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Regulation of platelet-activating factor synthesis in human neutrophils by MAP kinases

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Abstract

Human neutrophils (PMN) are potentially a major source of platelet-activating factor (PAF) produced during inflammatory responses. The stimulated synthesis of PAF in PMN is carried out by a phospholipid remodeling pathway involving three enzymes: acetyl-CoA:lyso-PAF acetyltransferase (acetyltransferase), type IV phospholipase A_2 ($cPLA_2$) and CoA-independent transacylase (CoA-IT). However, the coordinated actions and the regulatory mechanisms of these enzymes in PAF synthesis are poorly defined. A23187 has been widely used to activate the remodeling pathway, but it has not been shown how closely its actions mimic those of physiological stimuli. Here we address this important problem and compare responses of the three remodeling enzymes and PAF synthesis by intact cells. In both A23187- and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated PMN, acetyltransferase activation is blocked by SB 203580, a p38 MAP kinase inhibitor, but not by PD 98059, which blocks activation of the ERKs. In contrast, either agent attenuated $cPLA_2$ activation. Correlating with these results, SB 203580 decreased stimulated PAF formation by 60%, whereas PD 98059 had little effect. However, the combination of both inhibitors decreased PAF formation to control levels. Although a role for CoA-IT in PAF synthesis is recognized, we did not detect activation of the enzyme in stimulated PMN. CoA-IT thus appears to exhibit full activity in resting as well as stimulated cells. We conclude that the calcium ionophore A23187 and the receptor agonist fMLP both act through common pathways to stimulate PAF synthesis, with p38 MAP kinase regulating acetyltransferase and supplementing ERK activation of $cPLA_2$.

Keywords: Neutrophil; Platelet-activating factor; MAP kinase; $cPLA_2$; Acetyltransferase; CoA-independent transacylase

1. Introduction

Platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; 1-*O*-alkyl-2-acetyl-GPC; PAF) is a potent, bioactive phospholipid that mediates diverse physiological and pathological responses. Polymorphonuclear leukocytes (PMN), a particularly well-studied cell type and a potential major source of PAF in vivo, produce this mediator via a phospholipid-remodeling pathway. Stimulated PAF synthesis is initiated by the formation of 1-*O*-alkyl-2-lyso-GPC (lyso-PAF) by either phospholipase A_2 (PLA_2) hydrolysis of 1-*O*-alkyl-2-arachidonoyl-GPC or by the CoA-independent transacylase (CoA-IT), which transfers the *sn*-2 acyl chain from 1-*O*-alkyl-2-acyl-GPC to an acceptor lyso-phospholipid. The lyso-PAF that is formed by either of these routes is then acetylated by acetyl-CoA: 1-*O*-alkyl-2-lyso-GPC acetyltransferase (acetyltransferase) to form PAF (for review, see Ref. [1]). Although the enzymes believed to be responsible for the remodeling pathway have been identified, their coordinated actions, regulatory roles(s), and the upstream signals that are critical to the conduct of this pathway have not been established. Con-

Abbreviations: PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; AA, arachidonic acid; PMN, polymorphonuclear leukocytes, neutrophils; PLA_2, phospholipase A_2; GPE, *sn*-glycero-3-phosphoethanolamine; GPC, *sn*-glycero-3-phosphocholine; CoA-IT, coenzyme A-independent transacylase; lyso-PAF, 1-*O*-alkyl-2-lyso-GPC; acetyltransferase, acetyl-CoA:1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine acetyltransferase; MAP kinase, mitogen-activated protein kinase; ERK, extracellular-signal regulated protein kinase; MEK, mitogen-activated protein kinase kinase; $cPLA_2$, 85 kDa cytosolic phospholipase A_2; TNFα, tumor necrosis factor alpha; PMA, phorbol-12-myristate-13-acetate

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sequently, despite many studies of PAF metabolism, the regulation of PAF synthesis is not well understood.

Two mitogen-activated protein kinase (MAP kinase) cascades, the extracellular signal-regulated kinases (ERKs) and p38 MAP kinases, have emerged as important elements in the regulation of PMN function [2–4]. These two kinase cascades appear to have overlapping but distinct functions with regard to both upstream activators and downstream targets. Both pathways are activated to a similar extent by chemotactic factors such as *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) [5], whereas p38 MAP kinase is preferentially or selectively activated by inflammatory cytokines and lipopolysaccharide (LPS) [6,7]. In downstream signaling, the ERK and p38 MAP kinase pathways can both activate the 85 kDa cytosolic PLA_2 ($cPLA_2$) [8,9], a PLA_2 believed to be essential for PAF synthesis [10], whereas only p38 MAP kinase appears to activate the acetyltransferase [11].

In spite of these advances in our understanding of the relationships between the MAP kinases and the individual enzymes of the remodeling pathway, the roles of p38 MAP kinase and ERKs in stimulus-induced PAF synthesis remain largely unexplored. In studies reported here, we examined the role of p38 MAP kinase and the ERKs in regulating all three individual enzymes of the remodeling pathway and assessed their respective roles in PAF synthesis in intact PMN. We focused on the actions of two agonists: the chemotactic oligopeptide fMLP and the calcium ionophore A23187, both of which are known to stimulate PAF synthesis and AA release. Because A23187 induces robust AA release and PAF formation, it has long been used as a model stimulus to study PAF metabolism. It is therefore important to determine whether these two different stimulus types, physiological and nonphysiological, elicit comparable responses in the remodeling pathway. Some of these results have been previously reported in abstract form [12].

2. Experimental procedures

2.1. Materials

1-*O*-[1,2-^{3}H]Hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine ([^{3}H]lyso-PAF; 56 Ci/mmol) was synthesized as described by Wyrick et al. [13]. The stock [^{3}H]lyso-PAF was mixed with unlabeled lyso-PAF in chloroform and methanol (4:1, v/v) when preparations of lower radiospecific activity were desired. 1-Stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (55 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). Na[^{3}H]acetate was obtained from American Radiolabeled Chemicals (St Louis, MO); phospholipid standards, including unlabeled lyso-PAF, phosphatidylcholine (PC), and PAF were from Avanti Polar Lipids (Birmingham, AL). MAP kinase kinase (MEK) inhibitor, PD 98059; p38 MAP kinase inhibitor, SB 203580; and calcium ionophore, A23187 (*Streptomyces chartreusensis*; free acid), were purchased from Calbiochem-Novabiochem Intl. (La Jolla, CA). Isolymph was from Gallard-Schlesinger (Carle Place, NY) and Dextran T-500 was from Pharmacia LKB Biotechnology (Uppsala, Sweden). Silica gel G plates were purchased from Analtech (Newark, DE). Leupeptin, pepstatin, fMLP and Dulbecco's phosphate buffered saline (PBS) were from Sigma (St Louis, MO).

