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Inhibition of PAF synthesis by stimulated human polymorphonuclear leucocytes with cloricromene, an inhibitor of phospholipase A_2 activation

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1 A phospholipase A_2 (PLA_2) represents the key enzyme in the remodelling pathway of platelet-activating factor (PAF) synthesis in human polymorphonuclear (PMN) leucocytes.

2 PLA_2 activation is also the rate-limiting step for the release of the arachidonic acid utilized for the synthesis of leukotrienes in stimulated leucocytes; however, it is unknown whether the PLA_2s involved in the two biosynthetic pathways are identical.

3 Cloricromene (8-monochloro-3-β-diethylaminoethyl-4-methyl-7-ethoxy-carbonylmethoxy coumarin) is an antithrombotic coumarin derivative which inhibits platelet and leucocyte function and suppresses arachidonic acid liberation by interfering with PLA_2 activation.

4 The aim of the present study was to assess whether chloricromene inhibits PAF synthesis by stimulated human polymorphonuclear leucocytes (PMNs).

5 Cloricromene (50-500 μM) inhibited in a concentration-dependent manner the release of PAF, as measured by h.p.l.c. bioassay, from A23187-stimulated PMNs. Significant inhibition (45%) of PAF-release was obtained with 50 μM cloricromene and the IC_{50} was 85 μM. Mepacrine (500 μM), a non-specific PLA_2 inhibitor, strikingly reduced PAF release.

6 The incorporation of [^{3}H]-acetate into [^{3}H]-PAF induced by serum-treated zymosan in human PMNs was also inhibited concentration-dependently by cloricromene, with an IC_{50} of 105 μM. Mepacrine also suppressed [^{3}H]-acetate incorporation into [^{3}H]-PAF.

7 Cloricromene did not affect the activities of the enzymes involved in PAF-synthesis acetyltransferase or phosphocholine transferase.

8 Our data demonstrate that cloricromene, an inhibitor of PLA_2-activation in human leucocytes, reduces the synthesis of PAF by stimulated PMNs. This finding has a twofold implication: the PLA_2s (or the mechanisms that regulate their activation) involved in PAF synthesis and arachidonate release in human leucocytes are either identical or else indistinguishable by their sensitivity to cloricromene; the inhibition of PAF release by activated leucocytes may contribute to the antithrombotic and anti-ischaemic activities exerted by cloricromene.

Keywords: Antithrombotic agents; arachidonic acid; leucocyte activation; leucocytes; leukotrienes; PAF; phospholipase A_2; tissue ischaemia

Introduction

Platelet-activating factor (PAF) is a phospholipid mediator of inflammation that has also been claimed to play a role in tissue injury and thrombosis (Koltai *et al.*, 1991). PAF is produced upon appropriate stimulation of a variety of cells including platelets, endothelial cells and leucocytes (Lotner *et al.*, 1980; Benveniste *et al.*, 1982; Zimmerman *et al.*, 1985).

PAF synthesis takes place either via the *de novo* pathway, which involves the transfer of phosphocholine from CDP-choline to 1-0-alkyl-2-acetyl-*sn*-glycerol by a cholinephosphotransferase (Renooij & Snyder, 1981), or via the remodelling pathway. In the latter case, 1-0-alkyl-2-acyl-*sn*-glycero-3-phosphocholine, present in cell membranes, is hydrolysed by a phospholipase A_2 (PLA_2) generating 1-0-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lysoPAF) which is then acetylated to PAF by a specific acetyltransferase (Wykle *et al.*, 1980). The burst of PAF synthesis in response to cell stimulation comes from the remodelling pathway (Alonso *et al.*, 1982; Garcia *et al.*, 1993) and thus the activation of a PLA_2 represents a crucial step in the control of PAF formation (Benveniste *et al.*, 1982; Chilton *et al.*, 1984). PLA_2 is also a key enzyme in the liberation of the arachidonic acid (AA) used as a substrate for the synthesis of leukotrienes in human leucocytes (Walsh *et al.*, 1981; Gresele *et al.*, 1993).

Considering, however, that different types of PLA_2 have been isolated in different tissues (Dennis, 1994; Diez *et al.*, 1994), the fact that PAF and leukotriene synthesis in polymorphonuclear leucocytes (PMNs) may depend on the activation of different PLA_2s cannot be excluded. Indeed, in leucocytes at least, two types of PLA_2 have been described (Albert & Snyder, 1983; Dennis, 1994) but the relative roles in PAF or leukotriene synthesis have not yet been clarified.

Data have been obtained that argue against the hypothesis that the PLA_2 involved in AA-liberation and leukotriene synthesis is the same as that involved in PAF-biosynthesis. For instance, it has been shown that the two biochemical pathways are differentially regulated by calcium in human PMNs (Garcia Rodriguez *et al.*, 1993) and by PAF or the 5-lipoxygenase inhibitor, zileuton, in rat alveolar macrophages (Shamsuddin *et al.*, 1995). On the other hand, the exposure of stimulated human neutrophils to recombinant PLA_2 has been shown to enhance the release of both leukotriene B_4 (LTB_4) and PAF (Steiner *et al.*, 1991) and initial studies on the simultaneous

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suppression of the synthesis of leukotrienes and PAF by novel, selective PLA_2 inhbitors have appeared (Glaser *et al.*, 1991; Marshall *et al.*, 1995). The subject is further complicated by the incomplete understanding of the mechanisms that regulate PLA_2 activation and it is interesting to note that not all the stimuli that release PAF also produce leukotrienes to the same extent, and vice-versa (Nieto *et al.*, 1988a; Fradin *et al.*, 1989; Leyravaud *et al.*, 1989).

