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CLORICROMENE INHIBITS LEUKOTRIENE FORMATION BY HUMAN POLYMORPHONUCLEAR LEUCOCYTES BY SUPPRESSING ARACHIDONATE RELEASE FROM MEMBRANE PHOSPHOLIPIDS

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Abstract—Cloricromene, an antithrombotic agent known to inhibit the release of arachidonic acid (AA) in stimulated human platelets, was tested for its effects on arachidonate release and metabolism in human polymorphonuclear leucocytes (PMNs). Cloricromene dose-dependently suppressed the release of leukotriene B_4 (LTB_4), as assessed by radioimmunoassay, from both isolated PMNs and human whole blood stimulated with the calcium ionophore A23187 or with serum-treated zymosan (STZ). The inhibitory effect was higher when the concentration of the stimulating agent was weaker. Cloricromene also inhibited dose-dependently the liberation of LTB_4, LTC_4, LTD_4 and 5-hydroxy-6,8,11,14-eicosatetraenoic acid as assessed by HPLC in the supernatant of A23187-stimulated PMNs. Finally, the drug was able to suppress the release of [^{3}H]AA from purified human PMNs prelabelled with the radioactive fatty acid and stimulated with either A23187 or with STZ. The A23187-induced decrease in the radioactivity of phosphatidylinositol, the phospholipid class mainly involved in AA release in stimulated PMNs, was also inhibited by cloricromene. Cloricromene suppresses leukotriene formation in human PMNs by reducing AA release from membrane phospholipids, possibly through interference with phospholipase A_2 activation; this activity may contribute to the leucocyte-inhibitory effects reported previously for cloricromene.

Leukotrienes (LTs§) represent the main product of the oxidation of arachidonic acid (AA) in human polymorphonuclear leucocytes (PMNs). AA is transformed by the enzyme 5-lipoxygenase into 5(*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE); the same enzyme also catalyses the further metabolism of 5-HPETE to LTA_4. 5-HPETE can also be reduced, by a peroxidase, to 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE). The unstable epoxide intermediate LTA_4 is hydrolysed by the enzyme LTA_4-hydrolase into LTB_4, a compound provided with strong proinflammatory activities. Alternatively, LTA_4 can be conjugated to reduced glutathione by the enzyme LTC_4-synthase to give LTC_4, a powerful spasmogenic substance. LTC_4 then undergoes a series of peptide cleavage reactions to give LTD_4 and LTE_4; LTD_4 is a potent vasospastic substance while LTE_4 is less active [1, 2].

The release of AA represents a rate-limiting step for the formation of AA metabolites, including LTs, in PMNs [3–5]. AA, the substrate for the 5-lipoxygenase in PMNs, is thought to be released from phospholipids, predominantly from phosphatidylinositol and phosphatidylcholine, mainly by the action of a phospholipase A_2 (PLA_2) [3–5].

The mechanisms regulating the activity of PLA_2 in human PMNs are not completely understood; however, Ca^{2+} mobilized from the endoplasmic reticulum by inositol-1,4,5-trisphosphate [6] probably plays a major role in the activation of this enzyme [7]. A receptor-coupled G-protein-mediated mechanism may also be involved [8] as well as protein kinase C regulated events [9].

LTs play an important role in the regulation of leucocyte function. LTB_4 is a powerful chemokinetic and chemotactic agent [1, 2] and it increases the adhesion of PMNs to endothelial cells [10]. On the other hand, AA itself can act as an excitatory second messenger in human neutrophils [11] and PLA_2 activation appears to be involved in the release of lysosomal enzymes and in superoxide formation independent of eicosanoid synthesis [12].

Increasing evidence shows that activated PMNs, besides inflammation, participate in thrombotic disorders and in tissue damage occurring during ischemia or shock [13–16].

Cloricromene, a coumarine derivative with antithrombotic and vasodilatory activities in animals [17, 18], has been reported to inhibit some human PMNs functions, including chemotaxis, adhesion

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§ Abbreviations: PMN, polymorphonuclear leucocyte; AA, arachidonic acid; LT, leukotriene; STZ, serum-treated zymosan; PLA_2; phospholipase A_2; 5-HPETE, 5(*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; HBSS, Hank's balanced salt solution; RIA, radioimmunoassay; PGB_2; prostaglandin B_2; RRT, relative retention time; PI, phosphatidyl inositol; PC, phosphatidyl choline.

and superoxide anion generation [19]. This drug prevents the release of AA in human platelets by interfering with the activation of PLA_2 [20]. Here we have assessed whether cloricromene interferes with the production of LTs in stimulated human PMNs and have tried to elucidate its mechanism of action.

