Influence of arachidonic acid on indices of phospholipase A₂ activity in the human neutrophil

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The present studies were conducted to understand better the regulation of phospholipase A₂ (PLA₂)-dependent mobilization of lipid mediators by arachidonic acid (C_{20:4}). After stimulation of human neutrophils, g.l.c./m.s. analysis of non-esterified fatty acids indicated that the quantity of C_{20:4} increased as a function of time after stimulation, from undetectable quantities to > 800 pmol/10⁷ cells. In contrast with C_{20:4}, the quantities of other free fatty acids such as oleic and linoleic were high in resting cells and did not change after stimulation. Some 15 % of the C_{20:4} released from cellular lipids remained cell-associated. To examine the effect of C_{20:4} on its own release, neutrophils were exposed to [³H]₈C_{20:4}, to differentiate it by g.l.c./m.s. from naturally occurring C_{20:4}. In A23187-stimulated neutrophils, low concentrations (5–10 μM) of [³H]₈C_{20:4} added just before A23187 increased the quantity of C_{20:4} produced by the cell, whereas higher concentrations (30–50 μM) decreased the quantity of C_{20:4} released from phospholipids. As other measures of PLA₂ activity, the effects of C_{20:4} on production of platelet-activating factor (PAF) and leukotriene B₄ (LTB₄) were assessed. C_{20:4} treatment just before stimulation of neutrophils blocked PAF

and LTB₄ production in a concentration-dependent manner (IC₅₀ 10–20 μM). The effect of C_{20:4} was not blocked by the cyclo-oxygenase inhibitor naproxene (10 μM), nor could it be mimicked by 1 μM LTB₄, 5-hydroxyeicosa-6,8,11,14-tetraenoic acid (5HETE), 5-hydroperoxyeicosa-6,8,11,14-tetraenoic acid (5HPETE) or 15-hydroxyeicosa-5,8,11,13-tetraenoic acid (15HETE). The 5-lipoxygenase (5LO) inhibitor zileuton induced a concentration-dependent decrease in PAF, with a maximal effect of a 50 % decrease at 10–50 μM. The decrease in PAF by the 5LO inhibitor could not be circumvented by addition of 1 μM 5HETE, 5HPETE and LTB₄, and may be attributed to the capacity of zileuton to increase the quantity of C_{20:4} in A23187-treated neutrophils. The inhibitory effect of C_{20:4} (20–40 μM) on PAF production could be antagonized by the protein kinase C inhibitor staurosporine (30 nM), but not by inhibitors of protein kinase A, tyrosine kinase or calmodulin kinase II. Taken together, these data demonstrate that C_{20:4} is selectively released from membrane phospholipids of A23187-stimulated neutrophils, and this C_{20:4} may play an important role in regulating the mobilization of C_{20:4} by altering PLA₂ activity.

INTRODUCTION

In inflammatory cells, including the human neutrophil, arachidonic acid (C_{20:4}) is a pivotal molecule involved in the production of lipid mediators. It serves as a substrate for a class of lipid mediators known as eicosanoids (members of this class include prostaglandins, leukotrienes, lipoxins and hydroxy-eicosatetraenoic acids (HETEs) [1,2]), and also plays a critical role in platelet-activating factor (PAF) production. For example, C_{20:4} is present in the major precursor molecule for PAF, 1-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (GPC) [3–5]. Moreover, if membrane phospholipids of inflammatory cells are depleted of C_{20:4}, there is a large decrease in the capacity of that cell to produce PAF [6,7]. Conversely, increasing the size of C_{20:4} pools in inflammatory cells enhances PAF production [8].

Recent reports have suggested an additional role for C_{20:4} as a direct modulator of cell functions, mediating phagocytosis in monocytes [9], adhesion of macrophages [10], altering Ca²⁺ influx in GH3 cells [11], modulating chloride channels [12] and interacting with GTP-binding protein in human neutrophils [13,14]. These reports suggest that C_{20:4} itself can have important effects on inflammatory cell activity. These observations are

supported by findings that C_{20:4} markedly enhances the activity of specific protein kinase C isoenzymes [15,16], suggesting a second-messenger role for C_{20:4}.

In view of these studies showing effects of C_{20:4} on a variety of enzyme systems, the present study was initiated to explore the effect of C_{20:4} itself on lipid-mediator production. We have examined whether there is selective release of C_{20:4} by stimulated human neutrophils, detailed the capacity of C_{20:4} to regulate the release of endogenous C_{20:4} and the production of PAF and leukotriene B₄ (LTB₄), and explored the mechanism of C_{20:4}-induced regulation of phospholipase A₂ (PLA₂) activity.

