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Factors that influence the proportions of platelet-activating factor and 1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine synthesized by the mast cell

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Recent studies have demonstrated that inflammatory cells can be divided into two groups depending on the type of 2-acetylated phospholipids [1-radyl-2-acetyl-*sn*-glycero-3-phosphocholine (GPC)] they produce: those that produce predominantly platelet-activating factor (PAF), and those that produce predominantly its 1-acyl analogue (1-acyl-2-acetyl-GPC; AAGPC) [Triggiani, Schleimer, Warner & Chilton (1991) J. Immunol. **147**, 660–666]. The present study has examined the factors that regulate the production of these two molecules in mouse bone marrow-derived mast cells (BMMC). Initial experiments indicated that PAF and AAGPC were catabolized by BMMC in a differential manner via two pathways: the first, exclusive for AAGPC, involved a 1-acyl hydrolase that removed the long chain at the *sn*-1 position of the molecule, and the second, common to AAGPC and PAF, involved acetylhydrolase that removed the acetate at the *sn*-2 position of the two molecules. Experiments were next designed to identify conditions where the differential catabolism of AAGPC and PAF could be eliminated in order to uncover other factors that regulate the proportions of AAGPC and PAF produced. Phenylmethanesulphonyl fluoride (PMSF) completely blocked the 1-acylhydrolase activity while having little or no effect on the acetyl hydrolase activity, thereby eliminating the influence of the catabolic pathway unique to AAGPC. Moreover, PMSF did not alter the release of arachidonic acid from phospholipid subclasses. PMSF-treated BMMC produced larger quantities of AAGPC than of PAF. The AAGPC/PAF ratio detected in PMSF-treated BMMC was very similar to the ratio of arachidonate contained in and released from 1-acyl-/1-alkyl-linked phosphatidylcholine (PC). BMMC supplemented with arachidonic acid in culture for 3 days increased their total arachidonic acid content in PC as well as the ratio of 1-acyl-2-arachidonoyl-GPC to 1-alkyl-2-arachidonoyl-GPC. These changes resulted in parallel and significant increases in both the total amount of 1-radyl-2-acetyl-GPC and the AAGPC/PAF ratio in BMMC. These data indicate that the AAGPC/PAF ratio produced by inflammatory cells is regulated by at least two factors: (1) differential catabolism of these two molecules, and (2) the distribution of arachidonate in 1-acyl- and 1-alkyl-2-arachidonoyl-GPC. These observations support the concept of a common pathway for AAGPC and PAF biosynthesis in which the two precursor molecules are 1-acyl-2-arachidonoyl-GPC and 1-alkyl-2-arachidonoyl-GPC, respectively.

INTRODUCTION

The generation of chemical mediators from the mast cell is a key element in the pathogenesis of inflammatory and allergic reactions. Mast cells have been shown to produce a number of lipid mediators, including arachidonic acid metabolites and platelet-activating factor [PAF; 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (GPC)] (for review see [1]). PAF is synthesized upon IgE- and non-IgE-mediated (ionophore A23187) activation of a variety of murine mast cells, such as bone marrow-derived mast cells (BMMC) [2] and the mast cell lines MC-9 [3] and PT-18 [4]. Moreover, we and others have shown that mast cells purified from human lung also produce PAF following stimulation with a monoclonal anti-IgE antibody [5,6]. However, the predominant 2-acetylated phospholipid produced by the human lung mast cell appears to be the 1-acyl analogue of PAF (1-acyl-2-acetyl-GPC; AAGPC) [5]. A similar finding has been reported in bovine endothelial cells [7]. We have recently extended this observation in a study which examined the relative proportions of AAGPC and PAF produced by different human cells involved in inflammatory reactions [8]. This study indicated that some cell types (mast cell, basophil, endothelial cell) produce predominantly AAGPC, whereas others (neutrophil, eosinophil, lung macrophage) produce predominantly PAF.

An obvious question that has arisen from the aforementioned studies is: what are the factors that determine the distribution of these 2-aceylated phospholipids in these two groups of cells? One of the factors that might contribute to the differential production of AAGPC and PAF in inflammatory cells is the rate of catabolism of these two molecules. For example, human neutrophils catabolize AAGPC at a rate 2–4-fold higher than that at which PAF is catabolized [9]. AAGPC, but not PAF, is catabolized through a pathway that involves the removal of the long chain at the *sn*-1 position of the molecule by an as yet uncharacterized 1-acylhydrolase [9]. Furthermore, both AAGPC and PAF are catabolized by a cellular acetylhydrolase that removes the acetate group from the *sn*-2 position of the two molecules [10,11]. Therefore different rates and different pathways of catabolism may regulate to a large degree the relative amounts of these phospholipids detected in inflammatory cells.

Abbreviations used: AAGPC, 1-acyl-2-acetyl-sn-glycero-3-phosphocholine; BMMC, mouse bone marrow-derived mast cell; DMSO, dimethyl sulphoxide; Dnp, dinitrophenyl; GPC, *sn*-glycero-3-phosphocholine; HBSS, Hanks buffered saline solution; n.i.c.i.-g.c./m.s., negative ion chemical ionization-gas chromatography/mass spectrometry; PAF, platelet-activating factor (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine); PMSF, phenylmethanesulphonyl fluoride.

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In support of this hypothesis is the observation by Sturk and colleagues that pretreatment of human neutrophils and platelets with phenylmethanesulphonyl fluoride (PMSF), a serine proteinase inhibitor, in order to block the catabolism of 1-radyl-2-acetyl-GPC, resulted in the production of larger amounts of 1-acyl-2-acetyl-GPC following cell activation [12].