MATERIALS AND METHODS

Blood sampling and isolation of PMNs. Heparinized blood (10 U/mL) was collected in plastic tubes by venipuncture from drug-free healthy volunteers. PMNs were isolated as described previously [21] using dextran sedimentation and Hypaque/Ficoll gradients according to Böyun [22]. Contaminating erythrocytes were removed by hypotonic lysis and PMNs were washed twice with Hank's balanced salt solution (HBSS), pH 7.35, without Ca^{2+} and Mg^{2+}, and containing 0.5% bovine serum albumin. PMNs were finally resuspended, at a concentration of 10^7 cells/mL, in HBSS containing Ca^{2+} and Mg^{2+} (0.6 and 0.8 mM, respectively).

These resuspensions contained 95.9 ± 0.6% PMNs and 4.1 ± 0.6% lymphomonocytes (N = 13) and the vitality, measured by Trypan blue, was 96.8 ± 0.2% (N = 18); the platelet to leucocyte ratio was 0.68 ± 0.1 (N = 18).

Stimulation of leukotriene synthesis by whole blood and isolated PMNs. The production of LTB_4 by stimulated whole blood was assessed by a method reported previously [21, 23]. Briefly, 0.5 mL aliquots of heparinized human blood (10 U/mL) were preincubated in a water bath at 37° in plastic tubes (8 × 80 mm) containing microlitre amounts of cloricromene (1–1000 μM, final concentration) or its vehicle for 10 min. The calcium ionophore A23187 (2, 7.5, 25 μM, final concentration) or serum-treated zymosan (STZ) (0.1, 0.5, 3 mg/mL) was then added in microlitre amounts and the samples were incubated for a further 60 min at 37° with gentle shaking. The reaction was stopped by centrifugation (12,000 *g* for 2 min) and the supernatant plasma was stored at −80° for subsequent assay. For the calculation of the results, on every sample used for whole blood stimulation a leucocyte and differential count was carried out.

For isolated leucocytes, 0.4 mL aliquots of isolated PMNs (10^7 cells/mL) were preincubated for 10 min at 37° in the presence of microlitre amounts of cloricromene or its vehicle and then were stimulated with either A23187 (2 and 10 μM, final concentration) or STZ (10 and 25 mg/mL). The reactions were stopped after 10 min by centrifugation at 12,000 *g* for 2 min and the cell-free supernatants were stored at −80° until assayed [21].

The concentrations of A23187 and STZ were selected from the dose–response curves to these stimuli in order to have either a weak or a strong stimulation; different concentrations of A23187 and STZ were employed in whole blood as compared with isolated PMNs because the potency of these stimuli is very different under these two conditions [21].

Measurement of LTB_4 by radioimmunoassay (RIA). Immunoreactive LTB_4 was measured in the supernatant of stimulated whole blood or PMNs by RIA with a highly specific antiserum kindly provided by Dr R. A. Forder (ICI, Pharmaceutical Division, Alderley Park, U.K.) [24] essentially as described previously [21, 23]. With this antiserum the RIA procedure can be reliably carried out directly in plasma [24]; thus, unextracted samples were diluted with 0.04 M phosphate-buffered saline, pH 7.4, containing 0.1% bovine γ-globulin (assay buffer) at three different dilutions, at least. The final volume of the assay mixture was 400 μL and consisted of ~3500 cpm of radiolabelled [^{3}H]LTB_4 (sp. radioact.: 196 Ci/mmol, Amersham International, Amersham, U.K.) and the specific rabbit antiserum diluted 1:3000 in assay buffer, giving final dilutions for the samples ranging from 1:4 to 1:800. Fifty percent displacement for unlabelled LTB_4 (IC_{50}) was at 1492 ± 135 pg/mL (N = 7) and the limit of detection (2 SD from zero) was 400 pg/mL. The slightly lower sensitivity of the assay in our hands as compared with the original description [24] is probably due to differences in the source materials (standard or labelled LTB_4 etc.). Cross-reactivities of the antiserum have been reported [24].