Phosphospecific antibodies against ERK (residues 196–209 of tyrosine phosphorylated human ERK-1) and against p38 MAP kinase (residues 171–186 of tyrosine phosphorylated human p38 MAP kinase) were from New England Bio-Labs (Beverly, MA). Rabbit polyclonal antibody against $cPLA_2$ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit antibody was purchased from Transduction Laboratories (Lexington, KY). Enhanced chemiluminescence detection reagents and polyvinylidene difluoride membranes (PVDF) were from DuPont NEN Research Products (Boston, MA).

2.2. Preparation of neutrophils

Neutrophils were prepared as previously described [14], with some modification. Briefly, heparinized blood containing 4 mM EDTA was sedimented using dextran to remove red blood cells. The blood cells were further separated by centrifugation over Isolymph, and contaminating red blood cells were removed by brief hypotonic lysis. Cell populations consisted of >95% PMN. Isolated neutrophils were suspended at 3.5×10^7 cells/1 ml in PBS containing 1.4 mM $CaCl_2$; however, for $cPLA_2$ activation experiments, cells were resuspended at 3.0×10^7 cells/3 ml.

2.3. SDS-PAGE and immunoblotting

For phosphospecific ERK and p38 MAP kinase immunoblotting, whole cell sonicates (2.5×10^5 cell equivalents) were resolved by SDS-PAGE (12% polyacrylamide), electrophoretically transferred to PVDF membranes overnight and immunoblotted. Detection of the phosphorylated forms of ERK and p38 MAP kinase was carried out according to the protocol provided by New England Bio-Labs.

$cPLA_2$ gel shift analysis was performed on PMN sonicate (100 μg protein) in which the nuclei and the granules, which are rich in proteases, were removed by centrifugation ($16{,}000 \times g$, 4 °C, 30 min). The sonicate was resolved by SDS-PAGE (7.5% polyacrylamide), transferred to PVDF membranes overnight and immunoblotted.

2.4. Acetyltransferase activity assay

Acetyltransferase activity was assayed as described [11,15] with some modifications. Briefly, PMN (3.5×10^7/

1 ml) in PBS containing 1.4 mM Ca^{2+} were incubated for 30 min at 37 °C in the presence or absence of 50 μM PD 98059 and/or 20 μM SB 203580; DMSO was used as a vehicle control. Cells were treated with or without 10 μM A23187 for 10 min. The reaction was stopped by addition of 35 ml chilled (4 °C) PBS. All subsequent manipulations were carried out at 4 °C. Cells were centrifuged for 10 min at 400 × *g* and the pellet resuspended in 1 ml of protection buffer (0.2 M Tris–HCl, pH 7.4, containing 50 μg/ml each of pepstatin A and leupeptin, 10 mM NaF, and 0.2 mM Na_3VO_4). Cells were sonicated twice for 8 s at a power setting of 2 with a probe sonicator (Heat System, Inc). The sonicate was centrifuged at 16,000 × *g* for 20 min to remove nuclei, granules and cellular debris, and the supernatant was centrifuged at 100,000 × *g* for 60 min to obtain a microsomal membrane pellet. The pellet was resuspended in 1 ml 0.2 M Tris–HCl (pH 7.4), and the protein concentration of the suspension was determined by the method of Bradford [16] using bovine serum albumin (BSA) as standard.

The acetyltransferase reaction was initiated by adding aliquots of the microsomal preparation (10 μg protein) to prewarmed tubes containing 100 μM acetyl-CoA and [^{3}H]lyso-PAF (16 μM, 0.1 μCi/tube; 1 ml final volume). After 15-min incubation at 37 °C, the reactions were stopped by extracting the lipids [17]. [^{3}H]PAF was measured after separation by TLC as described below.

2.5. CoA-IT activity assay

PMN (3.5×10^7/1 ml) in PBS containing 1.4 mM Ca^{2+} were stimulated with tumor necrosis factor alpha (TNFα, 3.16 nM, 10 min), A23187 (10 μM, 10 min), fMLP (10 μM, 10 min) or phorbol-12-myristate-13-acetate (PMA, 100 nM, 5 min). In some experiments, PMN were pretreated with or without the MAP kinase inhibitors, as described above, prior to stimulation. Microsomal fractions were isolated as described above, and protein concentration determined by the method of Bradford [16]. CoA-IT activity was measured using the acceptor assay described by Venable et al. [18], with some modification. Briefly, each reaction tube contained 900 μl 0.2 M Tris–HCl (pH 7.4) to which was added [^{3}H]lyso-PAF (0.2 μCi, 5 μM) dissolved in 50 μl of Tris buffer (pH 7.4) containing 2 mg BSA/ml. Tubes were prewarmed for 10 min (37 °C), and reactions were initiated by adding 5–10 μg microsomal membrane protein (1 ml final volume). Samples were shaken for 15 min (37 °C), and the reactions stopped by extracting the lipids [17]. 1-*O*-[^{3}H]hexadecyl-2-acyl-GPC was separated by TLC as described below and measured by liquid scintillation counting. Typically, 10–20% of added [^{3}H]lyso-PAF was acylated.

In some experiments, a donor assay was used to measure CoA-IT activity [18]. PMN were labeled with [^{3}H]lyso-PAF (60,000 dpm/3.5×10^7 cell/ml) in PBS without Ca^{2+} for 60 min at 37 °C. PMN were washed three times with PBS (4 °C) containing 2 mg/ml BSA and rinsed an additional time with PBS alone. Cells were resuspended (3.5×10^7/ml) in PBS containing 1.4 mM $CaCl_2$, allowed to rest for 20 min (4 °C), then warmed for 20 min (37 °C). Under these conditions, PMN convert >90% of incorporated [^{3}H]lyso-PAF to 1-*O*-[^{3}H]alkyl-2-arachidonoyl-GPC [19]. Cells were stimulated and microsomal membranes were prepared as described above. The same buffers and conditions were used as in the acceptor assay. The reaction was initiated by adding 30 μM unlabeled 1-*O*-alk-1′-enyl-2-lyso-GPE. The reaction was stopped by extraction, and the transacylase product, 1-*O*-[^{3}H]hexadecyl-2-lyso-GPC, was isolated by TLC and quantitated by liquid scintillation counting. Typically, 10–20% of the labeled PC was converted to 1-*O*-[^{3}H]hexadecyl-2-lyso-GPC.