Another potentially important factor in the regulation of the levels of AAGPC and PAF in inflammatory cells is the distribution of phospholipid precursors in these cells. In particular, we and others have demonstrated that the biosynthesis of PAF is linked to arachidonate metabolism through a common precursor molecule (1-alkyl-2-arachidonoyl-GPC) [13–15]. These studies have shown that, in the initial step of PAF biosynthesis, arachidonate is removed from 1-alkyl-2-arachidonoyl-GPC to form lyso-PAF, which is subsequently acetylated by an acetyltransferase to form PAF. This reaction was initially thought to be catalysed by a phospholipase A_2 [13,16]; more recently, however, it has been suggested that lyso-PAF can also be formed by a transacylase activity which transfers arachidonate from 1-alkyl-2-arachidonoyl-GPC to a lysophospholipid acceptor during cell activation [17–19]. Although there is evidence that both phospholipase A_2 and transacylase may be specific for arachidonate at the *sn*-2 position of the phosphatidylcholine precursor molecule, it is not clear whether these activities are specific for the type of linkage at the *sn*-1 position (acyl or alkyl) of the molecule.

The present study has examined whether the distribution of arachidonate in 1-acyl and 1-alkyl subclasses of phosphatidylcholine plays a critical role in determining the relative quantities of AAGPC and PAF produced by BMMC. The present study demonstrates that there is a correlation between arachidonate release from 1-acyl-2-arachidonoyl-GPC and 1-alkyl-2-arachidonoyl-GPC and the formation of AAGPC and PAF respectively. In addition, these experiments suggest that the mechanism by which arachidonate is removed from 1-radyl-2-arachidonoyl-GPC to form 1-radyl-2-lyso-GPC has no specificity for the 1-alkyl or 1-acyl linkage at the *sn*-1 position of the molecule.

EXPERIMENTAL

Materials

[^{3}H]Arachidonic acid (76 Ci/nmol), [^{3}H]PAF (40.1 Ci/nmol), 1-[^{14}C]palmitoyl-2-lyso-GPC (55 μCi/nmol) and [^{3}H]acetic acid (Na salt, 3.3 Ci/nmol) were obtained from New England Nuclear. Unlabelled arachidonic acid was from Nu-Check Prep (Elysian, MN, U.S.A.). Octadeuterated arachidonic acid ([$^2H_8$]AA) was from Biomol (Philadelphia, PA, U.S.A.). Mouse IgE anti-[dinitrophenyl (DNP)-HSA] were generously provided by Dr. D. W. MacGlashan Jr. (The Johns Hopkins University, Baltimore, MD, U.S.A.). Pentafluorobenzoyl bromide and diisopropylethylamine were from Pierce Chemical Co. (Rockford, IL, U.S.A.). All solvents were h.p.l.c. grade from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Mast cell preparations

BMMC were obtained from suspension cultures of bone marrow cells from CBA/J mice (Jackson Laboratories, Bar Harbor, ME, U.S.A.) in RPMI-1640 culture medium (GIBCO, Grand Island, NY, U.S.A.) supplemented with 10% (v/v) fetal calf serum, 2-mercaptoethanol (50 μM), L-glutamine (2 mM) and antibiotics. The medium was enriched with 3% (v/v) culture medium obtained from a cell line (S1572F-D11) which consistently produces mouse interleukin-3 [20]. Supernatant fluid obtained from this cell line was kindly supplied by Dr. Teruko Ishizaka, La Jolla Institute for Allergy and Immunology, La Jolla, CA, U.S.A. The cells were grown for 3 weeks at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were observed to be > 90% mast cells after 3 weeks and the viability was always higher than 90%, as determined by Trypan Blue exclusion. BMMC were passively sensitized by overnight incubation with 10 μg of mouse IgE anti-Dnp/ml.

In some experiments, BMMC were supplemented with arachidonic acid (100 nmol/20 ml of culture medium each day for 3 days) complexed with BSA, or with BSA alone (control). In the labelling experiments, BMMC were labelled by adding 1 μCi of [^{3}H]arachidonic acid (76 Ci/mmol) complexed to BSA to 20 ml of medium for 24 h, as previously described [21].

Incubation conditions

BMMC were washed (3 ×) in Ca^{2+}-free Hanks buffered saline solution (HBSS) containing 0.25 mg of BSA/ml and were resuspended in HBSS. The cells were incubated (37 °C, 15 min) with dimethyl sulphoxide (DMSO) (control) or PMSF (2 mM) and then washed twice in Ca^{2+}-free HBSS containing 0.25 mg of BSA/ml. Cell viability after incubation with PMSF was 90 ± 3%. The cells were resuspended [(3–5) × 10^6/ml] in HBSS containing 0.5 mg of BSA/ml and stimulated either with antigen (Dnp_7-HSA; 2 μg/ml) or with A23187 (2 μM).

