

Regulation and kinetics of platelet-activating factor and leukotriene C₄ synthesis by activated human basophils

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Summary

Background Allergic disease is the result of an interplay of many different cell types, including basophils and mast cells, in combination with various inflammatory lipid mediators, such as platelet-activating factor (PAF) and leukotrienes (LT). LTC₄ synthesis by human basophils has been studied quite extensively. However, not much is known about the synthesis of PAF by human basophils.

Objective In this study, we have made a comprehensive comparison between the kinetics of PAF and LTC₄ synthesis, in highly purified basophils, activated with different stimuli or with combinations of stimuli.

Methods Synthesis of PAF and LTC₄ by human basophils was determined with commercially available assay kits. The basophils were activated with C5a, fMLP, PMA, allergen or anti-IgE, in the absence and presence of IL-3 and/or in combination with elevation of cytosolic free Ca²⁺ by the sarcoplasmic reticulum Ca²⁺-ATPase inhibitor thapsigargin.

Results Most stimuli were found to induce both PAF and LTC₄ synthesis. PAF synthesis and LTC₄ release were enhanced by preincubation of the basophils with IL-3 or by elevation of cytosolic free Ca²⁺ by thapsigargin. Incubation of human basophils with IL-3 alone or thapsigargin alone did not result in detectable synthesis of PAF and LTC₄, whereas the combination of the two resulted in high amounts of PAF and LTC₄ synthesis. Depending on the stimulus used, LTC₄ release was 5–100-fold higher than PAF synthesis. In addition, PAF, but not LTC₄, was transiently detected, probably due to PAF degradation.

LTC₄ and PAF synthesis was strongly blocked by inhibitors of cytosolic phospholipase A₂, indicating that this enzyme is involved in PAF and LTC₄ synthesis by activated human basophils.

Conclusion This study provides a first comprehensive comparison of PAF and LTC₄ synthesis in highly purified human basophils, stimulated with a variety of stimuli.

Keywords cPLA₂, human basophils, LTC₄, PAF

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Introduction

Allergic disease is the result of many different cell types producing an abundant amount of inflammatory mediators, such as platelet-activating factor (PAF) and leukotrienes (LT). Basophils and mast cells are thought to be involved in the pathogenesis of allergy.

The cysteinyl leukotrienes LTC₄, LTD₄ and LTE₄ cause various events, e.g. sustained contraction of smooth muscle in the lung leading to airway obstruction, impaired mucociliary clearance, enhanced mucus secretion and increase in pulmonary vascular permeability causing oedema [1–3]. Additionally, cysteinyl leukotrienes append to the allergic inflammation by attracting eosinophilic granulocytes [1, 4]. The effect of PAF is also diverse, including vasodilatation, vascular permeability changes, chemotaxis, priming and activation of neutrophils

and eosinophils. Because of these effects, PAF is thought to play a role in e.g. sepsis and shock [5].

Clinically, inhaled LTC₄ and LTD₄ have been shown to be potent mediators (1000 times more potent than histamine) in causing airway obstruction in normal subjects, with a longer duration of action compared to histamine [6]. Furthermore, in patients with asthma, LTC₄ and LTD₄ increase bronchial hyper-responsiveness to histamine and methacholine [7]. Besides the clear effect of leukotrienes on pulmonary function, leukotrienes have been detected in plasma, urine, sputum and broncho-alveolar lavage fluids (BALF) from patients with exacerbations and after allergen challenge [8]. The role of PAF in allergic inflammation (e.g. allergic asthma) is illustrated by its contribution to the ventilation/perfusion mismatches in severe asthma attacks [9]. Involvement of PAF is also suggested by findings of increased PAF-acetylhydrolase (PAF-AH) activity in BALF of allergic subjects after allergen challenge [10] as well as in nasal lavage fluid of rhinitis patients [11]. Moreover, it has been shown that the severity of respiratory symptoms in asthmatic

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children was more prominent in children with a PAF-AH deficiency [12].

The synthesis of free arachidonic acid (AA, the precursor of LTC₄) and lyso-PAF (the precursor of PAF) is initiated by activation of phospholipase A₂ (PLA₂) and subsequent cleavage of the sn-2 fatty acid from phospholipids. AA is then converted into LTA₄ by a complex of 5-lipoxygenase and the 5-lipoxygenase-activating protein. LTA₄ is transformed by leukotriene C₄ synthase to LTC₄. LTC₄ can be further converted into LTD₄ and LTE₄. Lyso-PAF is derived from PLA₂-mediated cleavage of 1-alkyl-2-acylphosphatidylcholine, and is rapidly converted into PAF by acetyltransferase. On the other hand, PAF can be degraded to lyso-PAF by PAF-AH. Although LTC₄ and PAF may be derived from one and the same precursor, i.e. 1-alkyl-2-AA-phosphatidylcholine, AA can be liberated from any sn-2-AA-containing phospholipid, and lyso-PAF can be derived from any 1-alkyl-2-acyl-phosphatidylcholine.

Although many different forms of PLA₂ have been described (for a review, see [13]), the PLA₂ enzymes in humans involved in the release of AA and the generation of lyso-phospholipids can be divided into two types: an 85-kDa cytosolic PLA₂ (cPLA₂, group IV) and a so-called secretory 14-kDa PLA₂ (sPLA₂, group I, II or V). Several studies have indicated that cytosolic PLA₂ rather than sPLA₂ is the key phospholipase involved in PAF and LTC₄ in activated human basophils [14–17]. The activation of cPLA₂ is regulated by elevation of the cytosolic free Ca²⁺ concentration and phosphorylation [14, 18–20].

The main routes of basophil activation (e.g. histamine release) are stimulation via cross-linking of the IgE receptor (with allergen or anti-IgE), stimulation of G-protein-coupled receptors by agonists [such as C5a and formyl-methionyl-leucyl-phenylalanine (fMLP)] or by direct activation of PKC by PMA. Previously, we have found that in the presence of thapsigargin, an inhibitor of the sarco(endo)plasmic reticulum Ca²⁺-ATPase, also interleukins, such as IL-3, IL-5 and GM-CSF, are able to induce considerable histamine release [21].

LTC₄ synthesis by human basophils has been studied quite extensively [22–26]. However, not much is known about the synthesis of PAF by human basophils. Some earlier studies even failed to show PAF synthesis in activated human basophils [27, 28], although PAF synthesis by human basophils has been suggested in unpurified basophils and *in vivo* [29, 30]. Comprehensive comparisons between the kinetics of PAF and LTC₄ synthesis, in highly purified basophils, activated with a variety of different stimuli or with combinations of stimuli, have, to the best of our knowledge, not been described.

