and lyso-paf production were decreased by $33 \pm 11\%$, $57 \pm 4\%$ and $96 \pm 3\%$ respectively ($n = 3$ or 5). The PKC inhibitors chlorpromazine and staurosporine inhibited to a significant extent both cell degranulation and all steps leading to paf biosynthesis. Our data suggest that PKC-dependent mechanisms are operational during cell degranulation and contribute only in part to paf biosynthesis. The PKC-dependent signal directly generated by PMA or diacylglycerol is not sufficient to trigger the full cell response, which is obtained only through receptor-operated antigen challenge.

INTRODUCTION

It has been substantiated that the biosynthesis of paf-acether [paf; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (1-*O*-alkyl-2-acetyl-GPC)] in stimulated pro-inflammatory cells is regulated by the level and the degree of activation of 1-*O*-alkyl-GPC:acetyl-CoA acetyltransferase (EC 2.3.1.67) (reviewed in [1,2]). This tightly membrane-bound enzyme [3–5] is present in its 'pro' form in unstimulated cells [6] and is phosphorylated into the active form during specific cell stimulation [7]. It has been shown that mouse bone-marrow-derived mast cells passively sensitized with the monoclonal IgE anti-[dinitrophenyl (DNP)-BSA] were able to form paf [8] and to release the granular enzyme β -*N*-acetylhexosaminidase (β -Hex) during antigen challenge [9]. We correlated this antigen-induced paf formation with the activation of acetyltransferase, i.e. phosphorylation of the enzyme, and we showed that the treatment of cells with the protein kinase C (PKC) activator phorbol myristate acetate (PMA) increased acetyltransferase activity [7].

The membrane PKC activity is markedly increased after antigen challenge of the mast cell line PT-18 [10]. We show here that antigen challenge of cultured mast cells induces a rapid loss of cytosolic PKC, concomitant with the appearance of membrane-bound enzyme. Thus we compared paf biosynthesis

and β -Hex release (as an index of mast cell degranulation) triggered by either PMA or the diacylglycerol analogue 1,2-dioctanoyl-*sn*-glycerol (DAG) associated or not with antigen. We demonstrated a lack of paf formation and β -Hex release during PMA challenge; by contrast, treatment of mast cells with a low concentration of PMA before antigen challenge potentiated paf formation, acetyltransferase activation and β -Hex release. To assess the role of PKC during antigen challenge, mast cells were cultured for 18–24 h with PMA and paf biosynthesis was immunologically triggered. Our results suggest that PKC is required in signal transduction leading to β -Hex release, but only in part for paf synthesis, in immunologically stimulated mast cells.

This work has been presented in part as a preliminary report at the 71st Annual Meeting of The Federation of American Societies for Experimental Biology (Washington, DC, 29 March–2 April 1987) [11].

MATERIALS AND METHODS

Reagents

The following reagents were obtained as from the sources given: fetal calf serum, RPMI 1640, non-essential amino acids,

Abbreviations used: β -Hex, β -*N*-acetylhexosaminidase; DAG, 1,2-dioctanoyl-*sn*-glycerol; GPC, *sn*-glycero-3-phosphocholine; paf, paf-acether (1-*O*-alkyl-2-acetyl-GPC; originally described as platelet-activating factor); paf- $C_{16:0}$, 1-*O*-hexadecyl-2-acetyl-GPC; paf- $C_{18:0}$, 1-*O*-octadecyl-2-acetyl-GPC; lyso-paf, 1-*O*-alkyl-GPC; lyso-paf- $C_{16:0}$, 1-*O*-hexadecyl-GPC; lyso-paf- $C_{18:0}$, 1-*O*-octadecyl-GPC; PDD, 4 α -phorbol 12,13-didecanoate; PMA, 4 β -phorbol 12-myristate 13-acetate; PKC, protein kinase C; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DTT, dithiothreitol; DNP, dinitrophenyl.