Analysis of LTs by HPLC in the supernatant of stimulated PMNs. Supernatants of stimulated PMNs were extracted as described [25]. Briefly, samples were made up to 20 mL with distilled water and the pH was adjusted to 8 with NH_4OH. Sep-Pack C18 cartridges (Waters Associates, Milford, MA, U.S.A.) were first washed with 20 mL ethanol and 20 mL H_2O and then the sample (20 mL) was loaded; 10 mL ethanol: H_2O (10:90 v/v) and 5 mL methanol were used sequentially for elution. The methanol fraction was dried under vacuum in a Hetovac vacuum concentrator (Heto Lab Equipment A/S, Birkerod, Denmark) and resuspended in 200 μM of methanol: H_2O (60:35 by vol.) containing 0.06% acetic acid, pH 5.9 (HPLC eluting solvent).

The HPLC system consisted of a Perkin–Elmer 3B equipped with a Rheodine 7125 injector with a 100 μL sample loop and a Perkin–Elmer model LC85 UV absorbance detector; as an integrator a Gold Software System (Beckman Instruments, San Ramon, U.S.A.) was adopted. The column was a C8 with particle size of 10 μm and dimensions of 200 × 25 mm (Perkin–Elmer, Norwack, U.S.A.). The mobile phase (HPLC eluting solvent) was run at a flow rate of 1 mL/min in an isocratic system. A known amount of prostaglandin B_2 (PGB_2), as internal standard, was added to each sample before the extraction and the elution of LTB_4, LTC_4, LTD_4 and PGB_2 was monitored at A_{280} nm; after 35 min the wave-length was shifted to A_{235} nm for the detection of 5-HETE and the elution followed for an additional 75 min. The quantitation was done by comparing the peak areas of various metabolites with that of the internal standard PGB_2, essentially as reported previously [25]. Detection limits were: 0.9, 1.2, 1.0 and 0.96 ng/10^6 PMNs for LTD_4, LTC_4, LTB_4 and 5-HETE, respectively.

Identification of immunoreactive material released by stimulated whole blood. Immunoreactive material released by stimulated whole blood was identified by HPLC analysis coupled to UV detection (see above) in samples stimulated by A23187 25 μM.

Plasma (0.4 mL) was extracted with 0.75 vol. acetonitrile, passed on a C18 Sep-Pak (using acetonitrile:H_2O, 70:30, as eluting solvent), resuspended into methanol:H_2O (60:35 by vol.) containing 0.06% acetic acid, pH 5.9, and injected for resolution in reversed phase HPLC. Fractions (1 mL) were collected, evaporated to dryness in a Hetovac Concentrator and the residues dissolved in assay buffer. LTB_4 immunoreactive material was assayed by RIA as described.

In addition, plasma samples with [^{3}H]LTB_4 (~6000 cpm) added were extracted, purified and subjected to HPLC separation as described above; the fractions collected were assessed by liquid scintillation counting for identifying LTB_4 elution.

[^{3}H]*AA release in stimulated PMNs.* Isolated purified PMNs, resuspended at 0.4×10^7 cells/mL in HBSS with 0.6 mM Ca^{2+}, 0.8 mM Mg^{2+} and bovine serum albumin 0.35%, were incubated with [^{3}H]AA (0.5 μCi/mL) for 60 min at 37° [26]. Labelling was stopped by centrifugation (1000 *g* for 15 min at 4°) and the pellet was washed twice with HBSS with Ca^{2+}, Mg^{2+}, and finally resuspended in HBSS buffer at 2×10^7 cells/mL.

Aliquots of [^{3}H]AA labelled neutrophil suspensions (0.3 mL) were preincubated with cloricromene or its vehicle for 10 min at 37° and then stimulated with increasing concentrations of Ca^{2+} ionophore A23187 or with STZ for 10 min. The reaction was stopped by adding 90 μL EDTA (0.1 M) and the samples were extracted according to Bills *et al.* [27] using 4.8 vol. chloroform:methanol (1:2).