In this study, we have therefore investigated the synthesis of PAF and LTC₄ in highly purified human basophils (>95%) after activation of the cells with C5a, fMLP, PMA, allergen or anti-IgE. Activation of the cells was performed in the absence and presence of thapsigargin or IL-3. In addition, to confirm that cytosolic PLA₂ (cPLA₂) is the key phospholipase involved in PAF and LTC₄ synthesis in activated human basophils, we show that PAF and LTC₄ synthesis was completely inhibited by well-characterized and commonly used inhibitors of cPLA₂ (i.e. AACOCF₃ [31] and MAFP [32]).

Materials and methods

Materials

Recombinant human IL-3 was obtained from Pepro Tech Inc. (Rocky Hill, NJ, USA). Polyclonal anti-IgE (sheep-anti-human) was obtained from the CLB (Amsterdam, The Netherlands). Thapsigargin, C5a, fMLP, PMA, taurine (2-amino-ethane-sulphonic acid) and SOD (superoxide dismutase) were purchased from Sigma Chemicals (St Louis, MO, USA). Catalase was purchased from Boehringer Mannheim (Mannheim, Germany). FMLP and PMA were dissolved in DMSO and stored at –20 °C. C5a was dissolved in PBS with 0.5% (w/v) human serum albumin. Methyl-arachidonyl-fluorophosphonate (MAFP, a solution in methylacetate) and arachidonyl-trifluoromethyl-ketone (AACOCF₃, a solution in ethanol), both from Cayman Chemicals Company (Ann Arbor, MI, USA), were stored at –80 °C in aliquots. MAFP and AACOCF₃ were evaporated to dryness for every day used, and were dissolved in incubation medium with the use of a bath sonicator at a concentration of 500 µM. Human recombinant PAF-AH, produced by *Escherichia coli*, was a generous gift from Dr Dietsch (ICOS Corporation, Bothell, WA, USA). The incubation medium contained 132 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 1.2 mM potassium phosphate, 20 mM HEPES, 5.5 mM glucose and 0.5% (w/v) human serum albumin, pH 7.4.

Purification of basophils

Buffy coats were prepared from 500 mL of human blood from healthy donors without an allergic history (unless otherwise indicated) after informed consent had been obtained. The basophils were purified by successive isopycnic centrifugation and elutriator centrifugation, as described by de Boer and Roos [33] (purity approximately 75%). The basophils were further purified by immunodepletion of residual contaminating cells with various monoclonal antibodies (CD2, CD14, CD16, CD19, CD66b) and magnetic beads, as described [34]. The cells were counted electronically (Coulter counter model ZF; Coulter Electronics, Dunstable, UK). The percentage of basophils was determined by differential staining with Alcian blue [35] and from cytocentrifuge preparations stained with May–Grünwald/Giemsa. Viability was determined by trypan blue exclusion. The purity of the basophils in these preparations was >95%, with a viability of about 98%.

Measurement of LTC₄ release

Basophils (1 × 10⁶/mL) were resuspended in incubation medium in the presence of 50 U/mL SOD, 30 µg/mL taurine and 2500 U/mL catalase to prevent oxidative metabolism of LTC₄ [36, 37]. The additives, at the indicated concentrations, did not affect histamine release by activated human basophils, nor did they interfere with the LTC₄ ELISA (data not shown). After preincubation for 5 min at 37 °C, the basophils were activated, and LTC₄ release was measured in the cell-free supernatant with a commercially available competitive ELISA kit (Neogen Corporation, Lexington, KY, USA) exactly as recommended by the manufacturer. Cell supernatants, rather than total cell extracts, were used because of problems with background signals in the assay used.

However, measurement of LTC₄ release does reflect LTC₄ synthesis, due to transport of the product to the extracellular space. The amounts of LTC₄ were calculated by means of a standard curve constructed with known amounts of LTC₄. Recovery of an LTC₄ spike was more than 90%. All samples were tested within the linear range of the assay. The detection limit of the assay was 0.04 ng LTC₄/10⁶ cells. Unstimulated LTC₄ release amounted to 0.21 ± 0.12 (mean ± SD, *n* = 14) ng LTC₄/10⁶ basophils; release of LTC₄ by activated human basophils was not corrected for the spontaneous release.

Measurement of PAF synthesis

PAF was measured as described before [38] with a commercially available competitive radioimmunoassay (New England Nuclear, Boston, MA, USA). In short, basophils (1 × 10⁶/mL) were preincubated for 5 min at 37 °C before stimulation. The reaction was stopped by mixing 0.8 mL of cell suspension with 3 mL of methanol/chloroform (2:1) with 2% (v/v) acetic acid added to the methanol. After separation of the phases with 1 mL of chloroform and 1 mL of H₂O, the lower chloroform phase was stored at -70 °C under nitrogen. Subsequently, the chloroform phases were evaporated under a stream of nitrogen and processed for the radioimmunoassay according to the manufacturer's instructions. The amounts of PAF were calculated by means of a standard curve constructed with known amounts of PAF. The recovery of PAF during the whole procedure was more than 90%. All samples were tested within the linear range of the assay. Unstimulated synthesis of PAF was below the detection limit of the assay (< 0.05 ng PAF/10⁶ cells). Since the above-described assay kit is not manufactured anymore, the measurements of PAF depicted in Table 1 and Fig. 8 were analysed with another PAF assay kit, from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Careful examination of the two PAF assay kits demonstrated a comparable detection of PAF, with identical detection limits. According to the manufacturers, both PAF assay kits do not detect lyso-PAF (cross-reactivity < 0.01%) or the 1-acyl analogue of PAF (cross-reactivity < 0.1%).

Table 1. Comparison of synthesis of PAF between monocytes and basophils

Addition	PAF synthesis (ng/10 ⁶ cells)	
	Monocytes	Basophils
None	0.13	< 0.05
PMA	2.29	0.10
fMLP	0.70	0.49
IL-3/fMLP	2.88	1.48
C5a	0.20	< 0.05
Thapsigargin	10.1	0.21
Thapsigargin/IL-3	14.6	6.5

After 5 min of preincubation at 37 °C, monocytes (1 × 10⁶/mL) and basophils (1 × 10⁶/mL) were activated with medium, PMA (100 ng/mL), fMLP (1 µM), IL-3 (500 pM, 5 min)/fMLP (1 µM), C5a (10 nM), thapsigargin (0.5 µM) or with thapsigargin (0.5 µM, 2 min)/IL-3 (500 pM) for 10 min. PAF synthesis was measured as described in Materials and methods. Results are expressed as the mean of 1–2 experiments, performed in duplicate.