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L-glutamine, penicillin and streptomycin (Biochrom Kg, Berlin, Germany); 2-mercaptoethanol, gelatin, concanavalin A, acetyl-CoA, fatty acid-free BSA, phosphocreatine, creatine, *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide, PMA, 4 α -phorbol 12,13-didecanoate (PDD), DAG, phospholipase C from *Clostridium welchii*, 1-*O*-hexadecyl-2-*O*-methyl-GPC, histone H₁, phosphatidylserine, diolein, leupeptin, dithiothreitol (DTT), staurosporine, chlorpromazine, EGTA, EDTA and sucrose (Sigma Chemical Co., St. Louis, MO, U.S.A.); heptafluorobutyric anhydride (Pierce, Rockford, IL, U.S.A.); 2,4-dinitrobenzenesulphonic acid (Eastman Kodak Co., Rochester, NY, U.S.A.); aspirin (lysine salt) (Aspegic; Egic, Amilly, France); 1-*O*-octadecyl-2-acetyl-GPC (paf-C_{18:0}), 1-*O*-hexadecyl-2-acetyl-GPC (paf-C_{16:0}) and 1-*O*-octadecyl-GPC (lyso-paf-C_{18:0}) (Bachem, Bubendorf, Switzerland); fura-2/AM (Calbiochem, San Diego, CA, U.S.A.); [*N*-methyl-¹⁴C]paf-C_{16:0} (58 μ Ci/mmol), [³H]acetyl-CoA (6 Ci/mmol) and Aquasol (New England Nuclear, Boston, MA, U.S.A.). [γ -³²P]ATP (20 Ci/mmol) was purchased from Amersham International. DEAE-cellulose (DE 52) was obtained from Whatman. DNP-BSA antigen was prepared according to [12] and stored at -20 °C. The concentration of DNP-BSA and the extent of substitution (DNP₁₃-BSA) were calculated using the molar absorption coefficient of DNP-L-lysine at 360 nm (17350) [12]. The monoclonal DNP-specific IgE-secreting hybridoma (line 2682-1) was grown as ascites as in [13].

Bone-marrow-derived mast cells

Bone marrow cells from tibias and femurs of BALB/c mice (Iffa Credo, L'Arbresle, France) were cultured in a 1:1 (v/v) mixture of conditioned medium and RPMI supplemented with 10% (v/v) fetal calf serum, 0.1 mM non-essential amino acids, 2 mM-L-glutamine, 50 μ M-2-mercaptoethanol, 50 units of penicillin/ml and 50 μ g of streptomycin/ml (enriched medium) [14]. The conditioned medium used for the differentiation of bone marrow cells into mast cells was the supernatant of 2-day mixed lymphocyte cultures (C57BL₆/C_{3H}/He) performed in the presence of 2 μ g of concanavalin A/ml as in [14]. Cells were passaged weekly, and by 21 days of culture were composed of a homogeneous mast cell population. To replicate tubes, each containing 100 μ l of the cell suspension (1×10^6 cells), was added 100 μ l of a 1:100 dilution of ascites fluid containing monoclonal IgE anti-DNP-BSA. After a 1 h incubation at 37 °C in a humidified atmosphere of air/CO₂ (19:1), the cells were washed twice by centrifugation at 400 *g* for 5 min in a modified Tyrode's solution containing 1.3 mM-CaCl₂, 0.3 mM-MgCl₂ and 0.1% (w/v) gelatin. The cells were finally resuspended in 0.45 ml of a modified Tyrode's solution containing 1.3 mM-CaCl₂, 0.3 mM-MgCl₂ and 0.25% (w/v) BSA. Sensitized mast cells were added with defined doses of DNP-BSA, and non-sensitized cells were challenged with PMA, DAG or PDD. Incubations were carried out for given time intervals and stopped by cooling at 4 °C. Cell suspensions were centrifuged and supernatants were collected for assay of β -Hex release. Cell pellets were resuspended in 150 mM-NaCl and sonicated at 4 °C prior to β -Hex and acetyltransferase determinations. In other experiments, passively sensitized mast cells were pretreated for 3 min at 37 °C with 5 ng of PMA/ml or for 15 min with chlorpromazine (10 μ M) or staurosporine (1 μ M) prior to antigen challenge. When paf and lyso-paf were to be measured, cell suspensions were extracted with 4 vol. of absolute ethanol. After centrifugation (1100 *g*, 10 min), pellets were discarded and supernatants were divided into two parts and brought to dryness under an air stream at 40 °C. The dry residues were either resuspended in 150 mM-NaCl containing 0.25% BSA (pH 7.4) or ethanol (60%) to assess paf content, or kept dried (-20 °C) until further analysis of paf molecular species of lyso-

paf content. In some experiments, mast cells replenished with fresh culture medium (35 ml/flask) were treated with PMA (200 ng/ml) or vehicle (0.2% dimethyl sulphoxide) for 18–24 h. Such cells were depleted of PKC [15].