2.6. $cPLA_2$ activity assay

PMN (3.0×10^7/3 ml) in PBS containing 1.4 mM Ca^{2+} were incubated for 30 min (37 °C) with or without 50 μM PD 98059 and/or 20 μM SB 203580; DMSO was used as an inhibitor vehicle control. Cells were then sequentially treated with or without 10 μM A23187 for 2 min, diluted with 35 ml chilled (4 °C) PBS, centrifuged, suspended in 1 ml protection buffer and sonicated. The protection buffer contains 10 mM HEPES (pH 7.5) containing 100 mM KCl, 1 mM EDTA, 1 mM EGTA, 50 μg/ml each of leupeptin and pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 0.2 mM Na_3VO_4, 4 mM dithiothreitol, and 1 mM diisopropylfluorophosphate and is necessary to protect $cPLA_2$ from the proteases present in the granules of PMN. For experiments using fMLP as a stimulus, 100 nM fMLP was used, and cells were stimulated for 1 min. The sonicate was centrifuged for 10 min at 16,000 × *g* to remove granules, nuclei and cellular debris, and the supernatant was further centrifuged for 1 h at 100,000 × *g*. In these procedures, the $cPLA_2$ is recovered in the cytosol since EDTA and EGTA chelate Ca^{2+} and release $cPLA_2$ that is membrane bound. The 100,000 × *g* supernatant was isolated and stored at 4 °C after adding 200 mg glycerol/1 ml until $cPLA_2$ activity was assayed. $cPLA_2$ activity of the samples remained unchanged after 1 week of storage; however, samples were typically assayed the following day. Soluble $cPLA_2$ activity was determined as previously described using 1-stearoyl-2-[^{14}C]arachidonoyl-GPC as the substrate and measuring [^{14}C]AA release [20]. Lipids were extracted by adding 4 volumes of $CHCl_3$/MeOH/acetic acid (2:1:0.01, v/v) + 0.05% butylated hydroxytoluene. Labeled AA was separated from phosphatidylcholine by TLC using a solvent system consisting of hexane/ethyl ether/formic acid (30:20:1, v/v); products and standards were visualized for scraping and counting by exposing the developed TLC plate to iodine vapors. Specific activities were based on protein concentration, as determined by the method of Bradford [16], using BSA as a standard.

Experiments to evaluate the effect of the kinase inhibitors on the phosphorylation state of $cPLA_2$ in stimulated PMN

were performed by Western blot analysis. Cells were treated the same way as those that were used for $cPLA_2$ activity measurement except that supernatant from the 30 min, $16{,}000 \times g$ spin was used rather than cytosol. We must emphasize that although the PMN sonicate contains a variety of protease and phosphatase inhibitors, it is important to centrifuge the PMN sonicate for at least 30 min to remove granular material to minimize degradation of the $cPLA_2$. Phosphorylation of $cPLA_2$ was determined by the appearance of a slower migrating band (gel shift) that was immunoreactive to the $cPLA_2$ antibody.

2.7. PAF formation assay

Stimulus-induced PAF accumulation was quantitated using two different methods. PAF production induced by fMLP stimulation was measured using a modification of the procedure described by Chilton et al. [19]. PMN were labeled as described above, and cells (3.5×10^7 cells/1 ml) were incubated with or without 50 μM PD 98059 and/or 20 μM SB 203580 (30 min, 37 °C), as detailed in the figure legends; DMSO was the inhibitor vehicle control. PMN were then treated with or without 10 μM fMLP for 10 min. The incubations were terminated by adding 3 ml chloroform/methanol (1:2, v/v) to the cell suspensions. Lipids were extracted, and [^{3}H]PAF was isolated by thin-layer chromatography (TLC) as described below.

A23187-stimulated PAF formation was quantitated as described [15]. Briefly, PMN were preincubated with and without kinase inhibitors, as described above, and treated with or without 10 μM A23187 for 10 min at 37 °C. Fifteen seconds after stimulation, 10 μCi [^{3}H]acetate was added to each reaction tube. Reactions were stopped by adding 3 ml chloroform/methanol (1:2, v/v) to the cell suspensions; lipids were extracted and products measured as described below.

2.8. Extraction and chromatography of lipids

Lipids were extracted from reaction mixtures using a modification of the Bligh and Dyer [17] method; the aqueous phase was acidified with acetic acid to lower the pH to ≤ 4. Authentic PC and PAF standards (50 μg of each) were added during extraction as carrier lipids to aid in recovery and chromatographic separation. The organic phases were evaporated under a stream of nitrogen and lipids dissolved in 100 μl chloroform/methanol (4:1, v/v). Lipids were separated using Silica gel G TLC plates developed in chloroform/methanol/acetic acid/water (50:25:8:4, v/v). Separated products and phospholipid standards were visualized by exposing the developed TLC plate to iodine vapors. In A23187-stimulated samples, radiolabeled lipids were detected using a radiochromatogram imaging system (Bio-Scan Inc., Washington, DC). Regions of the silica corresponding to labeled, individual lipid classes were scraped, and the radioactivity was quantitated by liquid scintillation counting (Minaxi Tri-Carb 4000 series). Since lower amounts of [^{3}H]PAF were formed upon fMLP stimulation, 5 mm zonal scraping was used to assure identification of products.

2.9. Statistical analysis

Data from experimental sets were averaged and reported as the mean ± S.E. Data were subjected to a paired, two-tailed Student's *t*-test to determine significance. Data comparisons that generated P values ≤ 0.05 were considered significantly different.