Measurement of histamine release

Basophils (~1 × 10⁵/mL) were activated with indicated agents after preincubation for 5 min at 37 °C in an incubation medium. The reaction was stopped by centrifugation for 10 s at 12 000g, and supernatants were collected. Supernatants (350 µL) were mixed with 200 µL of 12% (w/v) perchloric acid and 750 µL of saline (0.9% NaCl w/v), and stored at 4 °C. For measurement of the total amount of histamine, the same number of cells used for the incubations was lysed in perchloric acid. Histamine was measured by fluorometric analysis as described by Siraganian [39]. Histamine release was calculated as the percentage of the total amount of histamine in the cells.

Measurement of sPLA₂ activity

sPLA₂ activity was measured with a commercially available assay kit (Cayman Chemicals Company, Ann Arbor, MI, USA), exactly as recommended by the manufacturer, with the 1,2-dithio analogue of diheptanoyl phosphatidylcholine, which serves as a substrate for most sPLA₂, but not for cPLA₂.

Results

Since basophil preparations are often contaminated with monocytes, we first compared the amount of PAF synthesized by pure monocytes (>95%) and pure basophils (>95%). The results of these experiments are depicted in Table 1. This table clearly shows that the amount of PAF detected in human monocytes is substantially higher than in human basophils for all stimuli tested. Subsequent experiments were therefore performed with bead-isolated basophils (purity >95%). For all stimulation conditions, basophils were stimulated with concentrations of stimuli that were known to induce optimal histamine release.

PAF and LTC₄ synthesis by human basophils activated either with fMLP or C5a

Next, the kinetics of PAF synthesis and LTC₄ release were determined after activation of human basophils in the absence and presence of IL-3. Fig. 1 shows the fMLP-induced synthesis of PAF (Fig. 1a) and release of LTC₄ (Fig. 1b) by human basophils in the absence and presence of IL-3. In the absence of IL-3, fMLP induced significant amounts of PAF and LTC₄. On the other hand, the fMLP-induced PAF synthesis and LTC₄ release were clearly enhanced by preincubation of the cells with IL-3 (500 pM). IL-3 itself did not induce synthesis of PAF or LTC₄. Remarkably, the levels of PAF that we detected decreased in time, whereas the levels of LTC₄ were sustained. The amount of LTC₄ detected was approximately 30-fold higher than that of PAF.

Next, C5a-induced PAF synthesis and LTC₄ release by human basophils in the absence and presence of IL-3 were determined (Fig. 2). In contrast to fMLP, C5a alone was not capable of inducing PAF or LTC₄ synthesis in basophils above our detection level. However, if the basophils were preincubated with IL-3, significant PAF synthesis (Fig. 2a)

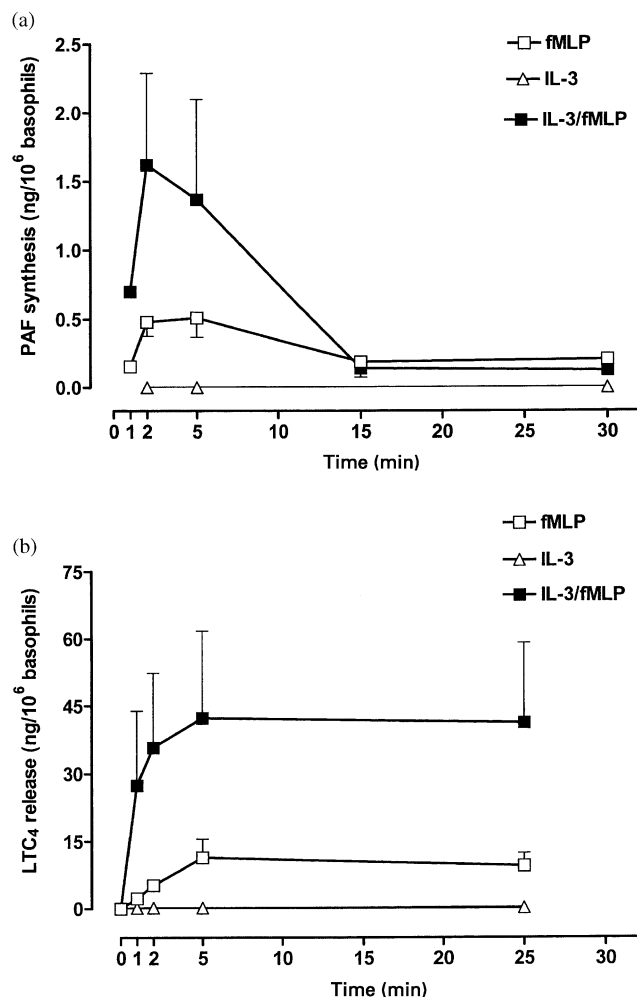


Fig. 1. Time course of PAF synthesis and LTC₄ release by basophils activated with fMLP in the absence or presence of IL-3. After 5 min of preincubation at 37 °C, basophils (1×10^6 /mL) were incubated with solvent or IL-3 (500 pM) for 5 min and were subsequently activated with solvent or fMLP (1 μ M) for the indicated times. PAF synthesis (a) and LTC₄ release (b) were measured as described under Materials and methods. Results are expressed as mean $\pm$ SEM of three independent experiments.

and LTC₄ release (Fig. 2b) were observed after C5a addition. Again, PAF was transiently detected, and the levels of PAF were approximately 30-fold lower than those of LTC₄.

PAF and LTC₄ synthesis by activated human basophils stimulated with PMA or IL-3

Figure 3 shows the PMA-induced synthesis of PAF and release of LTC₄ in the absence and presence of thapsigargin. Stimulation of human basophils with PMA did not result in detectable synthesis of PAF (Fig. 3a) or release of LTC₄ (Fig. 3b). Because PMA does not induce a rise in cytosolic free Ca²⁺ and because activation of cPLA₂ (a key enzyme in the synthesis of PAF and LTC₄ in activated human basophils) requires elevation of cytosolic free Ca²⁺, the effect of thapsigargin on PMA-induced PAF synthesis and LTC₄ release was studied. In the past, we have demonstrated that thapsigargin induces emptying of the Ca²⁺ stores in human