EXPERIMENTAL

Materials

[³H]Acetic acid (sodium salt; 50–100 Ci/mmol) and [³H]acetyl-CoA (1–5 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Histopaque-1077 and common laboratory chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Silica-gel G plates were from Analtech (Newark, DE, U.S.A.). Essentially fatty-acid-free BSA was obtained from Calbiochem (San Diego, CA, U.S.A.). Silica-gel

Abbreviations used: C_{20:4}, arachidonic acid (eicosa-5,8,11,14-tetraenoic acid); C_{20:2}, eicosa-11,14-dienoic acid; C_{18:1}, oleic acid (octadec-9-enoic acid); C_{18:2}, linoleic acid (octadec-9,12-dienoic acid); 1-alkyl, 1-O-alkyl; 1-alkenyl, 1-O-alk-1'-enyl; GPC, *sn*-glycero-3-phosphocholine; 5HETE, 5(S)-hydroxyeicosa-6,8,11,14-tetraenoic acid; 15HETE, 15(S)-hydroxyeicosa-5,8,11,13-tetraenoic acid; 5HPETE, 5(S)-hydroperoxyeicosa-6,8,11,14-tetraenoic acid; 5LO, 5-lipoxygenase; LTB₄, leukotriene B₄; PAF, platelet-activating factor (1-alkyl-2-acetyl-GPC); lyso-PAF, 1-alkyl-2-lyso-GPC; PLA₂, phospholipase A₂.

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columns were from Baker (Phillipsburg, NJ, U.S.A.). [$^2\text{H}_8$] $\text{C}_{20:4}$ was from Cambridge Isotope Laboratories (Woburn, MA, U.S.A.). All other fatty acids were from Nu-Chek Prep (Elysian, MN, U.S.A.). H-7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride], A-3 [*N*-(2-aminoethyl)-5-chloronaphthalene-1-sulphonamide hydrochloride], erbstatin, lavendustin, [$^2\text{H}_4$]LTB $_4$, HETEs and hydroperoxyeicosatetraenoic acids (HPETEs) were from Biomol (Plymouth Meeting, PA, U.S.A.). Staurosporine was from Kamiya Biomedical Co. (Thousand Oaks, CA, U.S.A.). KT5720 and KN62 were from Calbiochem. LTB $_4$, zileuton {Abbott A-64077; *N*-hydroxy-*N*-(1-benzo[*b*]thien-2-ylethyl)urea} and Ly223982 ((*E*)-5-(3-carboxybenzoyl)-2-[[6-(4-methoxyphenyl)-5-hexenyl]oxy]benzene-propanoic acid) were synthesized by the Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals.

Preparation of human neutrophils

Neutrophils were prepared from heparinized venous blood collected from healthy donors and isolated by the procedure of Boyum [17], by using the Histopaque-1077 technique as previously described [18]. The final leucocyte preparation was suspended in PBS composed of 137 mM NaCl, 8.8 mM Na $_2$ HPO $_4$, 1.5 mM KH $_2$ PO $_4$, 2.7 mM KCl (Dulbecco's; Gibco Laboratories, Long Island, NY, U.S.A.), and was of greater than 95% viability and purity, as determined by Trypan Blue exclusion and histological examination.

Assay for PAF production

The incorporation of [^3H]acetate was used to quantify PAF biosynthesis [19]. Neutrophils produce acetylated derivatives of phosphatidylcholine that are > 85% 1-alkyl-2-acetyl-GPC, with the balance being 1-acyl-2-acetyl-GPC [20]. Neutrophil suspensions [(10–20) $\times 10^6$ cells/ml] in PBS containing 1 mM Ca $^{2+}$ and 1.1 mM Mg $^{2+}$ were incubated with compounds or vehicles at 37 °C in a volume of 950 μl . All compounds were added in a volume of 1–5 μl at the times indicated in the Figure legends. The vehicles were dimethyl sulphoxide for staurosporine, A-3, H-7, KN62, KT5720, lavendustin, erbstatin and zileuton, and ethanol for the fatty acids and eicosanoids. Then 50 μl of a solution containing [^3H]acetic acid (20–30 μCi) with A23187 (2 μM final concn.) in PBS with Ca $^{2+}$, Mg $^{2+}$ and 1 mg/ml BSA was added to the cell suspensions. After 10 min at 37 °C, the reactions were terminated by addition of 1 vol. of chloroform/methanol (1:2, v/v) and the lipids were extracted [21]. After extraction of total lipids, individual phospholipids were separated by t.l.c. on silica gel G plates developed in chloroform/methanol/acetic acid/water (50:25:8:4, by vol.), localized by radioscanning (Bioscan, Washington DC, U.S.A.), and the area corresponding to PAF was scraped and quantified by liquid-scintillation counting.