3. Results

3.1. Activation of MAP kinases and acetyltransferase

ERK-1, ERK-2, p38 MAP kinase and acetyltransferase are activated in PMN stimulated by fMLP [11,21,22]. To determine if stimulation by A23187 has similar effects, we first examined ERKs and p38 MAP kinase by Western blot analysis using whole cell sonicates obtained from control and A23187-stimulated PMN. Both ERKs and p38 MAP kinase underwent apparent gel shifts after treatment with A23187 (data not shown), suggesting that the MAP kinases had been phosphorylated. To confirm this, Western blots using tyrosine phosphospecific antibodies against phosphorylated ERKs and p38 MAP kinase were performed (Fig. 1). Panel A presents a blot of control and stimulated PMN sonicates using antibodies that selectively recognize ERKs phosphorylated on the tyrosine of their regulatory TEY sequence. Stimulation with A23187 induced phosphorylation of ERK-1 (p44), ERK-2 (p42) and p40, an ERK-like species that is likely a breakdown product of

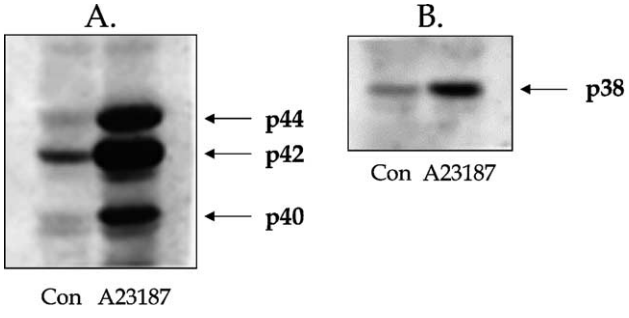

Fig. 1. Activation of MAP kinases by A23187. PMN (3.0×10^7 cells/3 ml) were stimulated with 10 μM A23187 for 10 min. Cells were sonicated in the presence of a protease and phosphatase protection buffer, and samples (2.5×10^5 cell equivalents) were analyzed by Western blot analysis as described in Experimental procedures. (A) Western blot of control and stimulated PMN sonicate using ERK antibodies that recognize ERKs phosphorylated at their regulatory TEY sequence. (B) Western blot analysis of control and stimulated PMN using antibodies that recognize p38 MAP kinase phosphorylated at its regulatory TGY sequence. Blots shown are representative samples from three independent experiments.

p44 (J.S. Owen and R.L. Wykle, unpublished observations). Phosphorylated p38 MAP kinase was also observed using an antibody that recognizes its tyrosine-phosphorylated regulatory TGY sequence (panel B). In a final set of experiments, we examined the impact of Ca^{2+} ionophore on acetyltransferase activity. A23187 (10 μM; 10 min) increased PMN acetyltransferase activity four-fold (Fig. 2). These results indicate that stimulation of PMN with either A23187 or fMLP [11,21,22] leads to the activation of ERK-1, ERK-2, p38 MAP kinase and acetyltransferase.

3.2. Effects of ERK and p38 MAP kinase inhibitors on acetyltransferase activity

We [5] and others [4,23,24] have shown that treatment of PMN with 50 μM PD 98059 or 20 μM SB 203580 for 30 min selectively inhibits ~ 80% of their respective ERK or p38 MAP kinase responses to test stimuli [21]. PMN treated at these concentrations of inhibitors, alone or in combination, for 30 min and then challenged for up to 20 min showed no change in their exclusion of Trypan blue (data not shown), did not leak cytosolic lactic acid dehydrogenase [5], and therefore remained intact. While SB 203580 has been shown to have nonspecific inhibitory effects on several enzymes [25], 50 μM PD 98059 has no inhibitory effect on p38 MAP kinase, and conversely, 20 μM SB 203580 has no effect on ERK activity [21].

We measured acetyltransferase activity in A23187-stimulated PMN that had been treated for 30 min with SB 203580 and/or PD 98059 to assess the relative contribution of the kinase pathways to the enzyme's activation. A basal level of acetyltransferase activity was detected in unstimulated PMN (22.8 ± 0.01 nmol PAF/mg/min), which was unaffected by preincubation with SB 203580 and/or PD 98059 (Fig. 2). However, SB 203580 completely inhibited the stimulatory effect of A23187 on acetyltransferase activity ($P < 0.05$). In contrast, PD 98059 had no significant effect under the same conditions. A combination of both inhibitors reduced stimulated acetyltransferase activity to the same level as that measured with the p38 MAP kinase inhibitor alone. Hence, p38 MAP kinase but not ERK-1 or ERK-2, appears critical for the activation of acetyltransferase induced by either a receptor agonist [11] or Ca^{2+} ionophore.

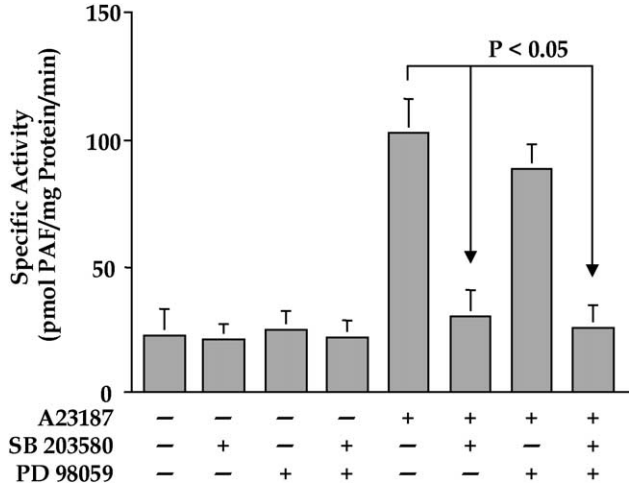

Fig. 2. Effect of kinase inhibitors on acetyltransferase activity. PMN (3.5×10^7 cells/1 ml) were incubated for 30 min with 20 μM SB 203580, 50 μM PD 98059, both drugs, or DMSO vehicle. Cells were then stimulated with 10 μM A23187 for 10 min. Microsomal fractions were prepared, and acetyltransferase activity was assayed as described in Experimental procedures. Results shown are the means ± S.E. of three experiments. Statistically significant differences (paired, two-tailed, Student's *t* test) are indicated.