Cloricromene is a coumarine derivative which possesses vasodilator, antithrombotic and antiischaemic activity in animals (Aporti *et al.*, 1987; Prosdocimi *et al.*, 1985; Cirillo *et al.*, 1992; Lidbury *et al.*, 1993) and which reduces several leucocyte functions, including chemotaxis, adhesion, superoxide anion generation, and tumour necrosis factor α (TNF-α) release (Bertocchi *et al.*, 1989; Squadrito *et al.*, 1991; Tranchina *et al.*, 1994).

We have previously shown that cloricromene inhibits leukotriene formation by stimulated human polymorphonuclear leucocytes due to the suppression of arachidonic acid release by interfering with the mechanisms that regulate PLA_2 activation (Gresele *et al.*, 1993).

The aim of the present study was to ascertain whether cloricromene can also impair the synthesis of PAF by stimulated, human polymorphonuclear leucocytes. A preliminary account of some of the present results has been published in abstract form (Ribaldi *et al.*, 1989).

Methods

Blood sampling and isolation of polymorphonuclear leucocytes

Blood anticoagulated with sodium heparin (10 u ml^{-1}) was collected in plastic tubes from drug-free healthy volunteers. Polymorphonuclear leucocytes (PMNs) were isolated using dextran sedimentation and Hypaque/Ficoll gradients as previously described (Gresele *et al.*, 1986). Contaminating erythrocytes were removed by hypotonic lysis and PMNs were washed twice with Hanks' balanced salt solution (HBSS), pH 7.35, without Ca^{2+} and Mg^{2+} containing 0.25% bovine serum albumin (BSA) and were finally resuspended in HBSS containing Ca^{2+} and Mg^{2+} (0.6 and 0.8 mM, respectively) and 0.25% BSA at a concentration of 10^7 cells ml^{-1}. Viability of the PMN was $97.2 \pm 0.2\%$ ($n=13$) by trypan blue dye exclusion test and purity was $96.8 \pm 0.3\%$ ($n=13$). Residual contaminating platelets in the final preparations were $3.9 \pm 0.06 \times 10^6$ ml^{-1} ($n=13$).

Stimulation of PAF-release by isolated PMNs

Aliquots of isolated PMNs (10^7 cells ml^{-1}) were stimulated with increasing concentrations of A23187 (1–50 μM) for different periods of time (5–60 min). Controls were carried out by treating PMNs with equivalent amounts of dimethylsulphoxide (DMSO) (final concentration never exceeded 0.5%).

In the studies with cloricromene, the PMNs, after 10 min of preincubation with the drug (50, 100, 200 or 500 μM) or its vehicle, were stimulated for 30 min at 37°C with 10 μM A23187. The reaction was stopped by centrifugation ($12,000 \times g$ for 2 min) in an Eppendorf microfuge (Eppendorf, Gerätebau, Germany) and the cell-free supernatant was stored at −80°C for subsequent assay. Complete dose-response curves were carried out on PMN preparations from every single blood donor in order to minimize variability due to the different individual response of PMNs to stimuli.

Extraction and assay of PAF

One volume of PMN supernatant was extracted by adding 3.2 volumes of chloroform/methanol 1:1 and 0.5 volumes saline (Pinckard *et al.*, 1979). The organic phase was dried under vacuum in a Hetovac vacuum concentrator (Heto Lab. Equipment A/S, Birkerod, Denmark) and resuspended into 100 μl of hexane: isopropanol (3:2, v/v) containing 5.5% H_2O (h.p.l.c. eluting solvent).

Purification of PAF was performed by high performance liquid chromatography (h.p.l.c.) in a system consisting of a Perkin Elmer 3B pump equipped with a Rheodine 7125 injector with a 100 μl sample loop at room temperature by using a silica spherisorb-Si (250 × 45 mm) with particle size of 5 μm. The mobile phase (h.p.l.c. eluting solvent) ran at a flow rate of 1 ml min^{-1} in an isocratic system. The peak corresponding to PAF was collected, dried under vacuum and resuspended in saline containing 0.25% BSA for subsequent bioassay. The retention time of PAF was estimated by injecting a known amount of [^{3}H]-C_{16}-PAF before each run. The radioactivity was always recovered between 19 and 23 min from injection.

PAF was then measured by bioassay by evaluating its ability to induce platelet aggregation and [^{3}H]-5-hydroxytryptamine (5-HT) release from aspirin-(100 μM) and apyrase-(150 μg ml^{-1}) pretreated washed rabbit platelets, as previously described (Pinckard *et al.*, 1979). Briefly, rabbit blood was collected into ACD (1:6 v/v, pH 4.5) from anaesthetized animals and [^{3}H]-5-HT (specific radioactivity: 17 Ci $mmol^{-1}$; 1 μl per ml of anticoagulated blood) was added and incubated at 37°C for 30 min with gentle shaking. The blood was then centrifuged at room temperature for 15 min at $190 \times g$ and the resulting platelet rich plasma was resuspended in 10 ml Tyrode's buffer pH 6.2, without Ca^{2+} and Mg^{2+} and containing EGTA 1 mM, and centrifuged for 15 min at $1100 \times g$. The platelet pellet was washed in the same medium without EGTA and platelets were finally suspended in Tyrode's buffer, pH 7.35, with Ca^{2+} and Mg^{2+} containing 0.25% BSA, apyrase and aspirin.