Assay for non-esterified fatty acids

Neutrophil suspensions [(5–10) $\times 10^6$ cells/ml] in PBS containing 1 mM Ca $^{2+}$ and 1.1 mM Mg $^{2+}$ were treated exactly as in the assay for PAF, except that [^3H]acetic acid was omitted. Before extraction of the lipids, C $_{20:2}$ or [$^2\text{H}_8$] $\text{C}_{20:4}$ (50–250 ng) was added as an internal standard, and the lipids were extracted by the method of Bligh and Dyer [21]. Solutions were removed under a stream of argon, and lipids were resuspended in hexane. Each sample in hexane was passed through a 500 mg silica solid-phase column, washed twice with hexane, and a fatty acid-enriched fraction was eluted with hexane/diethyl ether (1:1, v/v). Solvents were removed from the samples under a stream of nitrogen, and

samples were converted into pentafluorobenzyl esters as previously described [22,23], by using pentafluorobenzyl bromide and di-isopropylethylamine in acetonitrile. Solvents were removed and samples were suspended in hexane. Negative-ion chemical-ionization g.l.c./m.s. analysis was performed as previously described [22,23]. Selected ions were monitored for the carboxylate anions of oleic acid (C $_{18:1}$) (m/z = 281), linoleic acid (C $_{18:2}$) (m/z = 279), C $_{20:4}$ (m/z = 303), [$^2\text{H}_8$] $\text{C}_{20:4}$ (m/z = 311) and eicosa-11,14-dienoic acid (C $_{20:2}$) (m/z = 307). Mole quantities of each fatty acid were calculated from the signal intensity for each fatty acid by comparison with that of the internal standard (either [$^2\text{H}_8$] $\text{C}_{20:4}$ or C $_{20:2}$). In experiments measuring the influence of exogenously added C $_{20:4}$ on endogenous C $_{20:4}$ production, [$^2\text{H}_8$] $\text{C}_{20:4}$ was added as exogenous C $_{20:4}$ and C $_{20:2}$ was utilized as an internal standard.

Assay for acetyltransferase activity

Acetyltransferase activity was measured in broken neutrophils as described by Wykle et al. [24]. Human neutrophils [(10–20) $\times 10^6$ cells/ml] in PBS were treated with C $_{20:4}$ for 1 min, then stimulated for 10 min in the presence of 2 μM A23187. The neutrophils were broken by N $_2$ cavitation [5.2 MPa (750 lb/in 2)], centrifuged for 10 min at 500 *g*, and the resulting homogenates were assayed for acetyltransferase activity. Briefly, homogenate protein was incubated with 200 μM acetyl-CoA, 0.5 μCi of [^3H]acetyl-CoA and 10 μM lyso-PAF. After a 10 min incubation at 37 °C, the products were separated by t.l.c. and the amount of labelled PAF produced was determined as described above.

Assay for LTB $_4$

Human neutrophil suspensions [(5–10) $\times 10^6$ cells/ml] in PBS were exposed to increasing concentrations of [$^2\text{H}_8$] $\text{C}_{20:4}$ and A23187 for 10 min at 37 °C. The release of LTB $_4$ was halted by addition of 50 μl of 9% formic acid, the cells were removed by centrifugation, and the resulting supernatant was extracted with an equal volume of ethyl acetate. The ethyl acetate fractions were dried, and derivatives prepared as previously described [23] and assayed by g.l.c./m.s. for the derivatives of LTB $_4$ (m/z = 479), [$^2\text{H}_4$]LTB $_4$ (m/z = 483) and [$^2\text{H}_8$]LTB $_4$ (m/z = 487). The quantities of LTB $_4$ and [$^2\text{H}_8$]LTB $_4$ were calculated by using [$^2\text{H}_4$]LTB $_4$ (50 ng) as the internal standard.

RESULTS

Fatty acid release from stimulated human neutrophils

When human neutrophils are stimulated, either through activation of cell-surface receptors or by a Ca $^{2+}$ ionophore in the presence of extracellular Ca $^{2+}$, they respond with the release of C $_{20:4}$, which is presumed to occur by a PLA $_2$ -mediated activity [25]. To explore the extent and specificity of this PLA $_2$ activity, human neutrophils were isolated, washed and then stimulated with the Ca $^{2+}$ ionophore A23187, and the mole quantities of the non-esterified fatty acids C $_{18:1}$, C $_{18:2}$ and C $_{20:4}$ in the cell suspensions were determined (Figure 1). Before addition of A23187, the mole quantity of C $_{20:4}$ was very low compared with those of C $_{18:1}$ and C $_{18:2}$. The mole quantity of C $_{20:4}$ increased rapidly after A23187 addition, with half-maximal levels reached within 3 min, and reaching a plateau at approx. 825 pmol/10 7 neutrophils. In contrast with the results with C $_{20:4}$, the fatty acids C $_{18:1}$ and C $_{18:2}$ were present at high levels in unstimulated neutrophils and did not change dramatically as a function of time after stimulation (Figure 1).