The activity of lactate dehydrogenase in the supernatants from stimulated and unstimulated cells was less than 5% of the total, as measured by the method of Wroblewski & LaDue [16].

Acetyltransferase assay

The acetyltransferase activity in lysates obtained from cells stimulated or not with defined agonist was measured as in [6]. Briefly, the cell lysate (70 μ l), corresponding to 0.56×10^6 cells, was added to a reaction mixture (final volume 0.5 ml, pH 7.0) which contained 4.2 mM-Hepes, 137 mM-NaCl, 2.6 mM-KCl, 0.65 mM-CaCl₂, 0.5 mM-MgCl₂, 0.2% BSA, 200 μ M-[³H]acetyl-CoA (0.5 μ Ci/100 nmol) and 40 μ M-lyso-paf. The reaction was carried out for 15 min at 37 °C and then stopped by addition of 2 ml of methanol/dichloromethane (1:1, v/v) containing [¹⁴C]paf (3000 d.p.m.) as an internal standard. Specific activity was expressed in pmol of [³H]paf/min per 10^6 cells, with 100% of acetyltransferase activity at basal level (no stimulation) being 115 ± 11 pmol/min per 10^6 cells (mean $\pm$ S.E.M., *n* = 14).

Assay of PKC

PKC was isolated and assayed according to the method of Kraft *et al.* [17], as modified by Vilgrain *et al.* [18]. Briefly, mast cells (1.4×10^7) were homogenized in 500 μ l of ice-cold buffer (25 mM-Tris/HCl, pH 7.5, 2 mM-EDTA, 10 mM-EGTA, 0.25 M-sucrose, 2 mM-DTT and 50 μ g of leupeptin/ml) using a Potter-Elvehjem instrument with a Teflon pestle. The homogenate was centrifuged at 105 000 *g* for 45 min at 4 °C. After centrifugation, the supernatant (cytosol) and the pellet (membranes) were used for enzymic studies. For the PKC assay in the particulate fraction, the 105 000 *g* pellet was resuspended in 50 μ l of homogenization buffer containing 0.5% Triton X-100 at 4 °C for 30 min and then centrifuged at 10 000 *g* for 10 min. The cytosol and the pellet extract were applied to a DEAE-cellulose column (200 μ l) previously equilibrated with Tris/DTT buffer (20 mM-Tris/HCl, pH 7.5, 2 mM-EDTA, 2 mM-EGTA, 1 mM-DTT and 2% glycerol). After a column wash with 1 ml of the same buffer, the PKC was eluted with 400 μ l of Tris/DTT buffer containing 0.2 M-NaCl. The enzyme activity was assayed by measuring the transfer of ³²P from [γ -³²P]ATP to lysine-rich histone H₁. The reaction mixture (80 μ l) contained 0.01 mM-[³²P]ATP (specific radioactivity 1000 c.p.m./pmol), 20 μ g of histone/ml, 10 mM-MgCl₂ and 0.75 mM-CaCl₂. Phosphatidylserine (13 μ g) and diolein (0.8 μ g) were included or not in the assay, as indicated, using a lipid suspension in 100 mM-Tris/HCl, pH 7.5, previously sonicated at 30 °C for 5 min. The reaction was carried out at 30 °C for 10 min and stopped by addition of 12% trichloroacetic acid in the presence of casein (900 μ g) as a carrier. The protein precipitate was dissolved in 1 M-NaOH, and ³²P incorporation was measured by scintillation counting in Aquasol with a Kontron SL 3000 spectrometer.

Measurement of intracellular Ca²⁺ ([Ca²⁺]_i)

[Ca²⁺]_i was measured by using the Ca²⁺ indicator fura-2 as described by Grynkiewicz *et al.* [19]. Mast cells were loaded with 1 μ M-fura-2/AM for 30 min in Tyrode's buffer containing 0.25% BSA. Cells were then centrifuged and resuspended in the same buffer. Fluorescence measurements were performed at 37 °C in 1 ml mast cell samples with a spectrofluorophotometer (model RF-5000; Shimadzu, Kyoto, Japan), after addition of agonist, by the dual-wavelength (340 and 380 nm) excitation method. Cytoplasmic free Ca²⁺ concentrations were determined according to the ratio method [19].