Platelet aggregation was carried out in an Elvi 840 aggregometer (Elvi Logos, Milan, Italy). Aliquots (0.25 ml) of [^{3}H]-5-HT-labelled, washed rabbit platelets were stirred at 1,100 r.p.m. for 2 min at 37°C, then 5–25 μl of the test samples were added. The resulting aggregation wave was followed for 5 min and maximal amplitude was recorded. Specificity and recovery of PAF were assessed by comparing the effects of the PAF extracted and purified from the supernatant of stimulated PMNs with those of authentic C_{16}-PAF retrieved from h.p.l.c., authentic C_{16}-PAF not subjected to h.p.l.c. or control buffer, on platelet aggregation and 5-HT-release. In some experiments a known quantity of standard C_{16}-PAF was added to PMN resuspension buffer, extracted, purified by h.p.l.c. and bioassayed exactly as described for the PMN supernatant samples.

At the end of the stimulation period, the samples were immediately centrifuged at $10,000 \times g$ for 2 min and the supernatants removed and counted for radioactivity in a liquid scintillation counter. The secreted [^{3}H]-5-HT was expressed as a percentage of the total, as determined by platelet lysis with Triton X-100 (Pinckard *et al.*, 1979).

A standard curve of aggregation and [^{3}H]-5-HT release induced by several concentrations of synthetic C_{16}-PAF (1×10^{-10} to 1×10^{-7}) was prepared for each series of assays and used for the calculation of PAF content in the test samples. In some selected experiments, PAF released in the supernatant by A23187-stimulated PMNs was also measured by radioimmunoassay (RIA) with a commercially available PAF RIA-kit (NEN-Du Pont, Boston, MA, U.S.A.). Briefly, PMN supernatants were extracted with methanol/chloroform/H_2O, 5:5:3.5 v/v and resuspended in 1 ml of working assay buffer (0.1 sodium azide, 0.05% Tween 20, 50 mM sodium citrate). A known amount of [^{3}H]-C_{16}-PAF (2,000 c.p.m.) was added to each sample before extraction, to assess the percentage recovery. A standard curve for displacement of antibody-bound [^{125}I]-PAF by standard cold PAF was obtained and the amount of PAF in the samples was calculated from the linear portion of the calibration curve (30–3000 pg ml^{-1}) by correcting the results for recovery.

Incorporation of [^{3}H]-acetate into [^{3}H]-PAF

Incorporation of [^{3}H]-acetate into PAF was assessed by a method described by Sturk *et al.* (1989). Aliquots (0.4 ml) of isolated PMNs were stimulated for different time intervals (5–30 min) with increasing concentrations of serum-treated zymosan (STZ) (1–25 mg ml^{-1}). Just prior to stimulation, 10 mCi l^{-1} [^{3}H]-acetate (4 μl) was added. PMNs were preincubated with chloricromene, or its solvent, for 10 min before the addition of [^{3}H]-acetate and the leucocytes were stimulated with 5 mg ml^{-1} STZ for 15 min at 37°C.

Leucocyte stimulation was stopped by extracting the samples according to Bligh and Dyer (Bligh & Dyer, 1959). Briefly, 2.4 ml chloroform/methanol (1/2 v/v) was added to the samples which, after thorough mixing for 30 s, were filtered through glass-wool to remove particulate material; two phases were obtained by further addition of 0.8 ml water. The organic phase was washed three times with 3.2 ml of a 1 : 1 mixture of methanol/water, to remove excess [^{3}H]-acetate, and was then dried and the residue resuspended in 100 μl of chloroform/methanol (2/1 v/v).

Separation of [^{3}H]-PAF was carried out by thin layer chromatography (t.l.c.) on Silica Gel 60A plates developed in chloroform/methanol/acetic acid/water (50 : 25 : 8 : 4, v/v) (Nieto *et al.*, 1988a). Areas containing PAF were scraped from the plates and the radioactivity, determined by liquid scintillation counting, was measured and quenching corrected by external standardization (Packard Instrument Company, Meridien, CT, U.S.A.).

Results are expressed as d.p.m./10^7 PMNs. The recovery of [^{3}H]-PAF added to unstimulated PMNs suspensions, extracted and separated by t.l.c., was 43.7 ± 2.5% (n = 8).

Assay of lysoPAF acetyltransferase or PAF-synthesizing phosphocholinetransferase activities

PMNs (1 × 10^7), resuspended in 0.5 ml HBSS buffer containing 0.25% BSA and preincubated with either cloricromene or its solvent for 10 min at 37°C, were stimulated with the calcium ionophore A23187 or its vehicle at 37°C for various time intervals. Incubations were interrupted by immediately centrifuging the samples at 10,000 × g for 10 s at room temperature and the pellets, resuspended in 0.4 ml of 0.1 M Tris-HCl (pH 7.4), were sonicated (three pulses of 10 s) in an ice bath.

Acetyltransferase activity was assayed in the cell homogenate as previously described (Goracci & Francescangeli, 1991) by the method of Wykle *et al.* (1980). Briefly, the assay was carried out in a volume of 0.5 ml of 0.1 M Tris-HCl buffer medium containing the lysate (approximately 200 μg of protein in 100 μl), 30 μM of lysoPAF, 200 μM [^{3}H]-acetyl-CoA (0.5 μCi in 100 μl) and 200 μM unlabelled acetyl-CoA. The reaction was carried out for 10 min at 37°C and was stopped by adding 3 ml of chloroform/methanol 2 : 1; 1 ml of chloroform and 1 ml of water were then added to form two phases. The amounts of [^{3}H]-PAF recovered were quantified after separation by t.l.c. and were expressed in pmol mg^{-1} protein min^{-1}.