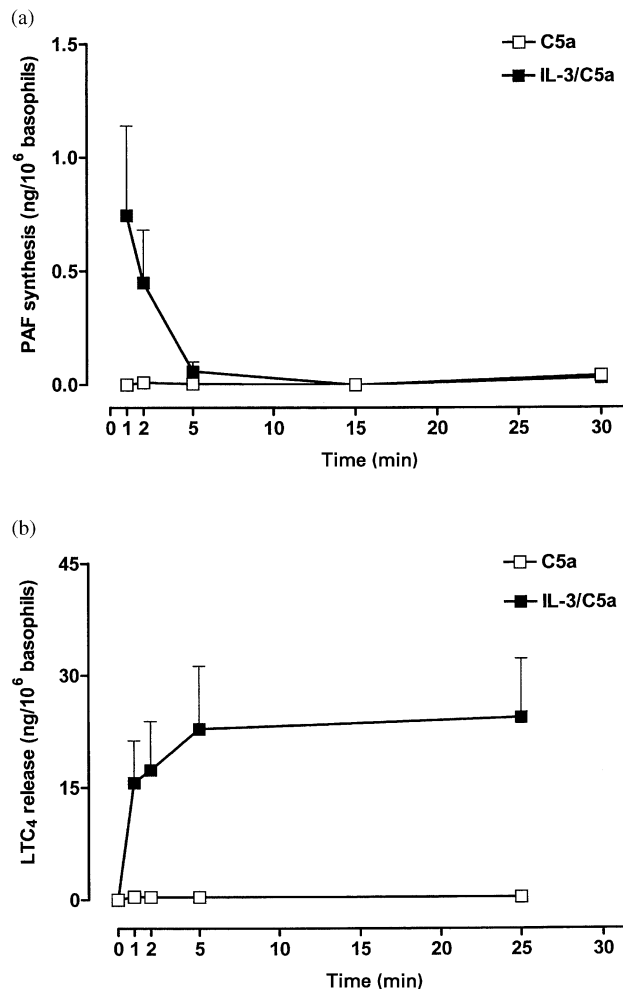


Fig. 2. Time course of PAF synthesis and LTC₄ release by basophils activated with C5a in the absence or presence of IL-3. After 5 min of preincubation at 37 °C, basophils (1×10^6 /mL) were incubated with solvent or IL-3 (500 pM) for 5 min and were subsequently activated with solvent or C5a (10 nM) for the indicated times. PAF synthesis (a) and LTC₄ release (b) were measured as described under Materials and methods. Results are expressed as mean $\pm$ SEM of three independent experiments.

basophils, resulting in a rise in cytosolic free Ca²⁺, partially caused by the influx of Ca²⁺ from the extracellular medium [21]. The addition of thapsigargin alone to human basophils did not result in synthesis of PAF or release of LTC₄ (Fig. 3). However, the combination of PMA with thapsigargin led to a relatively high production of PAF (Fig. 3a) and LTC₄ (Fig. 3b) by human basophils. The difference in the detected amounts of PAF synthesis and LTC₄ release under these conditions was 'limited' to a factor of 5.

As described above, incubation of human basophils with IL-3 does not result in the synthesis of PAF and/or LTC₄ (see Fig. 1). Because IL-3 does not induce elevation of cytosolic free Ca²⁺, human basophils were preincubated with thapsigargin, and subsequently activated with IL-3. Under these conditions, a considerable synthesis of PAF (Fig. 4a) and LTC₄ release (Fig. 4b) was observed. Again, the amount of PAF detected was 5-fold lower than those of LTC₄.

The delayed onset of PAF synthesis and LTC₄ release induced by either PMA or IL-3, compared to the relative

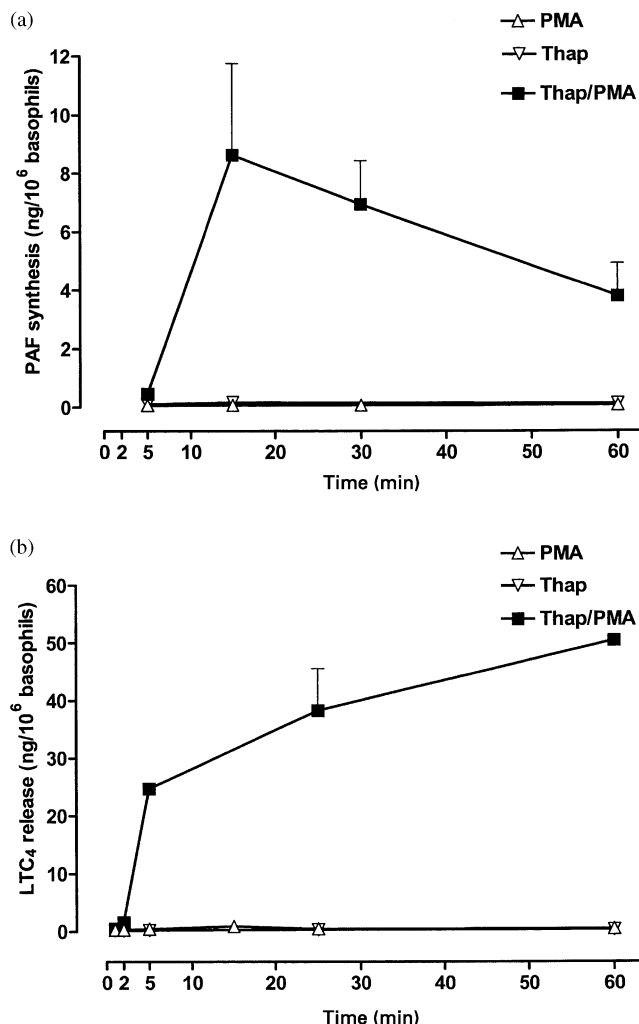


Fig. 3. Time course of PAF synthesis and LTC₄ release by basophils activated with PMA in the absence or presence of thapsigargin. After 5 min of preincubation at 37 °C, basophils (1×10^6 /mL) were incubated with solvent or thapsigargin (Thap, 0.5 μ M) for 2 min and were subsequently activated with solvent or PMA (100 ng/mL) for the indicated times. PAF synthesis (a) and LTC₄ release (b) were measured as described in Materials and methods. Results are expressed as mean $\pm$ SEM of three independent experiments.

rapid onset of fMLP- or C5a-induced PAF synthesis and LTC₄ release, resembles the kinetics of histamine release under these conditions [21, 40].