To address how much of the C $_{20:4}$ produced by the stimulated neutrophil remained associated with the cell, suspensions of

Figure 1 Fatty acid release from human neutrophils after stimulation

Human neutrophils were prepared as described in the Experimental section and stimulated with 2 μ M A23187 followed by incubation at 37 $^{\circ}$ C. At the indicated times after stimulation, the total contents of each tube were extracted into chloroform and the mole quantities of the indicated fatty acids were determined as described in the Experimental section. These data are means $\pm$ S.E.M. of three experimental determinations each performed in duplicate.

Figure 2 Effect of [²H₈]C_{20:4} on the release of C_{20:4} from human neutrophils

Human neutrophils were washed, suspended in PBS and the indicated concentrations of [²H₈]C_{20:4} were added, followed immediately by stimulation with 2 μ M A23187. After 10 min at 37 $^{\circ}$ C, the samples were extracted and the mole quantity of C_{20:4} was determined. These data are means $\pm$ S.E.M. of 4 experiments.

neutrophils (10^7) were stimulated with 2 μ M A23187 for 10 min and then removed from the buffer by centrifugation. The mole quantities of C_{20:4} located in the supernatant and in the total suspension (cells + buffer) were determined. Of the total C_{20:4} produced by the neutrophil (627 ± 4 pmol, $n = 3$ determinations in one experiment, representative of two experiments), most (518 ± 7 pmol; 83%) was found in the supernatant buffer. This resulted in an approximate concentration of 0.5 μ M of C_{20:4} in the total cell suspension. The balance of the C_{20:4} produced by the neutrophils (109 pmol) remained cell-associated. It is difficult to determine an exact concentration for this cell-associated C_{20:4}, but an estimate based on 10^7 cells with a radius of 4×10^{-6} m would result in an approximate concentration of 25 μ M. Addition of 1 mg/ml BSA to A23187-stimulated neutrophil suspensions resulted in an increased mass of total free C_{20:4} (1054 ± 12 pmol, $n = 3$) as well as increased free C_{20:4} that remained cell-associated (281 pmol/ 10^7 cells), suggesting that there is a considerable mass of C_{20:4} that remains cell-associated both with and without added BSA.

Influence of exogenous C_{20:4} on endogenous C_{20:4} release

The selectivity of C_{20:4} release and the relatively high concentration of C_{20:4} to which neutrophils were exposed during cell

activation raised the question as to the role of this C_{20:4} within the neutrophil. In particular, experiments were directed at understanding whether C_{20:4} itself could affect the enzyme which released it, PLA₂. [²H₈]C_{20:4} was used in these experiments, as an analogue of C_{20:4} that does not occur in nature and can be separated from naturally occurring C_{20:4} by g.l.c./m.s. analysis. Neutrophils exposed to concentrations of [²H₈]C_{20:4} up to 40 μ M in the absence of any other stimulation were not stimulated to release large amounts of endogenous C_{20:4} (26 and 63 pmol of C_{20:4} after 10 min of 0 and 40 μ M [²H₈]C_{20:4} respectively, $n = 2$). In contrast, when neutrophils were exposed to increasing concentrations of [²H₈]C_{20:4} just before stimulation with A23187 (Figure 2), two effects were observed. At low concentrations (below 20 μ M) there was an increase in the quantity of free C_{20:4} mobilized by the cell. At concentrations equal to or greater than 20 μ M there was a decrease in the quantity of free C_{20:4} produced by the cell.

Influence of exogenous C_{20:4} on PAF biosynthesis

The release of C_{20:4} from cellular phospholipids is taken to be a reflection of PLA₂ activity, as this enzyme produces a non-esterified fatty acid from the *sn*-2 acyl chain of phospholipids [25]. Another indicator of PLA₂ activity that can be employed is the production of PAF. This bioactive phospholipid is derived from a lysophospholipid intermediate 1-alkyl-2-lyso-GPC (lyso-PAF), which can be produced either by direct action of a PLA₂ [3] or indirectly by PLA₂ production of a lysophospholipid that then drives a CoA-independent transacylase to produce 1-alkyl-2-lyso-GPC [18,26,27]. Figure 3 illustrates that C_{20:4} could completely block the production of PAF by A23187-stimulated neutrophils, with an IC₅₀ of approx. 15 μ M. Other fatty acids, such as C_{18:1} and C_{18:2}, inhibited PAF production by A23187-stimulated neutrophils with similar potency (Figure 3).