Synthesis of PAF and AAGPC

The synthesis of PAF and AAGPC was determined by the incorporation of [^{3}H]acetate into the *sn*-2 position of these molecules [5,16]. The BMMC were stimulated with antigen or ionophore A23187 for 5 min in the presence of [^{3}H]acetate (~ 7 μCi, 2 μmol). At the end of the incubation, 3 vol. of methanol/chloroform (2:1, v/v) were added to the tubes and the lipids were extracted from the whole mixture (cells + supernatant) with a modification of the technique of Bligh & Dyer [22] in which sufficient formic acid was added to lower the pH of the aqueous phase to 3. Total 1-radyl-2-acetyl-GPC (includes both PAF and AAGPC) was isolated by t.l.c. as previously described [5,8]. Recovery of 1-radyl-2-acetyl-GPC after extraction and t.l.c. was 80.1 ± 2,3%. An aliquot of 1-radyl-2-acetyl-GPC was removed for liquid scintillation counting and the remaining was further separated into PAF and AAGPC by t.l.c. of the 1-radyl-2,3-diacetate derivatives. The 1-radyl-2,3-diacetate derivatives were prepared and analysed by t.l.c. as previously described [5,8]. The radioactivity in the areas of 1-alkyl-2,3-diacetate and 1-acyl-2,3-diacetate was detected by a Bioscan System 200 (Bioscan, Washington, DC, U.S.A.) and the silica was directly scraped into vials for liquid scintillation counting. In experiments not presented in this study, we have verified that the results obtained by the incorporation of labelled acetate into PAF and AAGPC in the BMMC correlate with those obtained by gas chromatography/mass spectrometry (g.c./m.s.).

Chromatography of lipids

Lipids were extracted [22] from the cell pellet (5 × 10^6 cells) and the choline-containing phosphoglycerols were isolated by using a normal-phase h.p.l.c. system [23]. An aliquot of purified phosphatidylcholine (equivalent to 10^6 cells) was prepared to determine the total arachidonate content by g.c./m.s. The remaining portion of phosphatidylcholine was further separated into 1-acyl, 1-alkyl and 1-alk-1′-enyl subclasses as described by Nakagawa *et al.* [24]. Briefly, phosphatidylcholine was hydrolysed to 1,2-diradylglycerols with phospholipase C (type XIII, from *Bacillus cereus*; Sigma) in 100 mM-Tris/HCl buffer, pH 7.4. The 1-radyl-2-acylglycerols were then extracted and converted into 1,2-diradyl-3-acetylglycerols by the addition of acetic anhydride and pyridine for 18 h at 37 °C. The 1,2-diradyl-3-acetylglycerols were then separated into 1-acyl, 1-alkyl and 1-

alk-1′-enyl subclasses by t.l.c. on layers of Silica Gel G developed in benzene/hexane/diethyl ether (50:45:4, by vol.). The lipids were extracted from the silica gel with diethyl ether/methanol (9:1, v/v) and prepared for g.c./m.s.

Negative ion chemical ionization (N.i.c.i.)-g.c./m.s. measurement of arachidonate

The mol quantities of unlabelled arachidonate were determined by hydrolysing arachidonate from the glycerolipids (intact phosphatidylcholine and isolated phosphatidylcholine subclasses) with 2 M-KOH in ethanol/water (3:1, v/v) for 30 min at 60 °C. [$^2H_8$]Arachidonic acid (50 ng) was added to the reaction mixture as an internal standard. After 30 min, additional water was added and the pH of the reaction mixture was adjusted to 3 with 6 M-HCl. Free arachidonic acid was extracted with hexane and converted to pentafluorobenzoyl esters as previously described [25], using pentafluorobenzoyl bromide and di-isopropylethylamine in acetonitrile. N.i.c.i.-g.c./m.s. was performed on Finnigan MAT TSQ 700 GC/MS/MS/DS (San Jose, CA,. U.S.A.) operated as a single quadrupole system, as previously described [25].

Release of [^{3}H]arachidonate from phosphatidylcholine subclasses

The release of arachidonate was determined in BMMC prelabelled with [^{3}H]arachidonate for 24 h. Previous experiments in this laboratory have shown that the labelled arachidonate is in equilibrium with endogenous stores of arachidonate in all phospholipid classes and subclasses after 24 h [21]. In these experiments, cells were then pretreated with DMSO (vehicle) or PMSF (2 mM) and subsequently stimulated with antigen (2 μg/ml) or A23187 (2 μM) for 5 min. At the end of incubation, cells were rapidly separated from the supernatant fluids by centrifugation (800 *g*, 2 min, 4 °C) and the lipids were extracted from the cell pellet [22]. Phosphatidylcholine was isolated by t.l.c. on Silica Gel G developed in chloroform/methanol/acetic acid/water (50:25:8:2.5, by vol.) and the radioactivity was determined by liquid scintillation counting. An aliquot of phosphatidylcholine was further separated into 1-acyl, 1-alkyl and 1-alk-1′-enyl subclasses as described above, and the radioactivity in each subclass was determined by liquid scintillation counting.

1-Acylhydrolase and acetylhydrolase assays

BMMC pretreated with DMSO (control) or PMSF (2 mM) were incubated at 37 °C for 5 min with buffer or antigen (2 μg/ml). At the end of the incubation, cells were removed from supernatant fluids by two centrifugation steps (400 *g*, 5 min, 4 °C). The cell pellets were resuspended in a Tris buffer containing sucrose (0.25 M) and sonicated (3 × 15 s, 50 % output; Microson, Heating Systems, Farmingdale, NY, U.S.A.). The cell homogenates were fractioned into membrane and soluble fractions by centrifugation (105000 *g*, 45 min, 4 °C). The soluble fraction was immediately used for the assay of 1-acylhydrolase and acetylhydrolase activity. The protein content of the soluble fraction was determined by the method of Bradford [26] and aliquots containing 50 μg of protein (1-acylhydrolase assay) or 2.5 μg of protein (acetylhydrolase assay) were used.