Paf and lyso-paf assay and characterization

Paf content was measured by the rabbit platelet aggregation assay [20,21] by comparison with known amounts of synthetic paf. Rabbit platelets were treated with 0.1 mM-aspirin for 15 min at room temperature to avoid aggregation due to contaminating arachidonic acid. Aggregation was carried out in the presence of the ADP scavenger complex phosphocreatine (0.7 mM)/creatine kinase (13.9 units/ml). Paf content was calculated using a calibration curve established with synthetic paf. Results are expressed as pmol of paf/ 10^6 mast cells. The specific paf antagonist BN 52021 ([22], Institut Henri Beaufour, Le Plessis-Robinson, France) inhibited platelet aggregation induced by cell ethanolic extracts. Lyso-paf was measured after chemical acetylation into paf [23]. Briefly, dry ethanolic residues were incubated overnight with 200 μ l of pyridine and 200 μ l of acetic anhydride. Pyridine was then eliminated by mixing the samples with 1 ml of dichloromethane. The samples were dried and the procedure was repeated twice; the dry residue was suspended in 150 mM-NaCl supplemented with 0.25% BSA or 60% ethanol and assayed for paf activity using the bioassay as described above.

In selected experiments, paf and lyso-paf were analysed by gas chromatography electron capture detection. Paf and lyso-paf isolated from cells were purified by h.p.l.c. prior to the digestion with phospholipase C from *Clostridium welchii*. The resulting 1-*O*-alkyl-2-acetyl-*sn*-glycerol- and 1-*O*-alkyl-*sn*-glycerol- species were converted into heptafluorobutyrate derivatives [24], which were dissolved in toluene and analysed by gas chromatography using an OV-1 fused capillary column (25 m $\times$ 0.25 mm i.d.; Touzart et Matignon, Vitry sur Seine, France). The yield after the derivatization, purification and chromatographic steps was calculated by measuring the recovery of 1-*O*-hexadecyl-2-*O*-methyl-GPC.

β -Hex assay

β -Hex was measured in supernatants and sonicated cell pellets by monitoring the hydrolysis of *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide, as described in [25]. The net percentage β -Hex

release was calculated using the following formula: $[(S - S_c) \times 100] / [(S + P) - S_c]$, where *S* is the β -Hex activity in the pellet of challenged cells, *P* is the activity in the pellet of challenged cells and *S_c* is the activity in the supernatant of unchallenged cells.

RESULTS

Paf biosynthesis and β -Hex release

We compared paf biosynthesis and β -Hex release triggered by PMA (a potent activator of PKC) or antigen. The antigen (40 ng/ml)-induced stimulation led to paf (Fig. 1a) and lyso-paf (Fig. 1b) formation, acetyltransferase activation (Fig. 1c) and β -Hex release (Fig. 2a). In contrast, when the cells were stimulated with PMA (100 ng/ml), only the acetyltransferase activity was augmented (Fig. 1c); neither formation of paf (Fig. 1a) or lyso-paf (Fig. 1b) nor β -Hex release was observed (results not shown). The maximal amount of paf synthesized by the antigen-challenged mast cells was reached by 5 min and slowly diminished thereafter (Fig. 1a). In 11 experiments, this maximal amount of paf formed at 5 min was 23 ± 3 pmol/ 10^6 cells (mean $\pm$ S.E.M.). In some experiments, PMA concentrations were varied between 5 and 50 ng/ml, but no β -Hex release or paf formation was observed (results not shown). Next we examined the time course of the synthesis of lyso-paf, the direct precursor of paf (Fig. 1b). The level of lyso-paf varied in unstimulated cells from 11 to 90 pmol/ 10^6 cells (35 ± 7 pmol/ 10^6 cells; mean $\pm$ S.E.M., *n* = 11). When mast cells were stimulated with 100 ng of PMA/ml (and also with between 5 and 50 ng/ml; results not shown) relatively minor changes in the initial total lyso-paf content were observed (Fig. 1b). By contrast, following antigen stimulation, lyso-paf content increased between 1 min and 30 min. The apparent decrease observed between 3 and 5 min was not significant (by Wilcoxon test). However, we observed that the level of lyso-paf had diminished at 5 min in 9 out of 23 experiments and at 3 min in 6 out of 13 experiments.