To determine if treatment with C_{20:4} altered the conversion of lyso-PAF into PAF, neutrophils were treated as described in Figure 3, then broken, and acetyltransferase activity was assessed as described in the Experimental section. Exposure of neutrophils to C_{20:4} or the 5-lipoxygenase (5LO) inhibitor zileuton [28] had no effect on broken-cell acetyltransferase activity [8003 ± 58 , 8046 ± 182 and 9985 ± 510 d.p.m. (means $\pm$ S.E.M., $n = 3$) after treatment with vehicle, 30 μ M C_{20:4} or 10 μ M zileuton respectively].

Influence of exogenous C_{20:4} on LTB₄ biosynthesis

Because lower concentrations of exogenous C_{20:4} increased the content of endogenous C_{20:4} (Figure 2) and decreased the synthesis of PAF (Figure 3), a third product from the PLA₂ reaction, LTB₄, was measured. In this experiment, [²H₈]C_{20:4} was provided as a source of exogenous C_{20:4} in A23187-stimulated neutrophils, allowing us to differentiate products from exogenous ([²H₈]LTB₄) and endogenous (LTB₄) sources of C_{20:4}.

Increasing the concentration of [²H₈]C_{20:4} caused a concentration-dependent decrease in LTB₄ synthesized from endogenous C_{20:4} (Figure 4). As might be expected, some of the [²H₈]C_{20:4} provided to the neutrophils was converted into [²H₈]LTB₄ (results not shown). Therefore the decrease in LTB₄ production in this assay may be due to [²H₈]C_{20:4}-induced inhibition of PLA₂ activity or to competition of [²H₈]C_{20:4} with endogenous C_{20:4} for 5LO, or both. However, there was significant inhibition of LTB₄ production at low concentrations of [²H₈]C_{20:4}, where little [²H₈]C_{20:4} was converted into [²H₈]LTB₄ and the total amount of 5LO products produced was well below the maximal capacity of the cells. This suggests that, at these low

Figure 3 Effect of $C_{20:4}$ of PAF production in human neutrophils

Human neutrophils were washed, resuspended in PBS and the indicated concentrations of $C_{18:1}$, $C_{18:2}$, $C_{20:4}$ or vehicle were added, followed immediately by $2 \mu\text{M}$ A23187 and 20–30 μCi of [^3H]acetic acid. After a 10 min incubation at 37°C , the lipids were extracted into chloroform and [^3H]PAF was measured as described in the Experimental section. These data are means $\pm$ S.E.M. of 4–5 experiments.

Figure 4 Effect of $C_{20:4}$ on LTB_4 production in human neutrophils

Human neutrophils were washed, resuspended in PBS and the indicated concentrations of [$^2\text{H}_8$] $C_{20:4}$ were added, followed immediately by stimulation with $2 \mu\text{M}$ A23187. After 10 min at 37°C , the cells were removed, the supernatants were extracted and the quantity of LTB_4 was determined as described in the Experimental section. These data are means $\pm$ S.E.M. of 3 determinations from one experiment and are representative of two experiments.

concentrations, the major influence of [$^2\text{H}_8$] $C_{20:4}$ is probably at the PLA_2 step.

Mechanism of $C_{20:4}$ -induced inhibition of PLA_2 activity

To explore further the mechanism by which $C_{20:4}$ inhibited PLA_2 activity, a number of experiments were performed to determine whether inhibition was due to $C_{20:4}$ itself or to a metabolite of $C_{20:4}$. Human neutrophils contain essentially no cyclo-oxygenase activity and, as might be predicted, the cyclo-oxygenase inhibitor naproxene had no effect on PAF production (8884 and 8442 d.p.m. in control and $100 \mu\text{M}$ naproxene-treated cells respectively). Neutrophils do possess 5LO activity, and therefore the effects of inhibiting 5LO activity were examined, by using the 5LO inhibitor zileuton. This 5LO inhibitor decreased PAF production in a concentration-dependent manner and had a maximal effect of blocking 50% of PAF production (Figure 5). The initial conclusion from this study was that some 5LO product was required for complete PAF production and that treatment with the 5LO inhibitor decreased the availability of that product. However, attempts to add back 5LO products had no restorative effect on PAF production. Specifically, addition of $1 \mu\text{M}$ 5HETE, 5HPETE, 15HETE or LTB_4 had no effect on basal PAF levels, A23187-stimulated PAF production nor on the