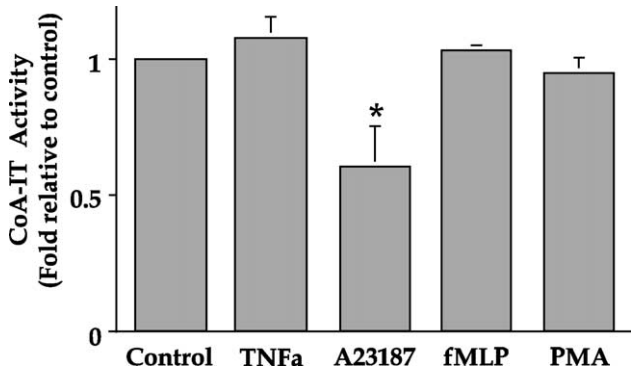

Fig. 3. Effect of various stimuli on CoA-IT activity. PMN (3.5×10^7 cells/1 ml) were stimulated with either TNFα (3.16 nM; 10 min), A23187 (10 μM; 10 min), fMLP (10 μM; 10 min) or PMA (100 nM; 5 min). Microsomal fractions were prepared, and CoA-IT activity was measured with an acceptor assay as described in Experimental procedures. Results are given as the means ± S.E. The control level of CoA-IT activity was 7.05 ± 0.46 pmol/mg/min. For each stimulus, the number of experiments performed is as follows: TNFα ($n = 22$), A23187 ($n = 7$), PMA ($n = 3$) and fMLP ($n = 3$). The *P* value from a two-tailed, paired, Student's *t*-test is as follows: $P < 0.05$ for unstimulated PMN versus A23187-stimulated PMN (*).

3.3. Effects of various stimuli on CoA-IT activity in PMN

CoA-IT has been shown to form lyso-PAF from 1-*O*-alkyl-2-arachidonoyl-GPC in the presence of lyso-PE, suggesting, along with other data [26], that it plays a role in PAF synthesis [27]. Two reports indicate that CoA-IT activity in cells rises modestly in response to stimulation of PMN with TNFα [28] or macrophages with LPS [29] and therefore may play a regulatory role in PAF synthesis. To test this in our system, CoA-IT activity was measured in microsomal membrane preparations obtained from PMN treated with a variety of stimuli (Fig. 3). Stimulation of PMN with TNFα, fMLP or PMA did not affect CoA-IT activity as measured in an in vitro system. (Typically, approximately 5–10% of the labeled substrate was converted to product.) In contrast, stimulation with A23187 reduced CoA-IT activity by 40% ($P < 0.05$). This last result was studied earlier in A23187-stimulated PMN and found to be due to the build-up of endogenous competing substrates (see Discussion). Moreover, pretreatment of PMN with

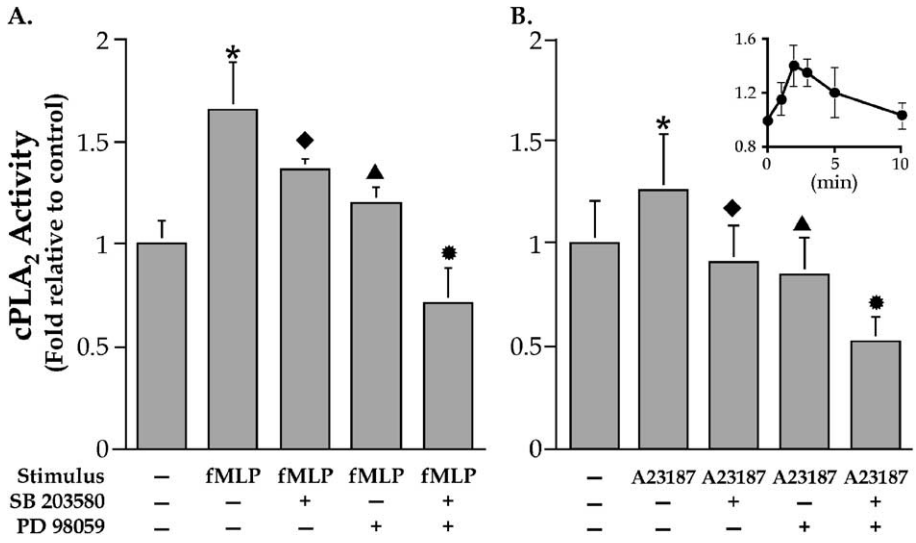

Fig. 4. Effects of the MAP kinase inhibitors on fMLP- and A23187-stimulated cPLA$_2$ activity. PMN (3×10^7/3 ml) were treated with either (A) fMLP (100 nM; 1 min), (B) A23187 (10 μM; 2 min) or vehicle at 37 °C. Cytosolic fractions were prepared and assayed for cPLA$_2$ activity as described in Experimental procedures. Data represent the means ± S.E. of three (fMLP) or five (A23187) separate experiments. *P* values from a two-tailed, paired, Student's *t*-test are as follows. (A) $P < 0.05$ for fMLP-stimulated PMN versus control (*) and versus fMLP-stimulated PMN treated with SB 203580 (♦), PD98059 (▲), or both inhibitors (✹); there is not a significant difference between control PMN and fMLP-treated PMN with both inhibitors; (B) $P < 0.05$ for A23187-stimulated PMN versus control (*) and versus A23187-stimulated PMN treated with SB 203580 (♦), PD98059 (▲), or both inhibitors (✹) $P < 0.05$ for * versus ✹. Inset: PMN (3×10^7/3 ml) were treated with 10 μM A23187 for the indicated time periods. Cytosolic fractions were prepared and cPLA$_2$ activity was measured as described. Maximal cPLA$_2$ activity occurred at 2 min. Consequently, the effects of A23187 on cPLA$_2$ activity and its phosphorylation state (Fig. 5) were measured after 2 min stimulation.

either or both kinase inhibitors had no effect on measured CoA-IT activity in control or treated PMN (data not shown). Finally, we were unable to activate CoA-IT in microsomal preparations in vitro using recombinant, active p38, ERK-1 and/or ERK-2 in the presence or absence of PMN soluble fraction (data not shown). Under similar cell-free conditions, p38 MAP kinase activated microsomal acetyltransferase [11] as well as recombinant cPLA$_2$, while ERK-1 and ERK-2 activated only recombinant cPLA$_2$ (J.S. Owen and R.L. Wykle, unpublished observations). We conclude that CoA-IT is not regulated by ERK1/2 or p38 MAP kinase and shows no stimulation of activity in PMN challenged with diverse stimuli.