PAF and LTC₄ synthesis by human basophils after cross-linking of the IgE receptor

A physiologic stimulus of human basophils is activation via the IgE receptor. Figure 5 shows the synthesis of PAF (Fig. 5a) and the release of LTC₄ (Fig. 5b) after stimulation of human basophils by cross-linking of the IgE receptor by anti-IgE or by an appropriate allergen. Stimulation of human basophils with 100 ng/mL of anti-IgE resulted in a low extent of LTC₄ release without significant amounts of PAF synthesized. Anti-IgE-induced PAF synthesis and LTC₄ release were strongly enhanced by preincubation with IL-3 or thapsigargin. The amount of LTC₄ detected under these

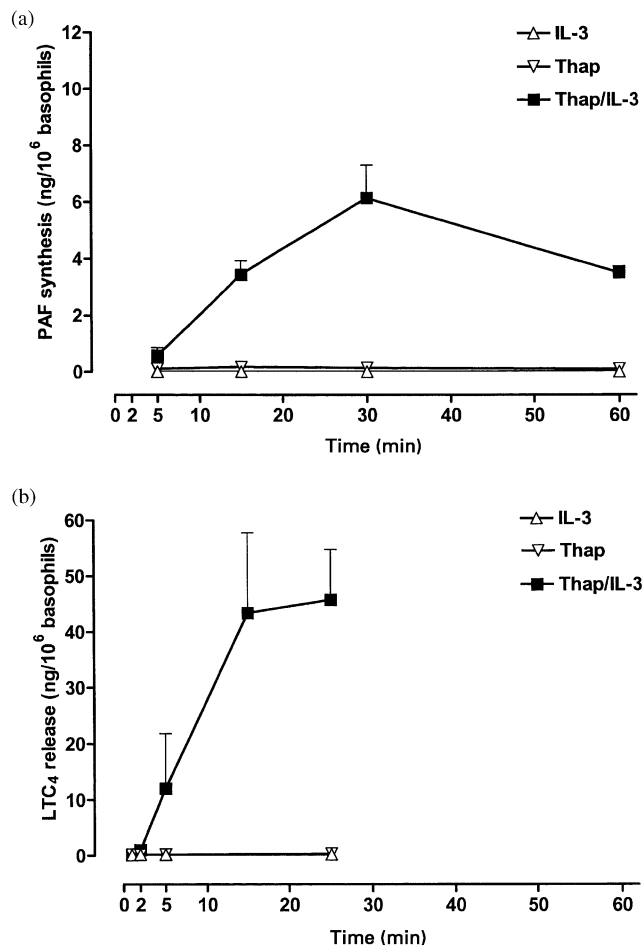


Fig. 4. Time course of PAF synthesis and LTC₄ release by basophils after activation with IL-3 in the absence or presence of thapsigargin. After 5 min of preincubation at 37 °C, basophils (1×10^6 /mL) were incubated with solvent or thapsigargin (Thap, 0.5 μ M) for 2 min and were subsequently activated with solvent or IL-3 (500 pM) for the indicated times. PAF synthesis (a) and LTC₄ release (b) were measured as described under Materials and methods. Results are expressed as mean $\pm$ SEM of three independent experiments.

conditions was approximately 100-fold higher than that of PAF. Again, PAF was transiently detected, whereas sustained levels of LTC₄ were measured. Histamine release under these conditions amounted to $29 \pm 15\%$ (anti-IgE), $72 \pm 16\%$ (IL-3/anti-IgE) and $67 \pm 14\%$ (thapsigargin/anti-IgE, mean $\pm$ SD, $n = 3$).

Activation of human basophils isolated from individuals with a high serum titre of specific IgE to cat dander, stimulated with cat dander allergen that caused histamine release ($52 \pm 2\%$, mean $\pm$ SD, $n = 3$), resulted in strong release of LTC₄, but hardly any PAF synthesis (Fig. 5).

Effect of inhibitors of cPLA₂ on PAF and LTC₄ synthesis by activated human basophils

To confirm the hypothesis that cPLA₂ is the key phospholipase A₂ involved in PAF and LTC₄ synthesis in activated human basophils [14–17], we measured the effect of two well-characterized and commonly used inhibitors of cPLA₂

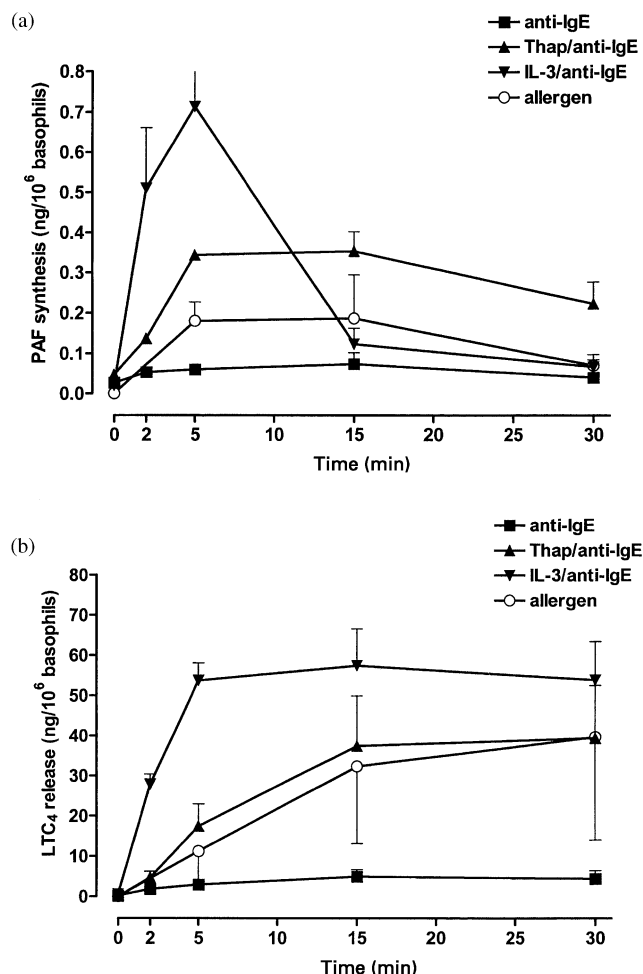


Fig. 5. Time course of PAF synthesis and LTC₄ synthesis by basophils after activation by cross-linking of the IgE receptor. After 5 min of preincubation at 37 °C, purified basophils (1×10^6 /mL) were incubated with solvent, thapsigargin (Thap, 0.5 μ M, 2 min) or IL-3 (500 pM, 5 min) and were subsequently activated with anti-IgE (100 ng/mL) for the indicated times. For allergen stimulation, basophils were isolated from individuals with high serum titres of specific IgE to cat dander and were incubated with cat dander allergen for the indicated times. A dilution of the cat dander extract of 1:60 was used, because at this concentration we observed optimal histamine release (data not shown). PAF synthesis (a) and LTC₄ release (b) were measured as described under Materials and methods. Results are expressed as mean $\pm$ SEM of three independent experiments.