Figure 5 Effect of zileuton on PAF production in human neutrophils

Human neutrophils were washed, resuspended in PBS, and the indicated concentrations of zileuton or vehicle were added, followed 5 min later by $2 \mu\text{M}$ A23187 (or vehicle) and 20–30 μCi of [^3H]acetic acid. After a 10 min incubation at 37°C , the lipids were extracted and [^3H]PAF was measured as described in the Experimental section. The A23187 control response is indicated, with the S.E.M. of the response indicated by the dashed lines. The response in the absence of A23187 is indicated by a dashed line at the bottom of the Figure. These data are means $\pm$ S.E.M. of triplicate determinations and are representative of three experiments.

Table 1 Effect of zileuton on $C_{20:4}$ release from human neutrophils

Human neutrophils were washed, resuspended in PBS, and the indicated concentrations of zileuton or vehicle were added, followed 5 min later by $2 \mu\text{M}$ A23187 or vehicle. After 10 min incubation at 37°C , the lipids were extracted and $C_{20:4}$ was measured as described in the Experimental section. The data are means $\pm$ S.E.M. of triplicate determinations and are representative of three experiments: *significantly different from no A23187; †significantly different from A23187 with zero zileuton; by ANOVA with Scheffe analysis.

Treatment		Result
Zileuton (μM)	A23187 ($2 \mu\text{M}$)	$C_{20:4}$ (pmol/ 10^7 neutrophils)
0	—	13 ± 10
50	—	3 ± 2
0	+	$690 \pm 16^*$
2	+	$1150 \pm 20^{*\dagger}$
10	+	$1120 \pm 30^{*\dagger}$
50	+	$910 \pm 100^{*\dagger}$

ability of the 5LO inhibitor to decrease PAF production (results not shown). Additional evidence that LTB_4 is not required for PAF production was obtained from data showing that PAF production was not altered in the presence of a LTB_4 -receptor antagonist, Ly 223982 [29] (35929 and 34660 d.p.m. in control and $0.1 \mu\text{M}$ Ly 223982-treated cells respectively). Taken together, these data suggest that lipoxygenase products are not required for PAF production and that another mechanism exists for 5LO-induced inhibition of PAF production.

Another possible explanation for the ability of zileuton to inhibit PAF production would be that this compound affects levels of $C_{20:4}$ by inhibiting its metabolism to 5LO products. To test this notion, neutrophils were treated with increasing concentrations of zileuton, and the effect of that treatment on basal and A23187-stimulated release of $C_{20:4}$ was assessed. The 5LO inhibitor did not stimulate release of $C_{20:4}$ by itself (Table 1). However, the quantity of free $C_{20:4}$ in neutrophils stimulated with A23187 was dramatically enhanced in the presence of the 5LO inhibitor (Table 1). These data demonstrated that inhibition of 5LO can result in increased quantities of free $C_{20:4}$, and

Figure 6 Effect of staurosporine on PAF production in human neutrophils

Human neutrophils were washed, and resuspended in PBS. For non-esterified fatty acids, the pretreatment scheme was staurosporine (300 nM), incubate 1 min, add 30 μ M of non-esterified fatty acid, incubate 1 min and then stimulate with 2 μ M A23187. For the 5LO inhibitor, the pretreatment scheme was 10 μ M zileuton, incubate 3 min, add staurosporine (stauro: 300 nM), incubate 2 min and then stimulate with 2 μ M A23187. PAF production proceeded in the presence of 20–30 μ Ci of [³H]acetic acid during a 10 min incubation at 37 °C, then the lipids were extracted and [³H]PAF was measured as described in the Experimental section. These data are means $\pm$ S.E.M. of triplicate determinations from one experiment and are representative of two or three experiments.

therefore the effects of 5LO inhibition on PAF production could be a result of enhanced levels of C_{20:4}.

From the aforementioned studies, the mechanism by which increasing levels of C_{20:4} could influence PLA₂ activity was unclear. One possible mechanism is stimulation of protein kinase activity by C_{20:4}. There is evidence for activation of protein kinase C by C_{20:4} [16], and this mechanism does not require metabolism of C_{20:4} to lipoxygenase products. The protein kinase inhibitor staurosporine [30] was employed to probe the involvement of kinase activity in this process. The effect of staurosporine on PAF production is illustrated in Figure 6. Staurosporine, in the absence of other treatments, enhanced PAF production induced by A23187. A23187-induced PAF production that was inhibited by C_{20:4} could be restored with staurosporine (Figure 6). In addition, staurosporine was able to antagonize zileuton-induced inhibition of PAF production (Figure 6). In contrast, staurosporine failed to antagonize C_{18:1}- or C_{18:2}-induced inhibition of PAF production (Figure 6). In addition, staurosporine (300 nM) restored endogenous C_{20:4} release that had been inhibited by 30 μ M [³H]₈C_{20:4} (270 and 710 pmol/10⁷ cells in the absence and presence of staurosporine, respectively).