The separation of free AA by TLC was performed using silica-gel 60A multistratified plates (Whatman International, Maidstone, U.K.) and petroleum ether:diethylether:acetic acid (70:30:1) as the solvent system [28]. Bands, identified by cochromatography with authentic standards, were scraped off the plate and AA radioactivity was measured by liquid scintillation counting (Emulsifier Safe, Packard Instrument B.V., Groningen, The Netherlands). Each sample was analysed in duplicate. Before TLC, 20 μL aliquots were counted directly for calculation of total lipid radioactivity. Individual phospholipids were separated by bidimensional TLC, lipids were visualized by exposure to iodine vapours and then scraped off the plates into counting vials [28]. Finally, in some selected experiments 5-lipoxygenase-labelled products were detected by HPLC in supernatants

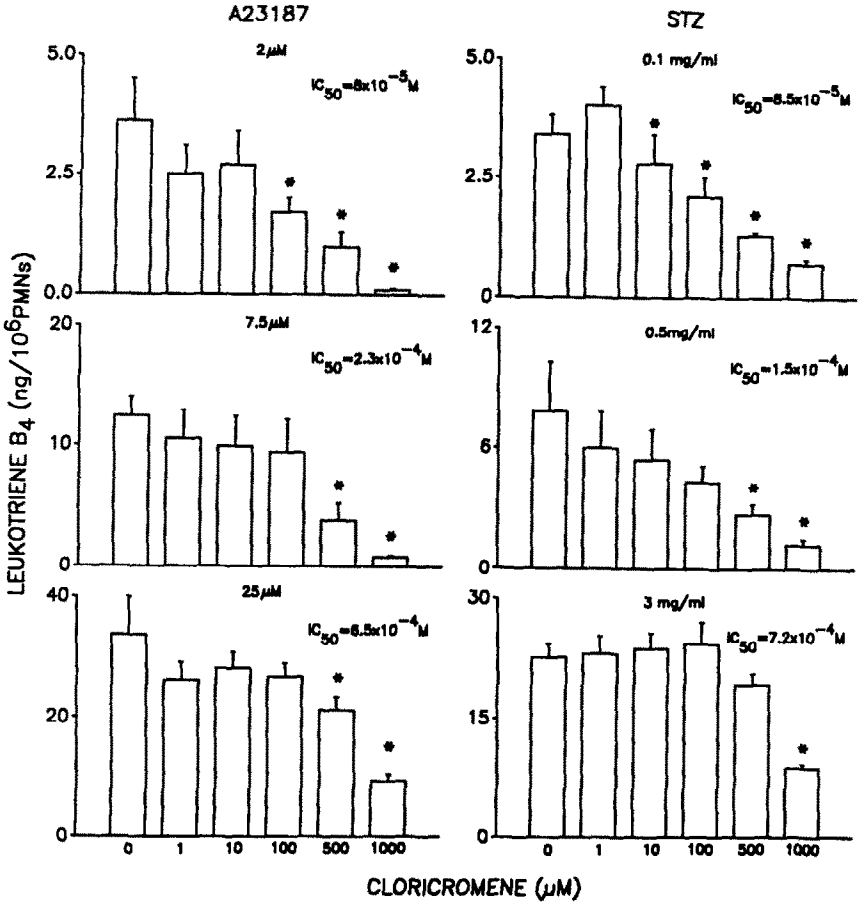

Fig. 1. Effect of cloricromene on LTB_4 production by human whole blood stimulated with different concentrations of A23187 or STZ. Data represent means ± SEM of four experiments, at least. Asterisks indicate a statistically significant difference as compared with control ($P < 0.05$, at least).