(AACOCF₃ [31] and MAFP [32]) on the synthesis of PAF and LTC₄, induced by the weak stimulus fMLP or by the strong stimulus thapsigargin/IL-3. The specificity of the inhibitors for cPLA₂ was confirmed by measuring the effect of MAFP and AACOCF₃ on sPLA₂ activity. sPLA₂ activity was measured as described in Material and methods. The activity of bee venom sPLA₂ (group III PLA₂, provided with the assay kit as a positive control) and the activity of sPLA₂ in eosinophil lysates (group IIa or group V PLA₂, see [41]) were not inhibited by 100 μ M of AACOCF₃ or by 100 μ M of MAFP (data not shown, $n=2$ performed in duplicate). AACOCF₃ and MAFP showed a dose-dependent inhibition of PAF synthesis induced by fMLP (Fig. 6a) or thapsigargin/IL-3 (Fig. 6b), with AACOCF₃ being more potent than MAFP. At low concentrations of AACOCF₃ and MAFP, there was a tendency of higher concentrations of PAF

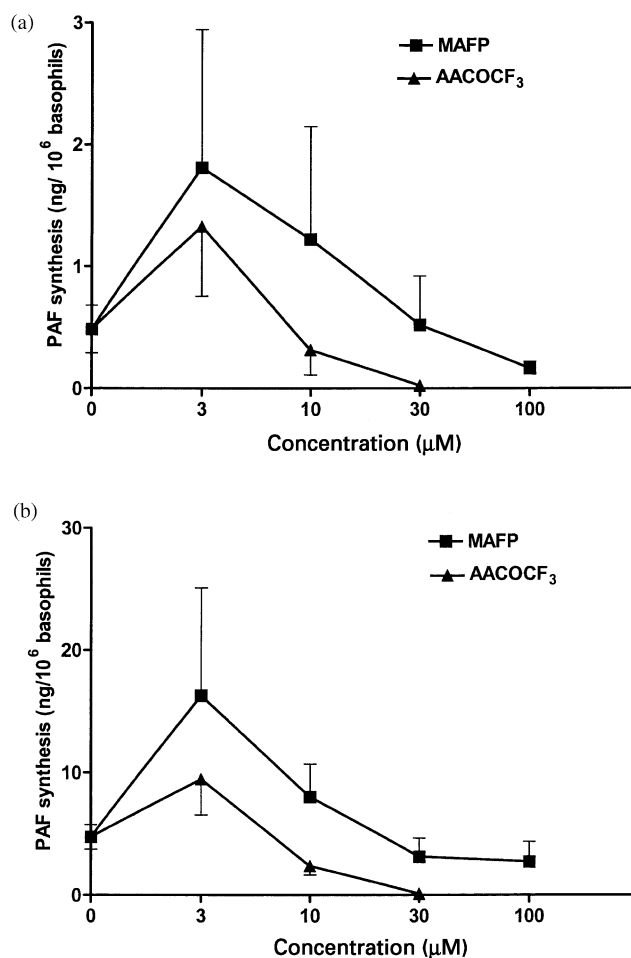


Fig. 6. Effect of the cPLA₂ inhibitors MAFP and AACOCF₃ on PAF synthesis by activated human basophils. After 5 min of preincubation at 37 °C, basophils (1×10^6 /mL) were incubated with solvent or with the indicated concentrations of MAFP or AACOCF₃ for 5 min and were subsequently activated with fMLP (1 μ M) for 5 min (a) or with the combination of thapsigargin (Thap, 0.5 μ M) and IL-3 (500 pM) for 30 min (b). Results are expressed as ng PAF/10⁶ basophils, and as mean $\pm$ SEM of three independent experiments.

measured, although this effect was not significant (see below, and the Discussion section).

Both inhibitors of cPLA₂ were also tested for their effect on LTC₄ release induced by either fMLP (Fig. 7a) or thapsigargin/IL-3 (Fig. 7b). LTC₄ release induced by fMLP or thapsigargin/IL-3 was strongly diminished by both inhibitors.

Effect of MAFP on PAF-AH activity

The tendency of enhanced detection of PAF in the presence of low concentration, especially of MAFP, led us to hypothesize that MAFP inhibits PAF-AH activity. Therefore, PAF was incubated with PAF-AH for 5 min at 37 °C in the absence or presence of 3- μ M MAFP, and thereafter PAF was measured in these samples. Figure 8 indicates that, indeed, MAFP acts as an inhibitor of PAF-AH under these conditions.

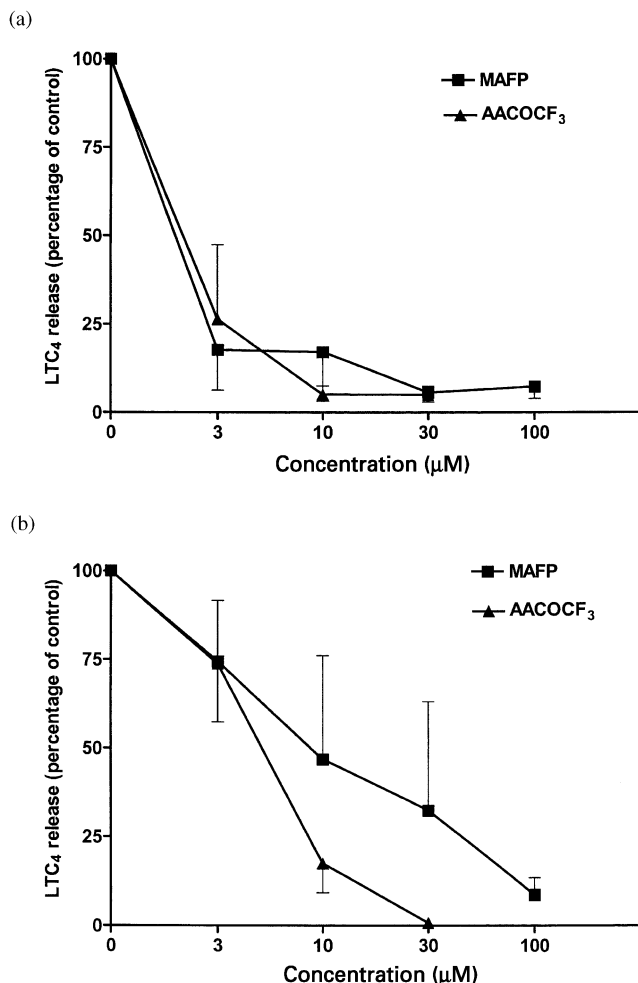


Fig. 7. Effect of the cPLA₂ inhibitors MAFP and AACOCF₃ on LTC₄ release by activated human basophils. After 5 min of preincubation at 37 °C, basophils (1×10^6 /mL) were incubated with solvent or with the indicated concentrations of MAFP or AACOCF₃ for 5 min and were subsequently activated with fMLP (1 μM) for 5 min (a) or with the combination of thapsigargin (Thap, 0.5 μM) and IL-3 (500 pM) for 30 min (b). Results are expressed as a percentage of LTC₄ release in the absence of inhibitors, and as mean $\pm$ SEM of three independent experiments. 100% values equal 11.3 ± 3.4 and 48.6 ± 11.7 (mean $\pm$ SEM, $n = 3$) ng LTC₄/10⁶ basophils for stimulation of human basophils with fMLP (a) and Thap/IL-3 (b), respectively.