3.4. Effects of ERK and p38 MAP kinase inhibitors on stimulated cPLA$_2$ activity

To measure the effects of the MAP kinase inhibitors on cPLA$_2$ activation, cells were stimulated in the presence or absence of SB 203580 and/or PD 98059, and the recovered cytosol was assayed for cPLA$_2$ activity (Fig. 4). Since calcium promotes cPLA$_2$ binding to membranes, EDTA and EGTA are included in the cell disruption mixture to remove free calcium and maximize recovery of the enzyme in the cytosolic fraction [20]. DTT is included in both the assay buffer and cell disruption mixture to destroy cPLA$_2$ activity. Using this procedure, >95% of the total cPLA$_2$

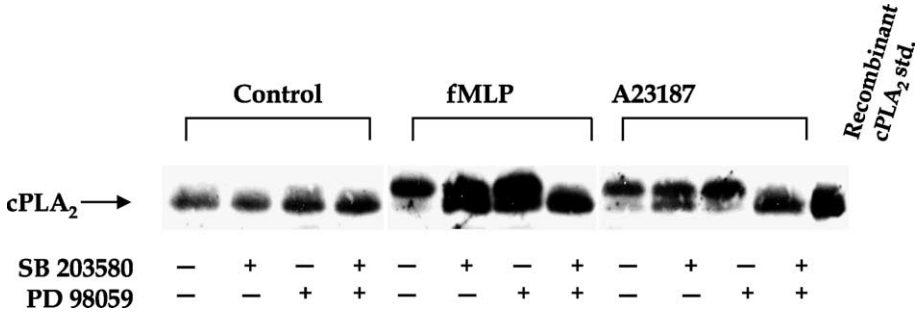

Fig. 5. Inhibition of cPLA$_2$ phosphorylation by SB 203580 and/or PD 98059. PMN were isolated and stimulated in the presence or absence of the MAP kinase inhibitors and then analyzed by Western blotting as described in Experimental procedures. Phosphorylation of cPLA$_2$ was determined by the appearance of a higher molecular weight band (gel shift) in the immunoblots. The blot shown is representative of six independent experiments.

activity is recovered in the cytosol [20]. Treatment of PMN with 100 nM fMLP increased $cPLA_2$ activity 65% relative to control (Fig. 4A; $P<0.05$). Use of either PD 98059 or SB 203580 alone partially inhibited (40–60%) this response ($P<0.05$), whereas a combination of both drugs totally ablated fMLP-induced $cPLA_2$ activation ($P<0.05$ for fMLP-stimulated PMN versus fMLP-stimulated PMN treated with either or both inhibitors). This pattern of inhibition is clearly seen in $cPLA_2$ Western blots (Fig. 5). $cPLA_2$ underwent an apparent gel shift in fMLP-treated PMN, which was partially reduced when the PMN were pretreated with either of the MAP kinase inhibitors alone. When both inhibitors were used, the fMLP-induced $cPLA_2$ gel shift was completely blocked. Stimulation of PMN with 10 μM A23187 likewise resulted in a rise in $cPLA_2$ activity. The effect peaked at 2 min and returned to control levels within 5 min (Fig. 4B; inset). Accordingly, we evaluated $cPLA_2$ activity at 2 min after stimulation. The stimulation with A23187 resulted in a modest but statistically significant 30% increase in $cPLA_2$ activity ($P<0.05$). Preincubation with SB 203580 or PD 98059 decreased to near control values the A23187-stimulated activation of $cPLA_2$, whereas the combination of both inhibitors resulted in a further decrease in $cPLA_2$ activity to below that observed in resting cells ($P<0.05$; Fig. 4B). Relative to the latter finding, we stress that cell viability under these experimental conditions was $\geq 97\%$, as measured by Trypan blue exclusion and furthermore that the inhibitors have no direct effect on $cPLA_2$ activity in enzymatic assays at the concentrations used here (data not shown). In some experiments, $cPLA_2$ Western blot analysis of PMN treated with A23187 showed

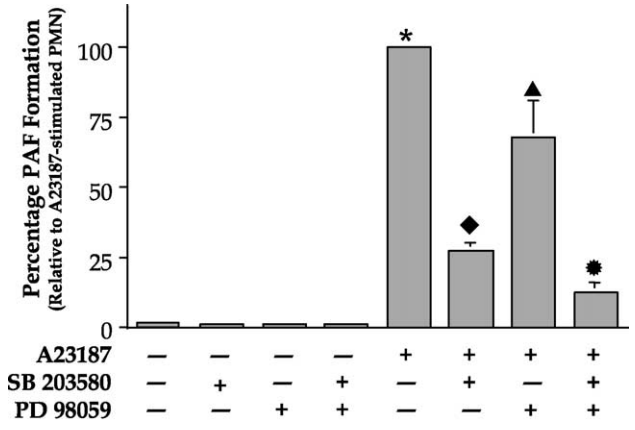

Fig. 6. Effect of kinase inhibitors on A23187-stimulated PAF formation. PMN (3.5×10^7 cells/1 ml) were incubated for 30 min with 20 μM SB 203580, 50 μM PD 98059, both drugs, or DMSO vehicle in PBS containing 1.4 mM Ca^{2+}. PMN were then stimulated for 10 min by adding 10 μM A23187 along with 10 μCi [^{3}H]acetate. PAF was extracted and quantitated as described in Experimental procedures. Results shown are the means ± S.E. of three experiments. Typically, 7000 dpm [^{3}H]acetate was incorporated into PAF in A23187-treated PMN. *P* values from a two-tailed, paired, Student's *t*-test are as follows: $P<0.05$ for A23187-stimulated PMN versus control (*) and versus A23187-stimulated PMN treated with SB 203580 (♦), PD98059 (▲), or both inhibitors (✹); $P<0.05$ for ♦ versus ✹.