The phosphocholinetransferase assay was carried out on the PMN-homogenates as previously described (Goracci & Francescangeli, 1991). Briefly, cell homogenates were incubated at 37°C for 15 min in 0.3 ml of a medium containing 60 mM Tris/HCl (pH 8.0), 0.5 mM 1-0-hexadecyl-2-acetyl-*sn*-glycerol, 0.005% Tween 20, 20 mM $MgCl_2$, 20 mM DTT, and 0.1 mM CDP (methyl [^{14}C]-choline). The reaction was started by adding the labelled compounds and stopped as described above for the acetyltransferase assay. The labelled phospholipid product, identified as [^{14}C]-PAF on t.l.c. by co-chromatography with unlabelled standard, was quantified by liquid scintillation counting and expressed in nmol mg^{-1} protein min^{-1}. Protein concentration were determined by the Folin reagent method.

Reagents

Cloricromene HCl (AD6 or 8-monochloro-3-beta-diethylaminoethyl-4-methyl-7-ethoxy carbonyl methoxy coumarin) was from FIDIA Res. Labs. (Abano Terme, Italy); the calcium ionophore A23187, zymosan A, bovine serum albumin (BSA), Tween 20, DTT, Triton X 100 and apyrase were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.); Lymphoprep was from Nycomed AS (Oslo, Norway); Dextran T 500 from Pharmacia (Uppsala, Sweden); HBSS from Biochron KG (Berlin, Germany). All h.p.l.c. solvents, from the highest grade available, were from Carlo Erba (Milan, Italy); [^{3}H]-acetyl-coenzyme A (spec. act. 2.4 Ci mmol^{-1}), CDP [methyl-[^{14}C]]-choline (spec. act. 1–2 nCi nmol^{-1}) and 5-hydroxy(G-[^{3}H]-tryptamine creatinine sulphate (spec. act. 17 Ci mmol^{-1}) were from Amersham (Little Chalfont, U.K.); 1-0-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lysoPAF) and 1-0-hexadecyl-2-acetyl-*sn*-glycero from Novabiochem (Länfelfingen, Switzerland); 1-0-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) from Bachem (Bubendorf, Switzerland); 1-0-hexadecyl-1′-2′-[^{3}H]-2-acetyl-*sn*-glycero-3-phosphorylcholine (spec. act. 56.7 Ci mmol^{-1}) and [^{3}H]-acetate, sodium salt (spec. act. 4.2 Ci mmol^{-1}) were from New England Nuclear (Boston, MA, U.S.A.); phospholipase C (PLC) from hog pancreas (Boehringer, Mannheim, Germany); silica gel 60A plates were from Whatman International Ltd, (Maidstone, U.K.). Sodium heparin was from Novo (Novo Nordisk, Denmark); acetylsalicylic acid, lysine salt was from Lirca Synthelabo (Milan, Italy); BN 52021 (9H-1.7α-(epoxymethano)-1H,6αH,cyclopenta[c]furo[2 - 3 - b]furo[3′,2′ : 3,4]cyclopenta [1,2 - d]furan-5,9,12-[4H]-trione,3-tert-butyl hexahydro-4,-7b,-11 hydroxy-8 methyl) was a gift from Dr P. Braquet (Institute Henri Beaufour) and was dissolved in DMSO, while L-659,989 ((±)-trans - 2 - (3 - methoxy - 5 - methylsulphonyl - 4-propoxyphenyl)-5-(3,4,5-trimethoxyphenyl)tetrahydrofuran), kindly given by Dr M. Goldenberg (Merck, Sharp & Dohme, Rahaway, N.J., U.S.A.), was suspended in DMSO. The final concentration of DMSO in the samples never exceeded 0.5%; controls were simultaneously run with buffer containing the same concentration of DMSO. Serum-treated (i.e. complement-coated) zymosan (STZ) for leucocyte stimulation was prepared as previously described (Gresele *et al.*, 1986).

Statistical analysis

All results are given as means ± s.e.mean. Student's t test for paired or unpaired data, one-way or repeated-measures ANOVA followed by Scheffe's multiple comparison test (Scheffé, 1959) were applied, as appropriate. The correlation between various parameters was assessed by linear regression analysis.

Results

Validation of the method for PAF separation and assay

A number of tests were carried out in order to validate the method used for the extraction and bioassay of PAF. The characterization of the platelet-activating material extracted from the supernatant of stimulated human PMNs as PAF (Nieto *et al.*, 1988a) was based on the chromatographic behaviour of the sample on high performance liquid chromatography, sensitivity of the biological activity to previous treatment with phospholipase C, and blockade of the platelet-activating activity by BN52021 and L-659,989, two specific PAF-receptor antagonists (Braquet *et al.*, 1985; Pompipom *et al.*, 1988).

When a known quantity (2 × 10^{-8} M) of PAF was added to PMN resuspension buffer, extracted and purified by h.p.l.c., the fractions containing the aggregation-inducing activity on rabbit platelets were found to elute at the same time as those containing standard [^{3}H]-PAF. In addition, the platelet aggregating activity recovered from extracted and purified su-

pernatants of stimulated PMNs was antagonized by L-659,989 (2.5×10^{-6} M) and by BN52021 (4×10^{-7} M) and completely abrogated by pretreatment with hog pancreas PLC. The recovery of [^{3}H]-PAF added to the PMNs resuspension buffer after extraction, SEP-PAK cartridge separation and purification by h.p.l.c. was 73.7±2.2% ($n=4$). In human plasma samples, the amounts of PAF measured by bioassay after extraction/purification correlated significantly with the amount (56 to 1120 pg ml^{-1}) added ($r=0.97$, $n=12$, $P<0.0006$).