The 1-acylhydrolase assay was performed by using 1-[^{14}C]palmitoyl-2-lyso-GPC (55 mCi/mmol) as a substrate. The use of 1-palmitoyl-2-lyso-GPC instead of AAGPC was necessary to avoid interference by acetylhydrolase. Previous studies have shown that the 2-lyso compound is comparable to AAGPC as a substrate for 1-acylhydrolase [9]. The substrate (100 pmol) complexed to BSA (final concentration 0.05 mg/ml) was incubated (37 °C, 15 min) with the soluble fractions in a final volume of 0.5 ml of HBSS. The reactions were terminated by the addition of 1.5 ml of methanol/chloroform (2:1, v/v). The samples were acidified (pH 3) with formic acid and the lipids were extracted [22]. The labelled products were isolated by t.l.c. using Silica Gel G plates developed in chloroform/methanol/acetic acid/water (50:25:8:4, by vol.). The radioactive areas were detected using a Bioscan System 200 and the silica was scraped directly into vials for liquid scintillation counting. The 1-acylhydrolase activity was expressed as pmol of [^{14}C]palmitate released from the substrate per min.

The acetylhydrolase assay was performed using labelled PAF (1-[^{3}H]hexadecyl-2-acetyl-GPC; 60 Ci/mmol) as a substrate. Because of the higher specific radioactivity of this substrate, the final amount (100 pmol/tube) was reached by adding unlabelled PAF. Incubation, lipid extraction and chromatography conditions were the same as those described for the 1-acylhydrolase assay. The activity of acetylhydrolase was expressed as pmoles of [^{3}H]PAF converted into [^{3}H]lyso-PAF per min.

Phosphorus determination

Total phosphatidylcholine content of the BMMC was determined after h.p.l.c. by the method of Rouser and colleagues [27].

Statistical analysis

Data are expressed as the means ± S.E.M. The *P* values were calculated by Student's *t* test for paired samples.

RESULTS

Effect of PMSF on the catabolism and synthesis of AAGPC and PAF by BMMC

Initial experiments were performed to evaluate the effect of PMSF on the catabolism of AAGPC and PAF in the mast cells. BMMC were preincubated with DMSO (vehicle) or PMSF (2 mM) and subsequently stimulated with antigen for 5 min. The cells were disrupted as described and the activities of 1-acylhydrolase and acetylhydrolase were measured in the soluble fraction. Table 1 shows that 1-acylhydrolase activity increased in BMMC stimulated with antigen, and that PMSF completely blocked the 1-acylhydrolase activity in both resting and antigen-stimulated cells. In contrast, acetylhydrolase activity was not significantly decreased in PMSF-treated cells. These data indicated that PMSF was able to block the catabolic pathway unique for AAGPC in the BMMC. This finding was further verified in intact cell preparations. In these experiments, cells were pretreated with PMSF and then incubated with labelled AAGPC or PAF. PMSF treatment completely blocked the release of [^{14}C]-

Table 1. Effect of PMSF on 1-acylhydrolase and acetylhydrolase activities in BMMC

BMMC were incubated (15 min, 37 °C) with DMSO or PMSF (2 mM), washed and then incubated with buffer or antigen (2 μg/ml) for 5 min. The cells were disrupted and the 1-acylhydrolase and acetylhydrolase activities were measured in the soluble fractions as described in the Experimental section. These data are expressed as the means ± S.E.M. of three experiments.

Treatment	Activity (pmol/min)	
	1-Acylhydrolase	Acetylhydrolase
Control (DMSO)	2.2 ± 1.1	5.6 ± 0.5
PMSF	0	5.2 ± 0.8
Antigen	3.0 ± 1.5	5.2 ± 0.8
PMSF + antigen	0	5.1 ± 0.9

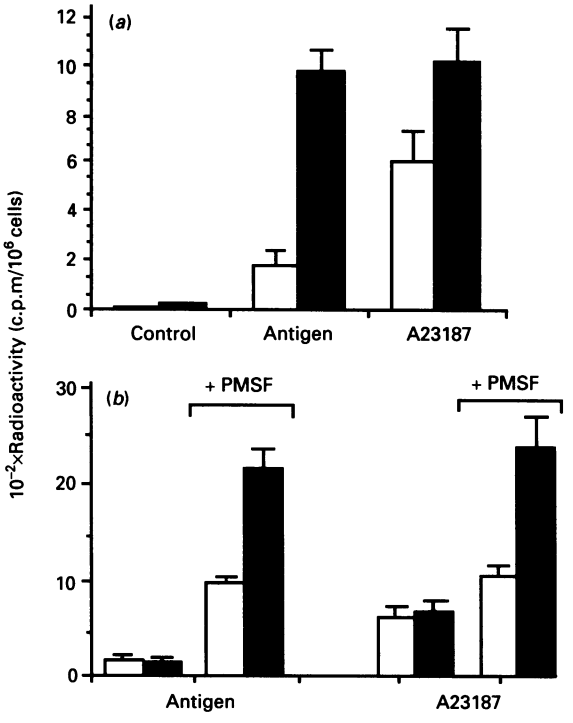

Fig. 1. Effect of PMSF on the synthesis by BMMC of (*a*) 1-radyl-2-acetyl-GPC and (*b*) PAF and AAGPC

(*a*) BMMC (5×10^6 cells/ml) were pretreated with DMSO (□) or with PMSF (■) and subsequently stimulated with antigen or A23187 for 5 min. The lipids were extracted and analysed by t.l.c. The radioactivity in the area corresponding to 1-radyl-2-acetyl-GPC was determined by liquid scintillation counting. These data are expressed as the means ± S.E.M. of three experiments. (*b*) The 1-radyl-2-acetyl-GPC obtained from BMMC stimulated with antigen or A23187 was isolated and converted to 1-radyl-2,3-diacetylglycerols by phospholipase C hydrolysis followed by acetylation. The diacetylglycerols derivatives of AAGPC (■) and PAF (□) were separated by t.l.c. and the radioactivity in the respective areas was determined by liquid scintillation counting. These data are expressed as the means ± S.E.M. of three experiments.