To characterize further the involvement of protein kinases in regulation of PLA₂ activity, a series of kinase inhibitors with

various profiles was examined. The effects observed with staurosporine were not mimicked by protein kinase A inhibitors KT5720 (1 μ M) [31] and A-3 (10 μ M) [32], tyrosine kinase inhibitors lavendustin (1 μ M) and erbstatin (25 μ M) [33] and calmodulin kinase II inhibitor KN62 (10 μ M) [34] (results not shown). Caution should be used in interpretation of these negative findings. However, these compounds were used at concentrations reported to have kinase-inhibitory activities in similar cell systems [35–37]. H-7 gave results similar to those of staurosporine, in that this compound at 300 μ M increased A23187-stimulated PAF production and partially restored PAF production that had been decreased by C_{20:4}.

DISCUSSION

Inflammatory cells produce large amounts of free C_{20:4} and its metabolites when stimulated. The effects of C_{20:4} are often assumed to be mediated via its bioactive metabolites, and there are reports that 5LO products can modulate PAF production in inflammatory cells [38–40]. However, recent reports indicate that C_{20:4} itself can play a direct role in the function of inflammatory cells [9,11]. In these experiments, we have examined stimulated human neutrophils and characterized the release of C_{20:4} and the potential role of that C_{20:4} in regulating PLA₂ activity.

The release of $C_{20:4}$ was examined in stimulated human neutrophils by g.l.c./m.s. and compared with the release of other fatty acids. These data indicate that levels of $C_{20:4}$ are selectively maintained low in resting cells and are selectively increased upon stimulation. The maintenance of low levels of $C_{20:4}$ in resting cells is probably accomplished through the activities of CoA-dependent and CoA-independent acyltransferase activities, which incorporate $C_{20:4}$ into phospholipids and remodel $C_{20:4}$ within specific phospholipid pools [41–44]. The CoA-independent transferase activity is very selective for $C_{20:4}$ [45–47], and there may also be $C_{20:4}$ -selective CoA synthetase activities [48,49]. The release of $C_{20:4}$ is presumably mediated by one or more PLA_2 activities. The present study suggests that the PLA_2 enzyme(s) which participates in this process is likely to be $C_{20:4}$ -selective. A $C_{20:4}$ -selective PLA_2 activity has been demonstrated in neutrophils [50]. However, it is not clear at present if the PLA_2 activity in neutrophils is due to an enzyme similar to that isolated and cloned from U937 cells [51,52].

When PLA_2 activity has been examined in broken-cell preparations and with isolated low-molecular-mass PLA_2 enzymes, non-esterified fatty acids, especially long-chain unsaturated fatty acids, have been shown to inhibit PLA_2 activity [53,54]. In fact, it is often difficult to measure PLA_2 activity in homogenates of human neutrophils unless non-esterified fatty acids have been removed [55]. One of the purposes of the present studies was to determine if the inhibition of PLA_2 activity observed with non-esterified fatty acids in systems *in vitro* would in fact extrapolate into the more complex intact-cell environment. These studies were possible by using [3H] $C_{20:4}$ as the exogenously applied $C_{20:4}$ to differentiate it from the endogenous pools of $C_{20:4}$.

The effects of $C_{20:4}$ on neutrophil function were explored by addition of exogenous $C_{20:4}$ to neutrophils and examining indices of PLA_2 activity, $C_{20:4}$ release, PAF production and LTB_4 formation. Interestingly, the influence of $C_{20:4}$ addition on endogenous $C_{20:4}$ release showed two effects. Higher concentrations of exogenous $C_{20:4}$ caused a decrease in the endogenous $C_{20:4}$ produced by the cell. However, at lower exogenous $C_{20:4}$ concentrations, the content of endogenous $C_{20:4}$ in cell suspensions was increased. This increase occurred at concentrations of exogenous $C_{20:4}$ that inhibited both PAF and LTB_4 production. A potential explanation for this different effect on mediator production and endogenous $C_{20:4}$ content is that, at lower concentrations of exogenous $C_{20:4}$, the exogenous $C_{20:4}$ competes with endogenous $C_{20:4}$ for re-incorporation back into complex lipids. This gives the appearance that more endogenous $C_{20:4}$ has been produced, even if PLA_2 activity has been decreased. Further evidence for this hypothesis comes from studies which examined $C_{20:4}$ release from broken-cell homogenates. Under these conditions, in which re-incorporation of $C_{20:4}$ into complex lipids is not measurable, no increase in $C_{20:4}$ release can be observed at low concentrations of exogenous $C_{20:4}$ (J. D. Winkler and F. H. Chilton, unpublished work).