The detection limit for PAF (2 s.d. from zero), as assessed by aggregation of washed rabbit platelets, was 89±6.3 pg ml^{-1} ($n=10$). Under the same experimental conditions intra-assay and inter-assay reproducibility studies showed a coefficient of variability of 9.8% ($n=9$) and 26% ($n=6$), respectively. Variability between individuals was instead larger due to a widely different capability of leucocytes from different individuals to release PAF upon stimulation.

Effects of cloricromene on PAF-release in the supernatant of stimulated PMNs

The stimulation of PMNs with increasing concentrations of A23187 (1 to 50 μM) for 30 min induced a dose-dependent release of PAF as measured by bioassay (Figure 1a). When measured by RIA, the amount of PAF-like material recovered in the supernatant of A23187 (10 μM)-stimulated PMNs was 69.3±3.8 ng per 10^7 PMNs ($n=5$), similar to that measured by h.p.l.c. bioassay.

The time course of PAF release in the supernatant of isolated purified human PMNs stimulated with the Ca^{2+} ionophore A23187 (10 μM), as measured by bioassay, is shown in Figure 1b. Detectable amounts of PAF appeared after 5 min of incubation with A23187, then tended to increase reaching peak levels at 30 min; thereafter a decrease of PAF in the PMNs-supernatant was observed at 1 h (down to 80.3% of the maximal amount released) (Figure 1b). On the basis of these results, the following experiments were carried out by using a concentration of A23187 of 10 μM and an incubation time of 30 min.

In a selected subset of experiments the amount of PAF produced by A23187 (10 μM)-stimulated PMNs was measured both in the supernatants and in the leucocyte pellets. Released PAF represented 6.9% of the total (20.0±5.4 vs 271±139 ng per 10^7 PMNs, $n=3$), in agreement with previously published data (Lynch & Henson, 1986). Cloricromene, preincubated for 10 min, inhibited the release of PAF into the supernatant of A23187-stimulated PMNs in a concentration-dependent manner; an inhibitory effect was already significant with 50 μM cloricromene (45.0% of inhibition). PAF release was further reduced by higher concentrations of cloricromene and the IC_{50} value was reached at 85 μM; a maximum reduction of around 78% was obtained with the highest concentration of the drug tested (500 μM) (Figure 1c). Preincubation of PMNs with the highest concentration of cloricromene tested (500 μM) did not affect their viability as assessed by trypan blue dye exclusion test (93±0.4%; $n=6$). Mepacrine (500 μM), a non-specific in-

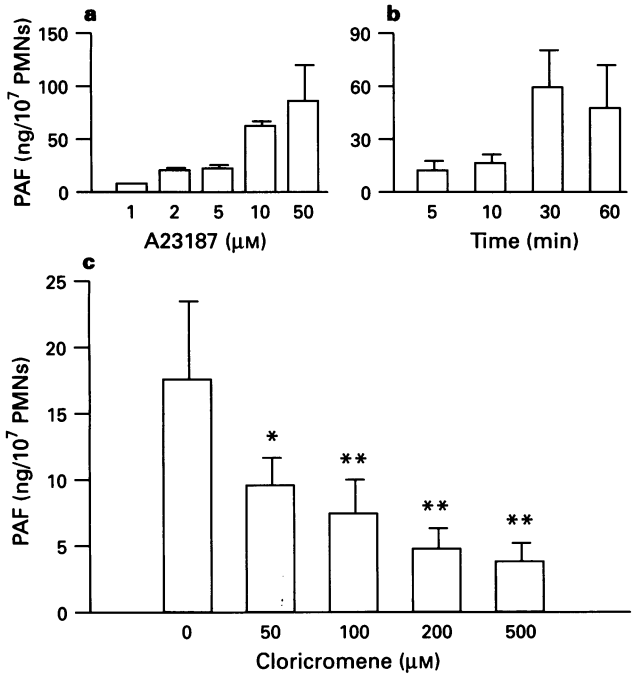

Figure 1 PAF measured in the supernatant of stimulated PMN suspensions by h.p.l.c. bioassay. (a) Dose-response curve of A23187-induced PAF synthesis. PAF was measured in the supernatant of PMN suspensions incubated with the stimulus for 30 min at 37°C. Data represent mean±s.e.mean of four separate experiments. (b) Time-course of A23187 (10 μM)-induced synthesis of PAF by human PMNs. Data represent mean±s.e.mean of four separate experiments. (c) Effect of cloricromene on A23187 (10 μM)-induced synthesis of PAF by human PMNs. Leucocyte susupensions, preincubated with increasing concentrations of cloricromene for 10 min at 37°C, were stimulated with A23187 for 30 min at 37°C. Data represent the mean±s.e.mean of eleven experiments. Asterisks indicate a statistically significant difference (ANOVA for repeated measures) as compared with control (*$P<0.05$; **$P<0.02$). A significant difference was also present between cloricromene 50 μM and cloricromene 100, 200, 500 μM ($P<0.009$) and between cloricromene 100 μM and cloricromene 200 and 500 μM ($P<0.01$).