palmitate from 1-[^{14}C]palmitoyl-2-lyso-GPC (from 0.9 ± 0.2 to 0 pmol/min), but had no effect on the removal of acetate from 1-[^{14}C]palmitoyl-2-acetyl-GPC (9.4 ± 2.6 and 10.4 ± 3 pmol/min respectively) or [^{3}H]PAF (10.3 ± 0.4 and 9.2 ± 0.6 pmol/min respectively). Furthermore, when the BMMC were preincubated with PMSF, AAGPC and PAF were catabolized at similar rates (13.8 ± 1.1 and 10.7 ± 0.7 pmol/min respectively). During the course of this study, we observed that acetylhydrolase, but not 1-acylhydrolase, could be released from BMMC. In addition, antigen stimulation of BMMC induced a significant increase in the release of acetylhydrolase into the supernatant fluid (control, 2.0 ± 0.7; antigen, 4.8 ± 1.1 pmoles/min per 10^6 cells; $P < 0.05$, $n = 4$). Pretreatment of BMMC with PMSF did not influence either spontaneous or antigen-induced release of acetylhydrolase. Taken together, these data indicate that PMSF completely blocks the 1-acylhydrolase pathway of catabolism which is specific for AAGPC, but only slightly affects the activity of acetylhydrolase, the catabolic enzyme common to both phospholipids.

In the next group of experiments, BMMC (PMSF-treated and non-PMSF-treated) were stimulated with antigen or A23187 and the production of PAF and AAGPC was examined. Fig. 1(*a*) demonstrates that PMSF treatment of BMMC increased the amount of 1-radyl-2-acetyl-GPC produced by the cells by approx. 5-fold after antigen stimulation and 2-fold after A23187 stimulation. The 1-radyl-2-acetyl-GPC produced by BMMC was then further analysed to determine the relative proportions of PAF and AAGPC. Fig. 1(*b*) shows that BMMC stimulated with both antigen and A23187 produced approximately equal amounts of PAF and AAGPC. Incubation of the cells with PMSF before stimulation resulted in the accumulation of AAGPC in larger quantities than PAF with both stimuli. Under these conditions, AAGPC accounted for approx. 70 % of the 2-acetylated phospholipids produced by the BMMC. It is interesting to note that the amount of PAF was also increased in PMSF-treated cells, although to a lesser extent than that of AAGPC. This finding may be explained either by the small decrease in acetylhydrolase activity induced by PMSF (Table 1), or by the large increase in AAGPC which would eventually compete with PAF for acetylhydrolase.

Effect of PMSF on arachidonate release from 1-alkyl-2-arachidonoyl-GPC and 1-acyl-2-arachidonoyl-GPC

The data shown in the previous section demonstrated that PMSF effectively blocked the catabolic pathway specific for AAGPC, thereby eliminating the influence of the differential catabolism of AAGPC and PAF. However, before comparing the distribution and release of arachidonate from phosphatidylcholine subclasses by the synthesis of AAGPC and PAF, it was necessary to determine if PMSF had any effect on arachidonate release from phosphatidylcholine subclasses. In these experiments, BMMC were labelled with [^{3}H]arachidonic acid in culture for 24 h. We have previously shown that, under these conditions, the labelled arachidonate is in equilibrium with the endogenous pools of arachidonate in all phospholipid subclasses [21]. However, we confirmed in the present study that the two major phosphatidylcholine subclasses (1-acyl and 1-alkyl) were labelled with the same specific radioactivity. In these experiments we measured both the radioactivity (by scintillation counting) and the mass (by g.c./m.s.) of arachidonate in phosphatidylcholine subclasses of BMMC labelled for 24 h. The ratios between labelling and mass of arachidonate, indicating the specific radioactivity, were 3.3 ± 0.6 and 3.1 ± 1.1 nCi/nmol for 1-acyl and 1-alkyl phosphatidylcholine, respectively (means ± S.E.M.; $n = 3$). These data indicated that the two subclasses were uniformly labelled. They also provide confirmation that, in BMMC labelled for 24 h, any loss of labelled arachidonate was a true representation of loss of endogenous arachidonate. Table 2 shows the release of arachidonate from the 1-alkyl, 1-acyl and 1-alk-1-enyl subclasses of phosphatidylcholine upon antigen and A23187 stimulation. In agreement with previous data [21], antigen or A23187 stimulation resulted in the release of arachidonate from the two major phosphatidylcholine subclasses. The majority of arachidonate was released from 1-acyl-2-arachidonoyl-GPC, with smaller quantities released from 1-alkyl-2-arachidonoyl-GPC. Table 2 also shows that pretreatment of BMMC with PMSF for 15 min prior to stimulation did not significantly influence either the quantity or the proportions of arachidonate released from phosphatidylcholine subclasses.