Discussion

In this study, we have determined the kinetics of PAF synthesis and LTC₄ release, after activation of highly purified basophils, with a variety of stimuli in the absence or presence of thapsigargin and/or IL-3. Optimal preincubation times of all stimuli were determined for histamine release, being a more or less standard read-out parameter for basophil activation. We cannot exclude that other preincubation times and/or other concentrations of stimuli are optimal for PAF synthesis and LTC₄ release.

The results presented have three important implications. First, it is clear that for all stimuli tested, PAF levels are only transiently detected, presumably due to the breakdown of PAF. The finding that the amount of PAF detected seems to

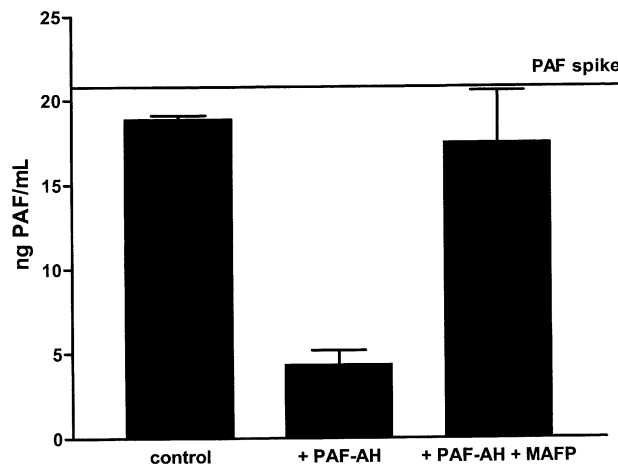


Fig. 8. Effect of MAFP on PAF-AH activity. PAF (13 ng/mL) in 400 μL of incubation medium was incubated with solvent or with PAF-AH (1 μg/mL) with or without preincubation for 5 min with 3 μM of MAFP. After 5 min at 37 °C, the reaction was stopped, and the samples were processed for the PAF measurement as described in Materials and methods. After chloroform extraction, the lipids were resuspended in 250 μL of incubation medium, and therefore the expected final concentration of PAF (provided there is 100% recovery) is 20.8 ng/mL, indicated with the line in the figure. Results are expressed as mean $\pm$ SEM of three different experiments.

be enhanced in the presence of low concentrations of MAFP (see Fig. 6), in combination with the finding that at this concentration MAFP acts as an inhibitor of PAF-AH (see Fig. 8), suggests that the degradation of PAF is caused by PAF-AH, resulting in the formation of lyso-PAF. In contrast, the levels of LTC₄ did not decline in time. Lyso-PAF is not detected in the PAF assay we used, and PAF-AH has been detected in mast cells [42]. Thus, when measuring PAF synthesis in activated basophils, one should always perform kinetic studies, because simultaneous synthesis and degradation of PAF may occur. Additional experiments are required to determine the extent of the observed breakdown of PAF.

Second, the very low production of PAF after IgE receptor activation accompanied by considerable LTC₄ production is remarkable. In the absence of IL-3 and thapsigargin, anti-IgE-induced PAF synthesis was hardly detectable, whereas LTC₄ was clearly detected [4.9 ± 3.0 (mean $\pm$ SD, $n = 3$) ng LTC₄/10⁶ cells]. Both PAF synthesis and LTC₄ release were enhanced by IL-3 and thapsigargin, although the amount of PAF detected was still very low compared to the amount of LTC₄. The difference between the amounts of PAF and LTC₄ released in response to anti-IgE compared with allergen is most likely caused by the fact that allergen leads to a multivalent, and more stronger, cross-linking of the IgE receptor, resulting in higher PAF synthesis (Fig. 5a), higher LTC₄ release (Fig. 5b) and higher histamine release. Alternatively, because the basophils used for the allergen stimulation were isolated from individuals with an allergic history, the higher PAF synthesis and LTC₄ release could be the consequence of *in vivo* priming of the basophils.

It should be noted that for *all* stimuli tested, on a molar base [the molecular weight of PAF (Mw of ~550) and LTC₄ (Mw = 626) are comparable] we observed much more LTC₄ release compared to PAF synthesis. Presumably, PAF and

LTC₄ are not synthesized (solely) from the common precursor 1-alkyl-2-arachidonyl-phosphatidylcholine. AA may also be derived from a phospholipid other than PC, resulting in a lyso-phospholipid that cannot be converted into PAF. Fonteh and Chilton [43] have described the mobilization of several arachidonate pools, with different pools involved in the generation of leukotrienes. Other investigators have shown that after cross-linking of the IgE receptor, mainly the 1-acyl analogue of PAF is synthesized [44]. This component is not detected in our PAF assay. Thus, if most of the AA is derived from 1-acyl-phospholipid, this would explain the relatively low PAF and high LTC₄ synthesis we observed. On the other hand, if the majority of PAF synthesized is quickly degraded, this could explain the difference in the amount of PAF and LTC₄ detected after activation of human basophils with all stimuli tested. In conclusion, it is likely that a combination of the two factors (i.e. fast metabolism of PAF and synthesis of the non-detectable 1-acyl-analogue of PAF) causes the low amounts of PAF detected.