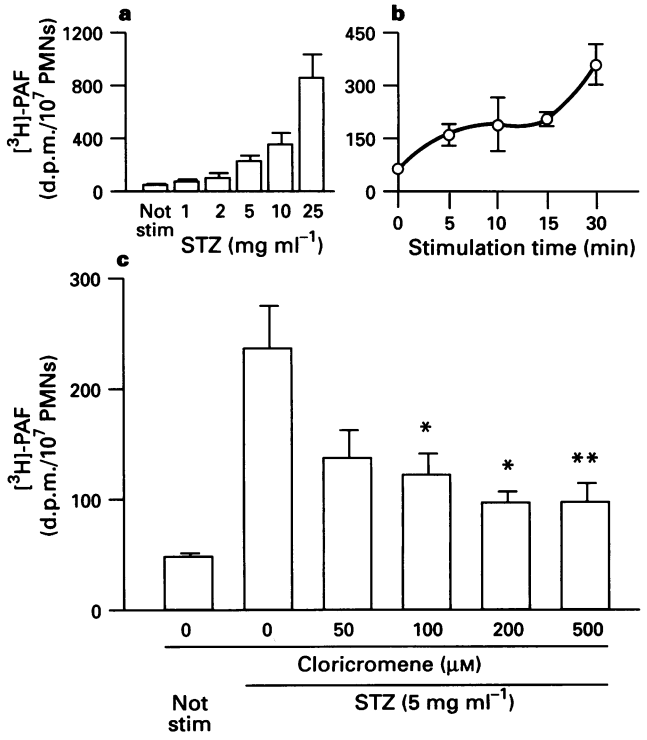

Figure 2 PAF measured by [^{3}H]-acetate incorporation into [^{3}H]-PAF. (a) Effect of increasing concentrations of STZ, preincubated with PMNs for 15 min at 37°C, on [^{3}H]-PAF formation. Data represent mean±s.e.mean of at least six separate experiments. Not stim = not stimulated. (b) [^{3}H]-acetate incorporation into PAF in leucocyte suspensions stimulated with 5 mg ml^{-1} STZ for different time intervals. Data represent mean±s.e.mean of at least six separate experiments. (c) Effect of cloricromene on [^{3}H]-acetate incorporation into PAF by leucocyte suspensions stimulated with STZ for 15 min at 37°C. Data represent mean±s.e.mean of six separate experiments. Asterisks indicate a statistically significant difference as compared with stimulated control (*$P<0.04$; **$P<0.02$).

Table 1 Acetyltransferase activity of resting and A23187 (5 μM)-stimulated polymorphonuclear leucocytes and the effect of preincubation with cloricromene

	LysoPAF-acetyltransferase activity (nmol mg^{-1} protein min^{-1}) *After A23187*					
	Basal	*3 min*	*5 min*	*10 min*	*20 min*	*30 min*
Control	0.50±0.11	1.48±0.34	1.61±0.47	0.88±0.37	0.26±0.21	0.21±0.13
Cloricromene (100 μM)	0.59±0.14	1.19±0.24	1.68±0.28	0.87±0.50	0.42±0.32	0.50±0.28

Data represent mean±s.e.mean ($n=4$, at least). No significant differences between control and cloricromene-pretreated samples were present at any time point.

hibitor of PLA_2 (Hoffman *et al.*, 1982; Stasi *et al.*, 1992), also suppressed A23187-induced PAF-release by 95% ($n=3$, $P<0.05$) (data not shown).

The effects of cloricromene on the release of PAF induced by STZ (1 mg ml^{-1}) were also evaluated. The release of PAF produced by STZ was low (0.36±0.18 ng per 10^7 PMNs, $n=3$) and cloricromene (100 μM) completely suppressed it (<0.089 ng ml^{-1}, $n=3$). Finally, a soluble agonist was also used as a stimulus (formyl-methionyl leucyl phenylalanine (fMLP, 100 nM) but the amount of PAF released by PMNs in the supernatant was below our detection limit.

Effects of cloricromene on the biosynthesis of PAF via acetyl-CoA: lysoPAF acetyltransferase

The production of PAF was also evaluated by measuring the incorporation of [^{3}H]-acetate into the fractions co-migrating with PAF on t.l.c. Unstimulated PMNs incorporated only minimal amounts of [^{3}H]-acetate into [^{3}H]-PAF (53.7±3.6 d.p.m./10^7 PMNs, $n=11$).

When human PMNs were stimulated for 15 min with increasing doses of STZ (1–25 mg ml^{-1}), the synthesis of [^{3}H]-PAF increased concentration-dependently, reaching a maximum 12 fold over basal at 25 mg ml^{-1} (Figure 2a). The time-course of the incorporation of labelled acetate into PAF in PMNs stimulated with STZ 5 mg ml^{-1} showed a detectable formation of [^{3}H]-PAF after 5 min and this did not increase significantly up to 15 min. A further increased incorporation of [^{3}H]-acetate into PAF was observed during the following 15 min (Figure 2b).

Preincubation of PMNs with cloricromene (50–500 μM) reduced concentration-dependently the incorporation of [^{3}H]-acetate into [^{3}H]-PAF induced by STZ (5 mg ml^{-1}); the estimated IC_{50} was 105 μM (Figure 2c) while the maximal inhibition attained was 58.1% as compared with control. When the stimulation of leucocytes with STZ (5 mg ml^{-1}) was carried out in cells preincubated with mepacrine (500 μM), incorporation of [^{3}H]-acetate into PAF was drastically reduced by 89.1±8.5% ($n=4$, $P<0.04$).

Incorporation of [^{3}H]-acetate into [^{3}H]-PAF was also assessed by using as a stimulus A23187 (1 μM). The amount of [^{3}H]-PAF produced was very high (4821±978 d.p.m./10^7 PMNs, $n=8$); cloricromene (500 μM) inhibited [^{3}H]-PAF production (by 47%, $P<0.081$) but to a lesser extent than when STZ was used as a stimulus. This result is in agreement with the previously reported finding that the inhibition by cloricromene of different cellular functions is inversely proportional to the strength of the stimulus (Prosdocimi *et al.*, 1986; Gresele *et al.*, 1993).