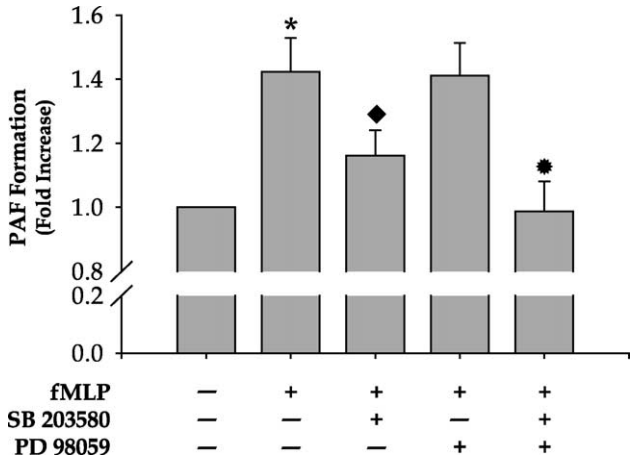

Fig. 7. Effect of kinase inhibitors on fMLP-stimulated PAF formation. PMN (3.5×10^7 cells/1 ml) were prelabeled with [^{3}H]lyso-PAF (60,000 dpm/tube) in PBS without Ca^{2+} for 60 min at 37 °C. PMN were then incubated for 30 min with 20 μM SB 203580, 50 μM PD 98059, both drugs, or DMSO vehicle in PBS containing 1.4 mM Ca^{2+}. PMN were subsequently stimulated for 10 min with 10 μM fMLP or with vehicle only as control; PAF was extracted and quantitated as described in Experimental procedures. Results shown are the means ± S.E. of eight independent experiments. Approximately 800–1500 dpm of labeled PC was converted to [^{3}H]PAF. *P* values from a two-tailed, paired, Student's *t*-test are as follows: $P<0.05$ for fMLP-stimulated PMN versus control (*) versus fMLP-stimulated PMN treated with SB 203580 (♦) or both inhibitors (✹); $P<0.05$ for ♦ versus ✹.

a decrease in immunoreactive $cPLA_2$ to below control levels (data not shown). This observation suggests that A23187 may induce degradation of $cPLA_2$, which may, in part, explain the decrease in $cPLA_2$ activity to below control levels. However, despite some loss of the enzyme, the Western blots do show that the phosphorylation state of $cPLA_2$ correlates with the activity data (Fig. 5). Either inhibitor alone partially attenuates the gel shift, and both inhibitors combined completely block $cPLA_2$ phosphorylation. These results suggest that as with fMLP, A23187-stimulated $cPLA_2$ activity is regulated by both ERKs and p38 MAP kinase.

3.5. Effects of ERK and p38 MAP kinase inhibitors on A23187- and fMLP-stimulated PAF formation

Unstimulated PMN synthesize little PAF (~ 110 dpm [^{3}H]acetate incorporated into PAF), and incubation with SB 203580, PD 98059 or both drugs did not significantly alter the basal PAF formation. Stimulation of PMN with A23187 resulted in a 68-fold increase in PAF formation (~ 7000 dpm; $P<0.05$); this dramatic increase was reduced 60% by SB 203580, 35% by PD 98059, and 85% using a combination of the drugs (Fig. 6). Cells stimulated with 10 μM fMLP exhibited a smaller but significant, 42 ± 11% increase in PAF formation relative to unstimulated cells (800–1500 dpm of labeled PC converted to PAF; $P<0.05$), which SB 203580 inhibited 60%. Although the ERK pathway inhibitor had no significant effect on fMLP-stimulated PAF produc-

tion, its combination with SB 203580 completely ablated stimulated PAF synthesis in PMN (Fig. 7). Overall, our studies indicate that the ERKs and p38 MAP kinase contribute to PAF formation induced by both stimulus types. In the absence of ERK activation, p38 MAP kinase appears to provide sufficient activation of $cPLA_2$ and acetyltransferase to support low levels of PAF formation.

4. Discussion

PAF synthesis has been implicated in a number of pathological conditions such as necrotizing entercolitis [30], ischemic reperfusion injury [31], multiple organ failure [32] and anaphylaxis [33]. Consequently, defining the regulation of stimulus-induced PAF formation could contribute to effective therapeutic strategies to control these and other inflammatory diseases. In many cells, including PMN, PAF synthesis proceeds through a remodeling pathway that appears to involve $cPLA_2$ [34], acetyltransferase [35,36] and CoA-IT [18,26]. Our present studies using the receptor agonist fMLP and the Ca^{2+} ionophore A23187 indicate that MAP kinase regulation of $cPLA_2$ and acetyltransferase, but not CoA-IT, govern stimulus-induced PAF synthesis.

Fig. 2 shows that SB 203580, a known inhibitor of p38 MAP kinase, blocks A23187-stimulated acetyltransferase activity, whereas PD 98059, a known inhibitor of the ERK pathway, has little effect. Combined with our findings in fMLP-stimulated PMN [11], these data indicate that p38 MAP kinase bears major, if not sole, responsibility for activating the acetyltransferase in PMN. As seen in Fig. 2, however, resting PMN exhibit appreciable acetyltransferase activity. Although this baseline activity can be somewhat further reduced to lower levels by alkaline phosphatase treatment of the microsomal membrane fractions (suggesting that the acetyltransferase may be partially activated during the PMN isolation process), there remains a significant acetyltransferase activity [11]. We speculate that the basal level activity is sufficient to allow PAF synthesis to proceed at a reduced rate, provided that lyso-PAF is available.

In addition to being released directly from its precursor by PLA_2, lyso-PAF can be formed by PLA_2 hydrolysis of plasmenylethanolamine and transacylation via CoA-IT [37]. Winkler et al. [28] reported a modest increase in CoA-IT activity in PMN stimulated with TNFα, and Svetlov et al. [29] reported an increase in CoA-IT activity, as measured by a donor assay but not by an acceptor assay, in a murine macrophage cell line, IC-21, challenged by LPS. We found no change in CoA-IT activity in PMN challenged with fMLP, TNFα or PMA (Fig. 3) as measured by either an acceptor or a donor assay. We are unable to explain why our findings differ from those of the earlier cited reports. The apparent decrease in CoA-IT activity in PMN treated with A23187 (Fig. 3) is explained by Venable et al. [27]. In these studies, we found that the apparent decrease in CoA-IT activity in Ca^{2+} ionophore-challenged PMN could be attributed to substrate competition by unlabeled lyso-phospholipid, i.e., 1-*O*-alk-1′-enyl-2-lyso-GPE, rather than to inhibition of the enzymatic activity per se. We found that washing microsomal membrane preparations from A23187-stimulated PMN with albumin, thereby removing competing unlabeled lyso-phospholipid, effectively restored CoA-IT activity to control levels [27]. Furthermore, addition of lyso-plasmenylethanolamine (at concentrations found in stimulated preparations) mimicked the apparent decrease in activity observed in the stimulated membrane preparations. Attempts to increase CoA-IT activity in PMN microsomal membranes using recombinant, active p38 MAP kinase, ERK-1 or ERK-2 in the presence or absence of PMN soluble fraction were unsuccessful (data not shown). Furthermore, preincubation of PMN with the MAP kinase inhibitors prior to stimulation with either TNFα or A23187 had no affect on CoA-IT activity, as determined in our in vitro assay (data not shown). We therefore conclude that under the conditions of our studies, CoA-IT is best viewed as a constitutively active enzyme whose contribution to lyso-PAF formation is passive, i.e., dependent upon the availability of PLA_2-generated 1-*O*-alk-1′-enyl-2-lyso-GPE.