In three separate experiments, stimulation of mast cells with PMA (100 ng/ml) for 15 min in the presence of lyso-paf [100 nM-

Fig. 1. Time course of paf (a) and lyso-paf (b) formation and acetyltransferase activation (c) in mast cells

Mast cells (1×10^6 in 0.5 ml) were challenged with antigen (40 ng/ml; ●), PMA (100 ng/ml; ▲) or PDD (100 ng/ml; □) at 37 °C. Results are means $\pm$ S.E.M. of three experiments, except for (b), where the numbers of experiments are indicated in parentheses. Where S.E.M. bars are omitted, the results are the means of two experiments. Control (100%) lyso-paf corresponded to unstimulated cells, which contained 35 ± 7 pmol/ 10^6 cells (*n* = 11). Control (100%) acetyltransferase activity was 115 ± 11 pmol/min per 10^6 cells (*n* = 14). In (b), the results showing antigen stimulation are significantly different from those with PMA stimulation; Wilcoxon analysis; * *P* < 0.001.

Fig. 2. Priming effect of PMA on antigen-induced β -Hex release (a), paf formation (b) and acetyltransferase activation (c)

Mast cells (1×10^6 cells in 0.5 ml) were incubated for 5 min at 37 °C with antigen (40 ng/ml) (●) or were pretreated with PMA (5 ng/ml) for 3 min at 37 °C and then challenged with antigen (40 ng/ml) for 5 min (○). Acetyltransferase activation, paf formation and β -Hex release were also measured in mast cells challenged with PMA (5 ng/ml) alone (□). Results are means $\pm$ S.E.M. of five or six experiments. The priming effect of PMA in all cases was significant (Wilcoxon test; $P < 0.001$).

Fig. 3. Acetyltransferase activation in mast cells by DAG

Mast cells (1×10^6 in 0.5 ml) were stimulated with the concentrations of DAG given for 10 min at 37 °C. Results are means $\pm$ S.E.M., $n = 3$.

10 μ M (the highest non-lytic concentration)] did not result in paf formation (results not shown). In contrast, acetyltransferase activity was increased; however, the time course of acetyltransferase stimulation with PMA was considerably slower than that observed following antigen challenge (Fig. 1c). The maximum of the PMA effect was reached at 10 min instead of 1–2 min (as for antigenic stimulation). The magnitude of PMA-induced stimulation was about twice that of the control, whereas antigen stimulated the enzyme activity by 3–4-fold. The inactive analogue of PMA, PDD (100 ng/ml), did not alter the acetyltransferase level (Fig. 1c), suggesting that the effect of PMA was specific for PKC activation. DAG, another PKC activator, stimulated acetyltransferase at concentrations between 50 and 150 μ g/ml (Fig. 3) without any associated paf formation or β -Hex release (results not shown).

Priming effect of PMA on paf biosynthesis and β -Hex release

The effect of pretreatment of passively sensitized mast cells with a low PMA concentration on acetyltransferase activity, lyso-paf and paf formation and release of β -Hex was studied. Incubation of cells with PMA (5 ng/ml) for 3 min did not result in an increase in acetyltransferase activity, paf synthesis or β -Hex release (Fig. 2). On challenging PMA-pretreated cells with an optimal concentration of antigen (40 ng/ml), we observed a potentiation of about 30% of acetyltransferase activity (Fig. 2c), paf formation (Fig. 2b) and release of β -Hex (Fig. 2a). In

Table 1. Effect of PMA (5 ng/ml, 3 min) pretreatment on antigen (40 ng/ml)-induced changes in lyso-paf content in mast cells

Lyso-paf was measured by gas chromatography. Results are expressed as % of control (no incubation; 44 ± 17 pmol/10⁶ cells) and are means $\pm$ S.E.M. ($n = 3$).

Time (s)	Lyso-paf content (% of control)	
	Antigen	PMA + antigen
30	77 $\pm$ 25	118 $\pm$ 29
60	130 $\pm$ 33	125 $\pm$ 16
180	139 $\pm$ 18	102 $\pm$ 49
300	83 $\pm$ 35	83 $\pm$ 30

Table 2. Distribution of PKC activity between cytosolic and particulate fractions during cell stimulation: comparison with acetyltransferase activity, paf and lyso-paf formation and β -Hex release

Sensitized mast cells were incubated with DNP-BSA (40 ng/ml) for 5 min or treated with PMA (200 ng/ml) for 18–24 h. PKC and acetyltransferase activities, paf and lyso-paf formation and β -Hex release were assessed as described in the Materials and methods section. Results are means $\pm$ S.E.M. of three to five experiments (* $n = 2$). Values given in parentheses are percentage inhibition, calculated as follows: $100 \times [1 - (S_i - N_i)/(S - N)]$, where S_i is the result from stimulated PMA-treated cells, N_i is from unstimulated PMA-treated cells, and S and N are from stimulated and unstimulated cells respectively (not PMA-treated).