Correlation between arachidonate distribution and release from 1-alkyl-2-arachidonoyl-GPC and 1-acyl-2-arachidonoyl-GPC and synthesis of AAGPC and PAF

The aforementioned data indicated that it was possible to compare the synthesis of AAGPC and PAF with the release of arachidonate from 1-acyl-2-arachidonoyl-GPC and 1-alkyl-2-arachidonoyl-GPC under conditions where AAGPC and PAF were not differentially catabolized (i.e. after PMSF treatment). Fig. 2 illustrates the relationship between arachidonate content and release and the proportions of AAGPC and PAF produced by antigen-stimulated BMMC. There is a striking correspondence between the percentage distribution of arachidonate in 1-acyl-2-

Table 2. Effect of PMSF on arachidonate release from phosphatidylcholine subclasses

BMMC were prelabelled with [^{3}H]arachidonate (1 μCi/20 ml of medium) for 24 h. The cells were incubated with DMSO (vehicle) or PMSF (2 mM) for 15 min, washed and stimulated with antigen (2 μg/ml) or A23187 (2 μM) for 5 min. Cells and supernatant fluids were separated by centrifugation and lipids were extracted from the cell pellet. Phosphatidylcholine was isolated by h.p.l.c., and subclasses were separated by t.l.c. as described in the Experimental section. The radioactivity was determined by liquid scintillation counting. These data are expressed as (c.p.m. in a given subclass from unstimulated BMMC) − (c.p.m. in that same subclass from stimulated BMMC) per 10^6 cells. Data are the means ± S.E.M. of three experiments.

	10^{-3} × Arachidonate release (c.p.m./10^6 cells)			
Subclass	Antigen	PMSF + antigen	A23187	PMSF + A23187
1-Acyl	5.2 ± 2.4	4.4 ± 1.5	13.4 ± 2.2	12.4 ± 3.3
1-Alkyl	1.4 ± 0.8	0.9 ± 0.1	5.0 ± 0.8	3.7 ± 0.7
1-Alk-1′-enyl	< 0.2	< 0.2	< 0.2	< 0.2

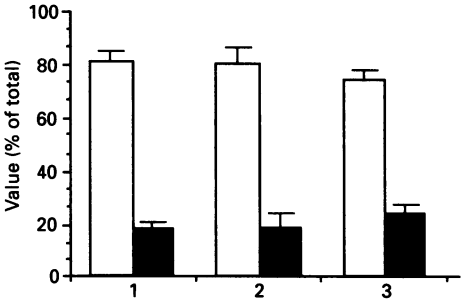

Fig. 2. Distributions of arachidonate in 1-acyl- and 1-alkyl-phosphatidylcholine, release of arachidonate from these two subclasses, and production of 2-acetylated phospholipids (AAGPC and PAF) induced by antigen stimulation

BMMC pretreated with PMSF (2 mM) were stimulated with antigen (2 μg/ml) for 5 min. At the end of the incubation, the lipids were extracted and analysed as described in the Experimental section. These data are expressed as the means ± S.E.M. of three experiments. □, 1-Acyl; ■, 1-alkyl. 1, Arachidonate content; 2, arachidonate release; 3, 2-acetylated products.

Table 3. Effect of arachidonate supplementation on 1-radyl-2-acetyl-GPC synthesis in BMMC

BMMC were supplemented in culture with BSA (no supplement) or arachidonic acid (100 nmol/day) for 3 days. The cells were then pretreated with PMSF (2 mM) and subsequently stimulated in the presence of [^{3}H]acetate with antigen or A23187 for 5 min. The lipids were extracted and analysed by t.l.c. The radioactivity in the area corresponding to 1-radyl-2-acetyl-GPC was determined by liquid scintillation counting. These data are expressed as the means ± S.E.M. of three experiments.

	1-Radyl-2-acetyl-GPC (c.p.m./10^6 cells)	
Stimulus	No supplement	Arachidonate supplement
None	136 ± 9	162 ± 16
Antigen	3880 ± 523	5526 ± 774
A23187	3104 ± 890	5404 ± 1,586

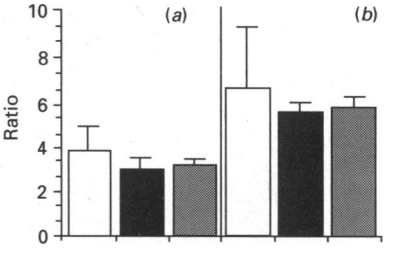

Fig. 3. Effect of arachidonate supplementation on the 1-acyl-2-arachidonoyl-GPC/1-alkyl-2-arachidonoyl-GPC and AAGPC/PAF ratios in antigen- and A23187-stimulated BMMC

BMMC were supplemented in culture with BSA (*a*) or arachidonate (100 nmol/day for 3 days; *b*). The cells were then pretreated with PMSF (2 mM) and subsequently stimulated with antigen or A23187 for 5 min. The lipids were extracted from the cell pellets, and arachidonate in the 1-acyl- and 1-alkyl-phosphatidylcholine was measured by g.c./m.s. Synthesis of AAGPC and PAF were measured in the whole mixture (cells + supernatant) as the incorporation of labelled acetate. These data are expressed as the means ± S.E.M. of three experiments. □, 1-Acyl-2-arachidonoyl-GPC/1-alkyl-2-arachidonoyl-GPC ratio; ■, AAGPC/PAF (antigen-stimulated); ■, AAGPC/PAF (A23187-stimulated).

arachidonoyl-GPC and 1-alkyl-2-arachidonoyl-GPC, the percentage release of arachidonate from these two subclasses, and the relative proportions of AAGPC (acyl) and PAF (alkyl) produced by the BMMC. Similar results were obtained in A23187-stimulated cells (results not shown). Therefore, by eliminating the differential catabolism of AAGPC and PAF, a direct correlation between the mobilization of arachidonate from the two major subclasses of phosphatidylcholine and the synthesis of the 2-acetylated products (AAGPC and PAF) can be revealed.