In contrast to acetyltransferase and CoA-IT, $cPLA_2$ activation in response to treatment with fMLP appears to involve both ERK and p38 MAP kinases. Individually, PD 98059 and SB 203580 partially inhibited $cPLA_2$ activation, while a combination of both drugs completely inhibited its activation (Fig. 4A). These results differ from the findings by Syrbu et al. [38] that neither PD 98059 nor SB 203580 inhibited fMLP-induced phosphorylation of $cPLA_2$ (a combination of the two drugs was not tested). However, the $cPLA_2$ Western blot data reported here (Fig. 5) indicate that

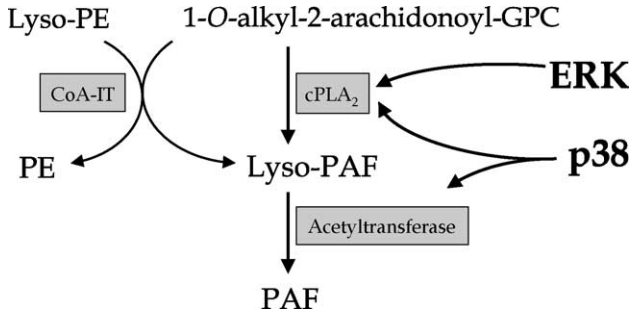

Fig. 8. Proposed regulation of stimulated PAF formation via the remodeling pathway in PMN. $cPLA_2$ can be phosphorylated by both ERKs and p38 MAP kinase. Once active, $cPLA_2$ hydrolyzes arachidonate from 1-*O*-alkyl-2-arachidonoyl-GPC, which can be converted to inflammatory eicosanoids, and the resultant lyso-PAF is subject to acetylation by acetyl-CoA:1-*O*-alkyl-2-lyso-*sn*-GPC acetyltransferase. CoA-IT also generates lyso-PAF if an appropriate acceptor lysophospholipid is present; however, the enzyme is not regulated by signaling. The constitutive activity of acetyltransferase can convert lyso-PAF that is generated by either pathway to PAF, but when acetyltransferase is activated by p38 MAP kinase, increased PAF formation is observed. Because PAF is such a powerful agonist, the lower amounts of PAF formed in the absence of acetyltransferase activation may be sufficient to induce biological effects.

individually, the two inhibitors do indeed partially block the $cPLA_2$ gel shift in fMLP- and A23187-stimulated PMN. Our results are consistent with those of Borsch-Haubold et al. [39] who reported that SB 203580 inhibited phosphorylation of $cPLA_2$. Moreover, our treatment of PMN with SB 203580 or PD 98059 reduced A23187-stimulated $cPLA_2$ activity to near unstimulated levels (Fig. 4, panel B). A combination of the drugs reduced $cPLA_2$ activity by ~ 50% in A23187-challenged (Fig. 4B) but not fMLP-challenged or resting PMN (Fig. 4A). Gel shift analysis (Fig. 5) to measure the phosphorylation state of $cPLA_2$ in A23187-stimulated PMN correlates with the activity data. However, some of the immunoblots also indicate that in A23187-stimulated PMN, the amount of $cPLA_2$ decreases as compared to control cells. This last observation may be explained by recent studies that show $cPLA_2$ can be degraded by caspase-3 in A23187- and TNFα-stimulated cells [40,41].

The results presented here indicate that both ERKs and p38 MAP kinases participate in the activation of $cPLA_2$. Although phosphorylation of the enzyme by p38 MAP kinase has been controversial [7,42–44], it is becoming increasingly clear that the kinase does modulate $cPLA_2$ activity. As shown in Figs. 4 and 5, the activation of $cPLA_2$, at least as it occurs in PMN treated with fMLP or A23187, is partially blocked by the addition of a p38 MAP kinase inhibitor. This result is compatible with studies by Kramer et al. [43,45], who demonstrated the incorporation of radiolabeled phosphate into recombinant $cPLA_2$ by a p38 MAP kinase-enriched column fraction from stimulated platelets. Recently, Borsch-Haubold et al. [46] reported direct phosphorylation of $cPLA_2$ by three human isoforms of p38 MAP kinase: p38α, β and δ. Experiments in our laboratory confirm this result (J.S. Owen and R.L. Wykle; unpublished observations). Since kinases downstream of p38 MAP kinase may also phosphorylate and activate $cPLA_2$ [47], we interpret our results as indicating that $cPLA_2$ activity is directly modulated by ERKs and either directly or indirectly by p38 MAP kinases.

Evaluation of the individual enzymes of the remodeling pathway suggests that both p38 MAP kinase and ERK cascades are intimately involved in stimulated PAF synthesis. However, the relative contribution of the two MAP kinase pathways to PAF formation cannot be determined in the cell-free systems used. Consequently, modulation of PAF formation was evaluated in intact PMN stimulated with A23187 or fMLP (Figs. 6 and 7). We found that in either A23187- or fMLP-stimulated PMN, blocking the p38 MAP kinase pathway significantly attenuates PAF synthesis. However, in order to optimally block stimulated PAF formation, the ERK pathway must also be inhibited. These results suggest a partial redundancy of MAP kinase signaling with respect to PAF synthesis. Furthermore, since both stimuli similarly activate the two MAP kinase pathways and give similar inhibition profiles, our results indicate that A23187 is indeed a suitable model stimulus for studies of PAF biosynthesis.

In summary, our results support a model in which both ERK and p38 MAP kinase cascades control flux through the remodeling pathway by activating two rate-limiting enzymes, $cPLA_2$ and acetyltransferase (Fig. 8). Although a role for CoA-IT in PAF synthesis is recognized, it appears that this enzyme is controlled not by the MAP kinases but by the availability of its lysophospholipid substrate, which is provided by a PLA_2. Furthermore, these results suggest that therapeutic strategies to control PAF-induced inflammation by inhibiting the regulatory mechanism of PAF formation would seem better aimed at blocking both the ERK and p38 MAP kinase pathways.

Acknowledgements

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