Agonist	PKC activity (pmol of ^{32}P /min per 10^7 cells)		Acetyltransferase activity (pmol/min per 10^6 cells)	Paf formation (pmol/ 10^6 cells)	Lyso-paf formation (pmol/ 10^6 cells)	Net β -Hex release (%)
	Cytosol	Membrane				
None	72 $\pm$ 14	15 $\pm$ 3	91 $\pm$ 10	0	31.0 $\pm$ 14	0
Antigen	7.3 $\pm$ 1.9	60 $\pm$ 19	305 $\pm$ 38	17.5 $\pm$ 4.4	58.1 $\pm$ 12.3	17 $\pm$ 3
PMA	0.3 $\pm$ 0.2	0.7 $\pm$ 0.2	97 $\pm$ 19	0	26.0 $\pm$ 7.4	0
PMA + antigen	0.4*	11*	239 $\pm$ 24 (33 $\pm$ 11)	7.3 $\pm$ 1.5 (57 $\pm$ 4)	27.4 $\pm$ 8.3 (96 $\pm$ 3)	2 $\pm$ 1 (90 $\pm$ 8)

contrast, the lyso-paf level was not modified by PMA pretreatment as compared to that obtained after antigen challenge alone (Table 1). These results suggest that there is a common step for acetyltransferase stimulation via antigen and PMA and that the increase in acetyltransferase activity is possibly the limiting factor in paf biosynthesis, as proposed previously [26].

Changes in $[\text{Ca}^{2+}]_i$ during cell stimulation

Changes in $[\text{Ca}^{2+}]_i$ were monitored using the fluorescent probe fura-2 in three independent experiments. When passively sensitized and fura-2/AM-loaded mast cells were challenged with antigen (40 ng/ml), $[\text{Ca}^{2+}]_i$ increased from 61 to 269 nM (one representative experiment). Preincubation of mast cells with EGTA for 1 min decreased the antigen-induced increase in fura-2 fluorescence from 61 to 136 nM. Exposure of cells to PMA alone (5–100 ng/ml) did not result in an increase in $[\text{Ca}^{2+}]_i$. Pretreatment of mast cells with PMA (5 ng/ml) before challenge with antigen (40 ng/ml) did not change the $[\text{Ca}^{2+}]_i$ increase as compared with antigen alone, since a variation of $[\text{Ca}^{2+}]_i$ between 60 and 253 nM was observed.

Changes in PKC activity in stimulated mast cells

The translocation of PKC from the cytosol to the plasma membranes observed during antigen challenge was used as an indicator of PKC activation. Unstimulated mast cells possessed a larger amount of cytosolic than membrane PKC (Table 2). Stimulation of mast cells with antigen (40 ng/ml) for 5 min induced a shift of the PKC activity from the cytosol to the membranes. Overnight treatment of cells with PMA (200 ng/ml) induced a total depletion of PKC activity in both cytosol and membranes. Antigenically induced acetyltransferase activation, paf formation and lyso-paf formation were inhibited by 33 $\pm$ 11 %, 57 $\pm$ 4 % and 96 $\pm$ 3 % ($n = 3$ or 5) respectively in the latter PMA-treated cells (Table 2). The antigenically initiated release of β -Hex by mast cells was completely abolished by PMA treatment. When the (overnight) PMA-treated cells were challenged with PMA (100 ng/ml) for 10 min, the stimulation of acetyltransferase was inhibited by 100 % ($n = 2$). Also, two inhibitors of PKC, chlorpromazine (10 μM) and staurosporine (1 μM), were extremely efficient in blocking the antigenic stimulation of mast cells (Table 3). Furthermore, the PMA (100 ng/ml)-induced activation of acetyltransferase in intact mast cells was inhibited by 91 $\pm$ 9 % ($n = 3$) by chlorpromazine (10 μM). Treatment of cells with these drugs did not result in lactate dehydrogenase leakage into the medium, suggesting that cell viability was not affected (results not shown).