Effect of arachidonate supplementation on the synthesis of PAF and AAGPC

To further test the hypothesis that the distribution of arachidonate in phosphatidylcholine subclasses influences the PAF/AAGPC ratio produced by stimulated BMMC, we studied the effect of arachidonate supplementation during cell culture on both the content of arachidonate in precursors and the formation of 1-radyl-2-acetyl-GPC. In these experiments, several protocols of supplementation were examined in an attempt to increase the total arachidonate content of phosphatidylcholine and simultaneously to modify the 1-acyl-2-arachidonoyl-GPC/1-alkyl-2-arachidonoyl-GPC ratio. However, the cells were somewhat resistant to changes in this ratio of precursor molecules. After testing different concentrations of arachidonate and different durations of the supplementation, the most satisfactory procedure was found to be a supplement of 100 nmol of arachidonate each day for 3 days. By using this protocol, the total arachidonate content in phosphatidylcholine increased from 0.6 ± 0.2 to 1.2 ± 0.1 nmol/10^7 cells ($P < 0.05$). In contrast, the total phosphorus in phosphatidylcholine remained constant (115 nmol/10^6 non-supplemented cells and 121 nmol/10^6 supplemented cells), indicating that the pool size of total phosphatidylcholine did not change. The 1-acyl-2-arachidonoyl-GPC/1-alkyl-2-arachidonoyl-GPC ratio also increased from 3.7 ± 1.1 nmol/10^6 cells (non-supplemented cells) to 6.4 ± 2.7 nmol/10^6 cells (arachidonate-supplemented cells). Table 3 shows that arachidonate supplementation of PMSF-treated cells induced a significant increase in the formation of 1-radyl-2-acetyl-GPC in both antigen- and A23187-stimulated BMMC. In addition to increasing the total amount of 1-radyl-2-acetyl-GPC produced by the cell, arachidonate supplementation also changed the

AAGPC/PAF ratio produced by the BMMC. The changes in the 1-acyl-2-arachidonoyl-GPC/1-alkyl-2-arachidonoyl-GPC and AAGPC/PAF ratios in both antigen- and A23187-stimulated cells are compared in Fig. 3. The AAGPC/PAF ratio increased from 2.9 ± 0.5 to 5.3 ± 0.4 in antigen-stimulated BMMC ($P < 0.05$) and from 3.0 ± 0.3 to 5.5 ± 0.5 in A23187-stimulated BMMC ($P < 0.01$).

DISCUSSION

Previous studies have shown that inflammatory cells can produce different amounts of PAF and AAGPC depending on the cell type examined [5,7,8]. The physiological significance of the production of both of these molecules has yet to be fully defined. For example, AAGPC has been shown to be 300–1000-fold less active than PAF in activating platelets and neutrophils, and to induce systemic hypotension *in vivo* [28,29]. However, other experiments have suggested that AAGPC may act as a naturally occurring antagonist for PAF in some systems where they are produced in similar quantities. For example, pre-incubation of human neutrophils with AAGPC inhibits the cytosolic Ca^{2+} increase and degranulation induced by PAF [29]. These data suggest that the differential production of these 2-acetylated phospholipids may influence the pro-inflammatory role of a given cell. Therefore the present study has examined the factors that regulate the synthesis of these two phospholipids in the mast cell.

It has been suggested that the differential catabolism of AAGPC and PAF may be a potential factor regulating the amounts of these two phospholipids in stimulated cells. The main evidence in support of this hypothesis is the existence of a catabolic pathway specific for AAGPC, in addition to the acetylhydrolase-dependent pathway common to AAGPC and PAF [9]. The AAGPC-specific pathway involves the removal of the 1-acyl moiety of the molecule by a 1-acylhydrolase to form 1-lyso-2-acetyl-GPC. This molecule can in turn be converted to more polar metabolites such as GPC and choline. In the present study, we have shown that it is possible to block the AAGPC-specific pathway in the BMMC by using PMSF. As expected from this effect on catabolism, the amount of AAGPC detected after cell activation was increased several-fold in PMSF-treated BMMC. These data are in agreement with the observation by Sturk *et al.* that PMSF potentiates the synthesis of AAGPC in the human neutrophil and platelet [12]. Although PMSF has been reported to inhibit acetylhydrolase activity in platelets [30], it had little effect on this enzyme in the BMMC. In experiments performed in the neutrophil in parallel with those in the BMMC, we observed that PMSF completely blocked the activity in the neutrophil (M. Triggiani, A. N. Fonteh & F. H. Chilton, unpublished work). At this time there is no explanation for this difference; however, other substantial differences between the acetylhydrolase in the BMMC and in the neutrophil have been found. These differences include (1) a higher specific activity of the enzyme in the BMMC compared with in the neutrophil, (2) the observation that in BMMC almost 50% of total cellular acetylhydrolase is associated with the cell membrane, and (3) the capacity of the BMMC, but not of the neutrophil, to release this enzyme upon stimulation (M. Triggiani, A. N. Fonteh & F. H. Chilton, unpublished work). Further studies will be required to determine whether these differences are only functional or are related to structural variants of acetylhydrolase. In any event, our data support a role for the differential catabolism of AAGPC and PAF in controlling the levels of these molecules in BMMC. They also indicate that it is possible to change significantly the AAGPC/PAF ratio by selectively blocking the AAGPC-specific catabolic pathway.