Effects of cloricromene on the activity of the enzymes acetyltransferase and phosphocholinetransferase in human leucocytes

The acetyltransferase activity was studied in PMNs stimulated with A23187 or with its vehicle. Leucocytes exhibited a rather low basal acetyltransferase activity that increased up to 5 fold upon stimulation with A23187 (5 μM), reaching a maximum activity between 3 and 5 min after stimulation. Preincubation with cloricromene (100 μM) did not modify significantly the acetyltransferase activity (Table 1).

Table 2 Phosphocholinetransferase activity of resting and A23187 (10 μM)-stimulated polymorphonuclear leucocytes and the effect of preincubation with cloricromene

	Phosphocholinetransferase activity (nmol mg^{-1} protein min^{-1})	
	Basal	*After A23187*
Control	0.25±0.008	0.28±0.04
Cloricromene (200 μM)	0.25±0.03	0.26±0.06

Data represent mean±s.e.mean ($n=3$, at least). Stimulations with A23187 were carried out for 30 min. No significant differences between control and cloricromene-pretreated samples were present.

Phosphocholinetransferase activity in unstimulated or A23187 (10 μM)-stimulated PMNs is shown in Table 2. No significant increase in this enzymic activity was evident after leucocyte stimulation, nor did the preincubation with cloricromene affect phosphocholinetransferase significantly (Table 2).

Discussion

Human polymorphonuclear leucocytes synthesize, and in part release, PAF and a series of bioactive metabolites of arachidonic acid upon appropriate stimulation. In activated PMNs the synthesis of PAF occurs mainly via the remodelling pathway which involves the activation of two enzymatic steps: the hydrolysis of 1-0-alkyl-2-(long chain)-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC) by a PLA_2 and the acetylation of 2-lysoPAF at the 2 position by a specific acetyltransferase (Wykle *et al.*, 1980). A phospholipase A_2 is also the rate-limiting step for the release of arachidonic acid utilized for the formation of leukotrienes in human PMNs (Walsh *et al.*, 1981; Gresele *et al.*, 1993). However, several forms of PLA_2 have been described with different calcium requirements, substrate specificities and mechanisms of regulation (Diez *et al.*, 1994; Dennis, 1994), and it is debated whether the PLA_2 participating in the remodelling pathway of PAF synthesis and the PLA_2 releasing arachidonate for leukotriene synthesis are identical (Benveniste *et al.*, 1982; Sisson *et al.*, 1987; Camussi *et al.*, 1990; Chilton *et al.*, 1991; Garcia Rodriguez *et al.*, 1993).

Indeed, while in some studies it was shown that some putative PLA_2 inhibitors produce a parallel suppression of the synthesis of PAF and arachidonic acid metabolites (Glaser *et al.*, 1991) and that PAF-synthesis is tightly coupled with LTB_4 production (Sisson *et al.*, 1987), other authors have shown that the increase in intracellular calcium produced by thapsigargin stimulates PAF production but not [^{3}H]-AA-release (Garcia Rodriguez *et al.*, 1993).

The picture is further complicated by the incomplete understanding of the mechanisms that regulate the activation of PLA_2. The increase in cytosolic Ca^{2+} concentration (Channon & Leslie, 1990), a G-protein-mediated mechanism (Silk *et al.*, 1989; Stasi *et al.*, 1992) and phosphorylation/dephosphorylation reactions (Wijkander & Sundler, 1992) have been proposed for the activation of PLA_2 in various cells (Dennis, 1994).

Cloricromene inhibits the release of arachidonate and the production of its oxygenated metabolites by stimulated human platelets and leucocytes (Porcellati *et al.*, 1990; Gresele *et al.*, 1993) by interfering with a G-protein-linked mechanism of PLA_2 activation (Stasi *et al.*, 1993). This drug, however, does not inhibit isolated PLA_2 or reduce the activity of other enzymes involved in phospholipid degradation, including PLC (Porcellati *et al.*, 1990; Stasi *et al.*, 1993).

Our data show that cloricromene is also able to inhibit the synthesis and release of PAF by stimulated human polymorphonuclear leucocytes. This inhibition is exerted at the level of the reaction catalyzed by phospholipase A_2 in the remodelling pathway. Indeed, cloricromene reduced the total release of PAF by activated leucocytes and it also suppressed [^{3}H]-acetate incorporation into [^{3}H]-PAF without affecting the activities of lysoPAF acetyltransferase or phosphocholine-transferase. The increased activity of acetyltransferase which follows the challenge of PMNs by STZ further supports the concept that the action of this enzyme is regulated by stimulation, probably through its phosphorylation (Nieto *et al.*, 1988b). The lack of any effect on this enzymic activity by cloricromene tends to exclude an unspecific effect of the drug on enzymes that may undergo activation and further points to a specific inhibition of the mechanisms involved in the regulation of PLA_2.