Gas chromatography analysis of paf molecular species

Paf and lyso-paf species formed by cells stimulated with antigen alone or by PMA-primed and antigen-stimulated mast cells were analysed using gas chromatography (Table 4). When sensitized mast cells were stimulated with antigen, the majority of paf contained $\text{C}_{16:0}$ molecules, and only about 20 % of paf was composed of $\text{C}_{18:0}$ molecules (Fig. 4). Traces of the $\text{C}_{18:1}$ analogue of paf were also detected (results not shown). When the cells were pretreated with 5 ng of PMA/ml before challenge with antigen, a similar paf composition was observed. Lyso-paf was composed of equal quantities of $\text{C}_{16:0}$ and $\text{C}_{18:0}$ molecules in unstimulated cells. Challenge of mast cells with antigen (40 ng/ml) alone or in combination with PMA (5 ng/ml) produced in both cases an increased formation of lyso-paf- $\text{C}_{16:0}$ in comparison with lyso-paf- $\text{C}_{18:0}$ molecules, though this did not reach the 80:20 ratio of paf.

DISCUSSION

As previously reported [7], antigen challenge of mast cells induced β -Hex release and acetyltransferase activation, i.e. phosphorylation of the enzyme and paf formation. PKC has been implicated in the acetyltransferase activation (phosphorylation) [7]. In the present study, we raised the question of the dependence of immunologically triggered paf formation on PKC activation, as shown in human neutrophils stimulated with a phagocytic stimulus [27]. Addition of PMA or of DAG, a synthetic analogue of endogenous diacylglycerol, the natural activator of PKC [28], to intact cells resulted in an increase of acetyltransferase activity. To assess whether acetyltransferase activation was mediated via PKC, we substituted for PMA the structurally similar phorbol ester, PDD, which does not activate PKC. The latter was ineffective in increasing the level of acetyltransferase activity. From these results, we concluded that PKC could be involved in the signal transduction leading to phosphorylation of acetyltransferase in mast cells. However, this event was not associated with the formation of paf or with the modulation of lyso-paf content in the cell. Further evidence for PKC involvement in cell activation was the following. Threshold concentrations of PMA potentiated the response of antigen challenge, as judged by the potentiation of β -Hex release and of the deacylation/acetylation pathway, i.e. acetyltransferase activation and paf formation. In contrast, we showed the absence of a priming effect of PMA on antigen-induced lyso-paf formation. The latter compound is the immediate precursor for paf

Table 3. Effects of PKC inhibitors on antigenic stimulation of mast cells

Cells (1×10^6 in 0.5 ml) were preincubated for 15 min at 37 °C with inhibitors prior to challenge with antigen (40 ng/ml) for 5 min. Acetyltransferase activity, paf and lyso-paf formation and β -Hex release were assessed as described in the Materials and methods section. Values in parentheses indicate percentage inhibition of response compared with control. ND, not determined. Results are means $\pm$ S.E.M. of three to six experiments (but * indicates $n = 2$).

Treatment	Acetyltransferase activity (pmol/min per 10^6 cells)		Paf (pmol/ 10^6 cells)		Lyso-paf (pmol/ 10^6 cells)		Net β -Hex release (%)	
	- Antigen	+ Antigen	- Antigen	+ Antigen	- Antigen	+ Antigen	- Antigen	+ Antigen
None	93 $\pm$ 14	273 $\pm$ 31	< 1	16.0 $\pm$ 2.3	57.7 $\pm$ 4.6	84.2 $\pm$ 24.0	8.1 $\pm$ 1.7	30.2 $\pm$ 5.8
Chlorpromazine (10 μ M)	128 $\pm$ 27	131 $\pm$ 26 (98%)	< 1	6.2 $\pm$ 3.4 (61%)	ND	ND	8.3 $\pm$ 0.1	18.1 $\pm$ 5.4 (41%)
Staurosporine (1 μ M)	87 $\pm$ 32	82 $\pm$ 20 (100%)	< 1	1.6 $\pm$ 1.0 (99%)	60.5*	41.7 $\pm$ 19.0 (100%)	9.9 $\pm$ 1.9	2.1 $\pm$ 1.2 (93%)

Table 4. Gas chromatography analysis of molecular composition of paf and lyso-paf formed by mast cells

Mast cells were pretreated or not with PMA (5 ng/ml) for 3 min and stimulated with antigen (40 ng/ml) for 5 min. Results are expressed as percentages of the total ($C_{16:0} + C_{18:0}$) and are means $\pm$ S.E.M., $n = 3$. *Tubes from three separate experiments (30 s to 5 min of stimulation with antigen) were pooled, and determination of molecular species was performed the same day. n.d., not detectable.