Third, the kinetics of PAF synthesis and LTC₄ release are comparable, and the synthesis of both PAF and LTC₄ is enhanced by IL-3 or thapsigargin. Elevation of cytosolic free Ca²⁺ by thapsigargin (partially caused by the influx of Ca²⁺ from the extracellular medium (see [21]) renders basophils susceptible to LTC₄ and PAF synthesis by stimuli that by themselves do not induce an increase in cytosolic free Ca²⁺, i.e. IL-3 or PMA. In addition, thapsigargin also enhanced the synthesis of PAF and LTC₄ induced by stimuli that by themselves already increase cytosolic free concentrations [e.g. fMLP, C5a (data not shown) and anti-IgE (see Fig. 5)]. The observed enhancement of PAF and LTC₄ synthesis by IL-3 is most probably mediated via activation of MAP kinase, followed by phosphorylation of cPLA₂ [17]. Moreover, it was demonstrated that the combination of elevation of cytosolic free Ca²⁺ concentrations by thapsigargin and activation of the MAP kinase by IL-3 results in a high synthesis of PAF and LTC₄. Because cPLA₂ is activated by submicromolar concentrations of Ca²⁺ and by phosphorylation via mitogen-activated protein (MAP) kinases [14, 18–20], these results support the concept that cPLA₂ is involved in PAF and LTC₄ synthesis in activated human basophils. It is noteworthy that other investigators have shown that thapsigargin alone, in contrast to A23187, does not induce the production of LTC₄ [23]. This is in partial contrast with findings in rat basophilic leukaemia (RBLs) cells, which produce LTC₄ after stimulation with thapsigargin but not after stimulation with DBHQ, another Ca²⁺-ATPase inhibitor [45]. It has been postulated that the inhibiting effect of hydrocortisone on the antigen-induced LTC₄ production in RBL cells is caused by reducing the intracellular Ca²⁺ increase [46].

The fast kinetics of appearance of PAF induced by fMLP or C5a in the presence of IL-3, compared to the relative late onset of PAF synthesis induced by PMA and IL-3 in the presence of thapsigargin, resemble the kinetics of histamine release [21, 40] and of inducing an increase in cytosolic free Ca²⁺, indicative of a role of Ca²⁺ in the synthesis of PAF.

There appear to be at least two distinct pathways for phosphorylation of cPLA₂, one via PKC and another via a PKC-independent route (induced by IL-3) in basophils [14]. On the other hand, other investigators have found a

potentiating effect of PKC inhibitors on anti-IgE-induced LTC₄ production, suggesting a negative regulation of PKC on cPLA₂ activation [47]. Moreover, Brunner et al. [48] have found that preincubation of basophils with PMA results in an inhibition of the LTC₄ production induced by the combination of IL-3 and C5a. Our data show that activation of PKC by PMA in the presence of elevation of Ca²⁺ by thapsigargin results in PAF and LTC₄ synthesis, which resembles the production induced by the combination of IL-3 and thapsigargin. In one preliminary experiment performed in duplicate, we found no inhibitory (or potentiating) effect of PMA on the LTC₄ synthesis induced by the combination of IL-3 and thapsigargin (data not shown).

Our results with the cPLA₂ inhibitors AACOCF₃ and MAFP are in agreement with the concept that cPLA₂ is the key phospholipase involved in PAF and LTC₄ synthesis in activated human basophils. We confirmed that these cPLA₂ inhibitors did not affect sPLA₂ activity (data not shown). On the other hand, it has been described that AACOCF₃ also inhibits 5-lipoxygenase and CoA-independent transacylase [49], essential enzymes in the synthesis of LTC₄ and PAF, respectively, and therefore results obtained with AACOCF₃ should be interpreted with care. In addition, interpretation of the results found with MAFP is hampered by the observation that on average a higher amount of PAF was detected in the presence of low concentrations of the cPLA₂ inhibitor. Although this effect was not significant ($P = 0.40$ for the fMLP-induced PAF synthesis (Fig. 6a) and $P = 0.10$ for the thapsigargin/IL-3-induced PAF synthesis (Fig. 6b), non-parametric Mann–Whitney test), the enhancement was observed in every individual experiment, but the extent of enhancement varied between the different experiments. The increased concentrations of PAF detected in the presence of low concentrations of MAFP are most likely due to an inhibitory effect of MAFP on PAF-AH activity (see Fig. 8). This fits with the observation that only enhanced concentrations of PAF, but not of LTC₄, were detected in the presence of low concentrations of MAFP.

Nevertheless, AACOCF₃ completely inhibited PAF and LTC₄ synthesis in activated human basophils, MAFP completely inhibited LTC₄ synthesis in activated human basophils, and the inhibition by 100 µM of MAFP on PAF synthesis induced by fMLP and thapsigargin/IL-3 was 60 ± 12% and 52 ± 24% (mean ± SEM, $n = 3$), respectively, when compared with results obtained in the absence of MAFP, and 85 ± 5% and 70 ± 25% (mean ± SEM, $n = 3$), respectively, when compared with the amount of PAF detected in the presence of 3-µM MAFP. Based on these results, it is likely that cPLA₂ is the key phospholipase A₂ involved in the synthesis of PAF and LTC₄ in activated human basophils, in agreement with observations from other laboratories [14–17]. In addition, it has been found that mice deficient in cPLA₂ showed a marked decrease in the production of eicosanoids and PAF (in peritoneal macrophages [50]). The anaphylactic responses of these animals were significantly reduced, as was their bronchial reactivity to methacholine [50]. However, other studies have demonstrated a role for sPLA₂ in PAF synthesis, both in human basophils [51] and in mast cells [52]. Nevertheless, in accordance with the complete inhibition of PAF and LTC₄ synthesis in activated human basophils by inhibitors of cPLA₂, findings in

our laboratory have demonstrated only very low (if any) amounts of sPLA₂ in human basophilic granulocytes [41].

A careful comparison of the results depicted in Figs 6 and 7 demonstrates that the cPLA₂ inhibitors AACOCF₃ and MAFP are more potent inhibitors of LTC₄ release than of PAF synthesis. When they inhibit a common step (i.e. cPLA₂) in the synthesis of both lipid mediators this is not expected, provided that cPLA₂ is, to the same extent, the rate-limiting step in PAF and LTC₄ synthesis. On the other hand, because both inhibitors are competitive inhibitors of cPLA₂, a different potencies of inhibition may be expected with different substrates of cPLA₂. Thus, different potency of inhibition could be explained if PAF and LTC₄ are not (exclusively) derived from the same precursor (i.e. 1-alkyl-2-AA-phosphatidylcholine).

In conclusion, we have examined the kinetics of PAF synthesis and LTC₄ release in highly purified basophil suspensions, activated with either fMLP, C5a, PMA or via cross-linking of the IgE receptor, in the absence and presence of IL-3 or thapsigargin. In the presence of either IL-3 or thapsigargin, enhanced PAF synthesis and LTC₄ release was observed with all stimuli tested. On a molar basis, much more LTC₄ release, compared to PAF synthesis, were observed for all stimuli. The detection of PAF was transient for all stimulation conditions tested. Finally, cPLA₂ is the predominant phospholipase involved in the synthesis of leukotrienes and PAF in human basophils, as was demonstrated by the strong inhibition of PAF and LTC₄ synthesis by well-characterized and commonly used inhibitors of cPLA₂.

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