When the differential catabolism was eliminated in the BMMC, it was possible to identify another factor that determines the AAGPC/PAF ratio produced by the BMMC. The proportions of AAGPC and PAF produced after stimulation of BMMC pretreated with PMSF was similar to the 1-acyl-2-arachidonoyl-GPC/1-alkyl-2-arachidonoyl-GPC ratio found in membrane phospholipids. However, it was not clear from previous studies if there is a preferential release of arachidonate from phosphatidylcholine subclasses during cell activation. In order to address this question, the assumption that PMSF had no significant effect on the release of arachidonate was verified. Experiments could then be performed to examine arachidonate release in a system free of the influence of the differential catabolism of the 2-acetylated phospholipids. Our data indicated that the majority of arachidonate in phosphatidylcholine is esterified in the 1-acyl-linked subclass (80%) followed by the 1-alkyl-linked subclass (19%). Stimulation by antigen or A23187 induced the release of arachidonate in a fashion proportional to its relative distribution in the two subclasses. Therefore approx. 80% and 20% of the total arachidonate released from phosphatidylcholine was mobilized from the 1-acyl and the 1-alkyl pools respectively. This in turn suggests that the enzymic system(s) involved in the mobilization of arachidonate from phosphatidylcholine have no specificity for the type of linkage at the *sn*-1 position of 1-radyl-2-arachidonoyl-GPC. Both of the enzymic activities that may mobilize arachidonate from phosphatidylcholine in this reaction, i.e. phospholipase A_2 [31–33] and the recently described CoA-independent transacylase [17–19,34,35], have been shown to be active on both 1-acyl-2-arachidonoyl-GPC and 1-alkyl-2-arachidonoyl-GPC. Although our data do not completely exclude the possibility of different enzymic activities acting on these two phosphatidylcholine subclasses, they are suggestive of a common mechanism that does not discriminate between the two arachidonate pools.

If arachidonate is released from 1-acyl-2-arachidonoyl-GPC and 1-alkyl-2-arachidonoyl-GPC in consistent proportions, it is reasonable to suppose that 1-acyl-2-lyso-GPC and 1-alkyl-2-lyso-GPC will be produced in the same proportions. These two lysophospholipids would then be available for conversion to AAGPC and PAF by acetyltransferase. There has been some controversy in the literature as to the selectivity of acetyltransferase for 1-acyl-2-lyso-GPC and 1-alkyl-2-lyso-GPC. However, studies performed in broken-cell preparations have shown that acetyltransferase has no specificity for the two lysophospholipids [36,37]. We have also demonstrated that, in the intact neutrophil pretreated with PMSF, AAGPC is readily formed by acetylation of 1-acyl-2-lyso-GPC [38].

The hypothesis that AAGPC and PAF may have a common biosynthetic pathway but two different precursor molecules, i.e. 1-acyl-2-arachidonoyl-GPC and 1-alkyl-2-arachidonoyl-GPC respectively, was further tested in experiments in which BMMC were supplemented with arachidonate in culture. As has been demonstrated in other cells, the supplementation of BMMC with arachidonate increased both the total content of arachidonate in PC and the synthesis of PAF [4,7,39,40]. Here we utilized a supplementation protocol which increased both the total 1-acyl-2-arachidonoyl-GPC and 1-alkyl-2-arachidonoyl-GPC found in the BMMC, and the ratio of 1-acyl-2-arachidonoyl-GPC to 1-alkyl-2-arachidonoyl-GPC. The synthesis of AAGPC and PAF was significantly enhanced by increasing the arachidonate content in phosphatidylcholine. Furthermore, changing the proportion of arachidonate in phosphatidylcholine subclasses resulted in a parallel and significant change in the ratio of the 2-acetylated products (AAGPC/PAF). These data demonstrate that arachidonate content and distribution in the 1-acyl-2-arachidonoyl-GPC and 1-alkyl-2-arachidonoyl-GPC have both quantitative (increase in the total production of 2-acetylated

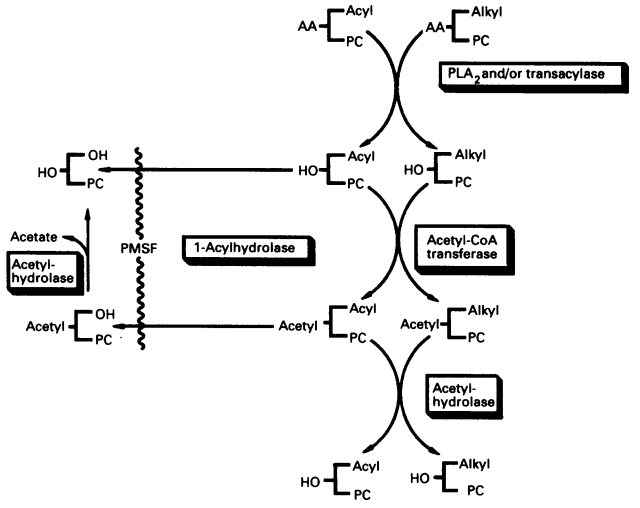

Fig. 4. Proposed metabolic pathway for AAGPC and PAF in BMMC

Abbreviations: PC, phosphatidylcholine; AA, arachidonic acid; PLA_2, phospholipase A_2.

products) and qualitative (change in the AAGPC/PAF ratio) effects.

In conclusion, the differential catabolism of AAGPC and PAF and the distribution of 1-acyl-2-arachidonoyl-GPC and 1-alkyl-2-arachidonoyl-GPC play a crucial role in determining proportions of AAGPC and PAF produced by BMMC. With the recognition of these two factors, it is possible to hypothesize about the biochemical steps that would lead to the synthesis of AAGPC along with PAF in inflammatory cells (Fig. 4). It is clear from this study that there are similarities and differences in the metabolic routes followed by AAGPC and PAF. The variable combination of these two factors in various inflammatory cells may be responsible for the different proportions of AAGPC and PAF produced upon cell activation.

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