Agonist	Paf		Lyso-paf	
	$C_{16:0}$	$C_{18:0}$	$C_{16:0}$	$C_{18:0}$
None	n.d.	n.d.	53 $\pm$ 5	47 $\pm$ 5
Antigen	84 $\pm$ 8	16 $\pm$ 8	70*	30*
PMA + antigen	79 $\pm$ 2	21 $\pm$ 2	69*	31*

Fig. 4. Gas chromatography analysis of paf species extracted from mast cells

Mast cells (1×10^6 in 0.5 ml) were incubated with antigen (40 ng/ml) for 5 min (a) or pretreated with PMA for 3 min before challenge with antigen for 5 min (b). 1, Internal standard (1-*O*-methyl-paf- $C_{16:0}$); 2, paf $C_{16:0}$; 3, paf- $C_{18:0}$. Similar unidentified peaks were also present in extracts from unstimulated cells (results not shown). Results are representative of three independent experiments.

biosynthesis via the acetyltransferase pathway. In a recent work, Nieto *et al.* [29] showed that PMA induced paf formation in human neutrophils *de novo* via a pathway involving DTT-insensitive CDP-choline:choline phosphotransferase. In light of our results, this pathway was not operational in mast cells stimulated with phorbol ester.

Mobilization of intracellular Ca^{2+} , as monitored by fluorescence probes, is detected very early after antigen challenge in basophils and mast cells, and appears to be one of the earliest intracellular changes after receptor bridging (reviewed in [30]). In contrast, PMA treatment of mast cells did not involve an increase in $[Ca^{2+}]_i$, in keeping with the idea that direct PKC agonists bypass all early biochemical events leading to cell activation.

Indirect activation of phospholipase A_2 by PMA was described in intact neutrophils [31], suggesting that this enzyme could be activated even in the absence of a Ca^{2+} signal. However, as pointed out above, we did not detect any change in the lyso-paf content after PMA challenge. This could suggest the importance of a Ca^{2+} signal in phospholipase A_2 stimulation in mast cells.

Unstimulated mast cells contained PKC which was located mostly in the cytosol. Antigen challenge of the cells induced a decrease of PKC in the cytosol, accompanied by an increase of activity associated with membranes. The overnight treatment of mast cells with PMA induced a total loss of PKC activity in both cytosol and membranes and a partial decrease in cell responsiveness to antigen challenge, i.e. decreases in acetyltransferase activation and paf formation. The net gain of lyso-paf was, however, totally inhibited. These results suggest either that PKC is not the only enzyme implicated in the signal transduction leading to paf biosynthesis or that alternative pathways are switched on in the absence of PKC. However, inhibitors of PKC (chlorpromazine and staurosporine) inhibited totally cell degranulation and all steps leading to paf biosynthesis. The discrepancy that we observed between the data obtained with PKC-depleted cells and those obtained with PKC inhibitors may be due either to the non-specific action of the latter [32] or to the differential down-regulation by PMA of PKC subtypes [33]. It was postulated recently that the PKC isoform ϵ does not phosphorylate histones [34], the commonly used substrates in PKC assay. Thus it is difficult at this stage to confirm the total depletion of all PKC isoenzymes on overnight incubation with PMA.

Previous reports have shown that, in a cell-free system, the acetyltransferase from rat spleen and exocrine glands was phosphorylated by a commercial cyclic AMP-dependent protein kinase [35,36] but not by a purified PKC [36]. It is possible, however, that connections exist between these two phosphorylation pathways, as discussed previously [37,38].

The molecular species of paf and lyso-paf formed by antigen-stimulated or PMA-primed plus antigen-stimulated cells were very similar, suggesting that the same precursors were implicated in paf synthesis following both types of stimuli. Interestingly, in stimulated cells lyso-paf was composed of predominantly $C_{16:0}$ molecules, in contrast with unstimulated cells, where equal quantities of $C_{16:0}$ and $C_{18:0}$ species were detected. This observation supports the idea of phospholipase A_2 specificity towards 1-*O*-hexadecyl-acyl-GPC or greater susceptibility of the latter to hydrolytic attack.

In conclusion, the present data allow us to consider PKC involvement in signal transduction during antigen challenge of mast cells, as shown by the PMA priming effect on β -Hex release and paf biosynthesis. However, studies with cells depleted of PKC suggest that signals leading to paf biosynthesis can be also transduced via pathways independent from PKC.

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