Further characterization of these effects of $C_{20:4}$ indicated that they were mediated by $C_{20:4}$ itself and not by a 5LO metabolite of $C_{20:4}$. A cautious interpretation would be that the effective molecule is not a 5LO metabolite of $C_{20:4}$, but could be $C_{20:4}$ or a non-5LO metabolite of $C_{20:4}$. Inhibition of PAF production by the 5LO inhibitor zileuton could not be reversed by re-introduction of 5LO products, and may be attributed to the increase in $C_{20:4}$ observed after treatment with zileuton. An increase in $C_{20:4}$ release in human neutrophils has been reported with the 5LO inhibitor BW755C [56]. In inflammatory cells, much of the released $C_{20:4}$ is not further metabolized, and remains as free $C_{20:4}$, e.g. approx. 50 % as free $C_{20:4}$ and 50 % as

5LO products in A23187-stimulated neutrophils [57]. These results suggest that some free $C_{20:4}$ is probably not destined for 5LO metabolism. Indeed, recent studies suggest that there may be different phospholipid precursor sources for free $C_{20:4}$ compared with $C_{20:4}$ which goes on to be metabolized by 5LO [58]. The massive and specific release of $C_{20:4}$ in inflammatory cells could indicate a functional role for $C_{20:4}$. This speculation is supported by observations that $C_{20:4}$ can mediate several functions in other cell systems, including modulation of Ca^{2+} flux [11,59,60], chloride channels [12], phagocytosis [9] and impairment of macrophage adherence [10].

The mechanism of action by which $C_{20:4}$ can perform these functions remains to be fully explored. There are several reports which suggest at least two mechanisms for $C_{20:4}$ -induced modulation of cell function. $C_{20:4}$ can bind to a GTP-binding protein in human neutrophils [13], and a recent paper suggests that PLA_2 activity can be modulated by a GTP-binding protein [14]. Some forms of protein kinase C are stimulated by fatty acids, especially $C_{20:4}$ [15,16,61], demonstrating that $C_{20:4}$ can modulate kinase activity. An earlier study showed data suggesting that protein kinase C activity appeared to be required for PAF production [62]. However, in these studies we observed that the kinase inhibitor staurosporine actually enhanced PAF production. Additionally, $C_{20:4}$ - and zileuton-induced inhibition of PLA_2 activity could be antagonized by the kinase inhibitor staurosporine. Further characterization of $C_{20:4}$ inhibition of PAF production revealed that the effect of staurosporine could not be mimicked by inhibitors of protein kinase A, tyrosine kinase or calmodulin kinase II. Whereas these data suggest a role for an isoenzyme of protein kinase C in $C_{20:4}$ -induced regulation of PLA_2 activity, certainly the involvement of other kinases cannot be ruled out [63,64], and regulatory mechanisms may vary between different inflammatory cells and different stimuli [65].

In contrast with the effects observed with $C_{20:4}$, inhibition of PAF production by $C_{18:1}$ and $C_{18:2}$ could not be antagonized by staurosporine. These data suggest that these other fatty acids may inhibit PAF production by a mechanism different from that of $C_{20:4}$. The recent observations by Wheelan and Clay [66] show that, similar to the current results, fatty acids such as $C_{20:4}$ and palmitic acid, but not stearic acid, inhibited PAF production. The authors speculated that some fatty acids could be used by acyl-CoA:lyso-PAF acyltransferase to compete with acetyl-CoA:lyso-PAF acetyltransferase for lyso-PAF. This increased availability of acyl-CoAs could thus decrease PAF production and could explain why inhibition of PAF production by $C_{18:1}$ and $C_{18:2}$ is not sensitive to staurosporine. Together with the observation that the concentrations of $C_{18:1}$ and $C_{18:2}$ do not change after stimulation, it indicates that these non-esterified fatty acids do not play a role in modulating PLA_2 activity in the stimulated neutrophil.

In summary, stimulated human neutrophils release large amounts of $C_{20:4}$ in a selective manner, resulting in high concentrations of cell-associated $C_{20:4}$. This $C_{20:4}$ can exert an inhibitory influence on PLA_2 activity in the neutrophils. This effect is not mediated by a 5LO metabolite of $C_{20:4}$, but appears to be mediated by $C_{20:4}$ -induced activation of kinase activity.

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