The inhibitory effect of cloricromene on PAF synthesis and release was already evident at the concentration of 50 μM. Although this concentration is relatively high it is conceivable that this action can be relevant to the effects that this drug exerts *in vivo* because it has been shown that concentrations as high as 15 μM can be achieved in plasma after the intravenous administration of 0.5 mg kg^{-1} to dogs (Prosdocimi *et al.*, 1985). In man the i.v. administration of 30 mg cloricromene has given peak plasma levels of the cloricromene acid metabolite of up to 10 μM in healthy volunteers, without side-effects (Prosdocimi *et al.*, unpublished observations). In addition, the drug accumulates actively in leucocytes, attaining intracellular concentrations much higher than those in plasma (Travagli *et al.*, 1989; Bertocchi *et al.*, 1989). It can be calculated that after 30 min incubation of PMNs with 20 μM cloricromene, 250 pmol of cloricromene acid and ester accumulate in 10^6 PMNs giving an estimated intracellular concentration of approximately 500 μM (Bertocchi *et al.*, 1989; Prosdocimi *et al.*, unpublished observations). Moreover, it has previously been shown that the inhibitory effects of cloricromene are inversely related to the strength of the stimulus used (Gresele *et al.*, 1993) and, in order to get a clearly detectable formation of PAF *in vitro*, relatively strong stimuli had to be used in our study.

Our data confirm the rate-limiting role played by PLA_2 in the synthesis of PAF by the 'remodelling' pathway (Benveniste *et al.*, 1982; Chilton *et al.*, 1984) and suggest that the PLA_2 that releases arachidonate for the production of leukotrienes in stimulated leucocytes may also be involved in the production of lysoPAF for the synthesis of PAF. In fact cloricromene inhibits the production of both lipid mediators in a similar manner (Gresele *et al.*, 1993; present data). The simultaneous impairment of both leukotriene B_4 and PAF-synthesis by leucocytes of patients with liver cirrhosis, a condition in which PLA_2 activity is altered (Laffi *et al.*, 1993), and the reduced production of both prostacyclin and PAF by stimulated endothelial cells in a patient with an antibody inhibiting PLA_2 (Schorer *et al.*, 1992), also support the similarity between the arachidonate-generating and the lysoPAF-generating PLA_2 activities. Alternatively, the enzymes might be different but could be joined by a similar, possibly G-protein-linked, mechanism of regulation affected by cloricromene (Stasi *et al.*, 1993). Indeed, it is interesting to note that the stimulation of isolated human PMNs with the G-protein activator AlF_4^- (20 mM) produces a detectable incorporation of [^{3}H]-acetate into [^{3}H]-PAF (basal = 51 $\pm$ 3 d.p.m., stimulated = 415 $\pm$ 3 d.p.m., $n = 3$) (data not shown).

Although the ability of cloricromene to reduce cytosolic calcium movements (Del Maschio *et al.*, 1990) or to raise intracellular cyclic GMP (Hakim *et al.*, 1988) may play a role in the inhibition of PLA_2 activity of the remodelling-pathway in leucocytes, this is not likely. In fact, calcium seems to exert its control on PAF-synthesis at the level of acetyltransferase in human PMNs (Garcia Rodriguez *et al.*, 1993) and this enzyme was found to be unaffected by cloricromene in our study. On the other hand, cyclic GMP is known to inhibit PLA_2 activity only indirectly, by interfering with the activation of PLC and the subsequent rise of intracellular calcium generated by the IP_3 produced (Stasi *et al.*, 1992). In our experiments, however, cloricromene was found to be effective even when the stimulus used was the calcium ionophore A23187. On the other hand, it has been suggested that cloricromene inhibits the production of tumour necrosis factor by modulating cyclic AMP levels (Squadrito *et al.*, 1994) and it is known that a rise of cyclic AMP inhibits the synthesis of PAF by neutrophils (Fonteh *et al.*, 1993). However, data on platelets indicate that cloricromene does not affect cyclic AMP either directly or indirectly (Prosdocimi *et al.*, 1986).

In addition, it is unlikely that the reduction of PAF synthesis is secondary to LTB_4 suppression because it has already been shown that LTB_4 is not essential for PAF synthesis (Glaser *et al.*, 1991) and because concentrations of cloricromene which almost totally suppress LTB_4 release from stimulated human PMN leucocytes (Gresele *et al.*, 1993) only partially inhibited PAF synthesis. The incomplete suppression of PAF release by cloricromene may partly be compatible with the persistence of the alternative metabolic pathway, the *de novo* pathway (Renooji & Snyder, 1981), which is unaffected by cloricromene.

It is conceivable that a drug simultaneously inhibiting the synthesis of both leukotrienes and PAF may be advantageous in a series of conditions where the interaction between activated leucocytes and the vessel wall plays a pathogenic role. PAF appears to modulate leucocyte adhesion to, or migration through, the endothelium (Zimmerman *et al.*, 1986) and it is tempting to speculate that the inhibitory activity of cloricromene on PAF-synthesis may in part contribute to the ability of this drug to inhibit some leucocyte functions, especially adhesion to the endothelium (Bertocchi *et al.*, 1989; Tranchina *et al.*, 1994).

It is interesting to note that PAF appears to be involved in tissue ischaemia, shock and in the release of TNF-α (Doebber *et al.*, 1985; Braquet *et al.*, 1987; Page & Spina, 1989; Pleszczynski, 1990) and recent data show that cloricromene exerts a protective effect on tissue ischaemia (Cirillo *et al.*, 1992; Lidbury *et al.*, 1993), shock and release of TNF-α *in vivo* (Squadrito *et al.*, 1991). It is therefore likely that some of the beneficial actions of this drug *in vivo* are related to PAF inhibition. In addition, the suppression of PLA_2 activity can lead to reduced leucocyte activation independent of eicosanoid synthesis (Bomalasky *et al.*, 1989; Abramson *et al.*, 1991). Whether the ability of cloricromene to inhibit PAF synthesis will offer a therapeutic advantage over the presently available antithrombotic agents will only be established by comparative, prospective clinical studies.

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