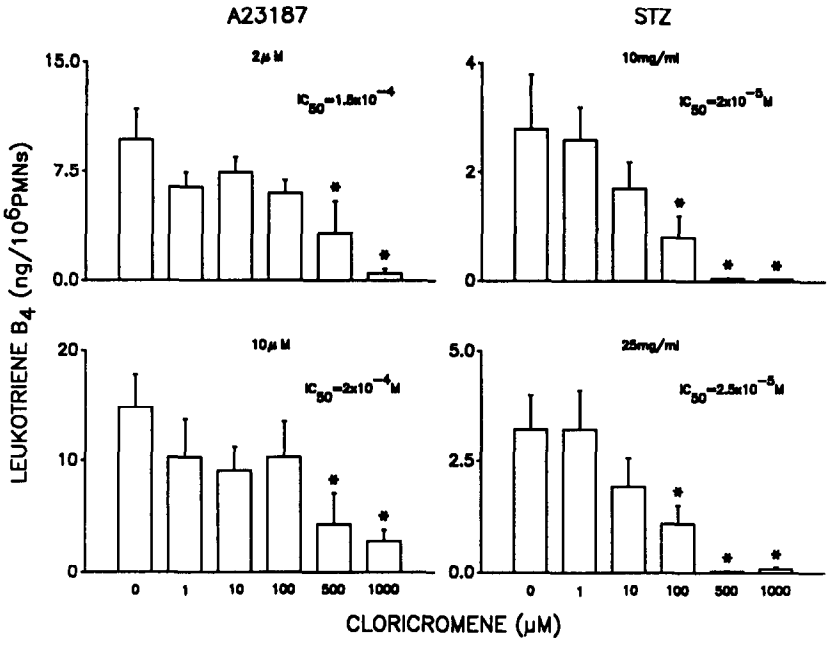

Fig. 2. Effect of cloricromene on LTB_4 production by isolated purified human PMNs stimulated with different concentrations of A23187 or STZ. Data represent means ± SEM of four experiments, at least. Asterisks indicate a statistically significant difference as compared with control ($P < 0.05$, at least).

of [^{3}H]AA-prelabelled PMNs stimulated with A23187.

Drugs and chemicals. Cloricromene HCL (AD6 or 8-monochloro-3-β-diethylaminoethyl-4-methyl-7-ethoxy carbonyl methoxy coumarin) (FIDIA Research Laboratories, Abano Terme, Italy) was dissolved in saline. A23187, Zymosan A, bovine serum albumin, LTB_4, LTC_4, LTD_4, 5-HETE and PGB_2 standards were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Lymphoprep was from Nycomed AS (Oslo, Norway); Dextran T500 from Pharmacia (Uppsala, Sweden); HBSS from Biochron KG (Berlin, Germany); [^{3}H]AA (sp. radioact.: 240 Ci/mmol) and [^{3}H]LTB_4 (sp. radioact.: 196 Ci/mmol) from NEN Dupont (Boston, MA, U.S.A.). All HPLC solvents, of the highest grade available, were from Carlo Erba (Milano, Italy).

Statistical analysis. All results are reported as means ± SEM. The statistical evaluation of the data was performed by one-way ANOVA followed by Shaffe's multiple comparison test. The correlation between various parameters was assessed by linear regression analysis.

RESULTS

Effects of cloricromene on LTB_4 production

The stimulation of heparinized human whole blood with increasing concentrations of A23187 (2, 7.5, 25 μM) or STZ (0.1, 0.5, 3 mg/mL) induced dose-dependent production of LTB_4 (Fig. 1).

The preincubation with cloricromene inhibited LTB_4 production in a dose-dependent fashion, both in A23187- and STZ-stimulated whole blood. The inhibitory effect of cloricromene appeared to be more potent when lower doses of both inducers were employed. Indeed, the IC_{50} of cloricromene for LTB_4 formation rose with the increase in strength of the stimulus used (Fig. 1). Of course, the absolute reduction in the total mass of LTB_4 secreted, under maximal suppression with cloricromene (1000 μM), was larger under conditions of strong stimulation than with a weaker stimulus (e.g. from 33.6 ± 6 to 9.4 ± 1.1 ng, i.e. a reduction of 24.2 ng of LTB_4 with A23187 25 μM, and from 3.6 ± 0.9 to 0.13 ± 0.08 ng, i.e. a reduction of 3.47 ng with A23187 2 μM).

A statistically significant inverse correlation was found between the amount of LTB_4 released and cloricromene concentration in whole blood samples stimulated with A23187 2, 7.5 and 25 μM ($r = -0.59$, $N = 36$, $P < 0.001$; $r = -0.50$, $N = 24$, $P < 0.05$ and $r = -0.60$, $N = 52$, $P < 0.001$, respectively) or with STZ 0.1, 0.5 and 3 mg/mL ($r = -0.53$, $N = 30$, $P < 0.01$; $r = -0.52$, $N = 30$, $P < 0.01$ and $r = -0.77$, $N = 35$, $P < 0.001$, respectively).

To validate the results obtained by RIA in whole blood, supernatants of A23187 (25 μM)-stimulated samples were separated by HPLC and RIA was performed on 1 mL fractions: the results showed that most of the immunoreactive material (88.8%) coeluted with LTB_4-standard. Minor peaks of cross-reactivity were observed in the fractions eluting between 25 and 29 min, i.e. at a retention time relative to PGB_2 (RRT) of 1.33–1.48 (8.5%), a fraction probably containing LTB_4 isomers [25], and between 88 and 90 min, i.e. at a RRT of 4.9–5.0 (0.3%). The recovery of [^{3}H]LTB_4 added to plasma samples after extraction and HPLC was 75.5 ± 0.7%;

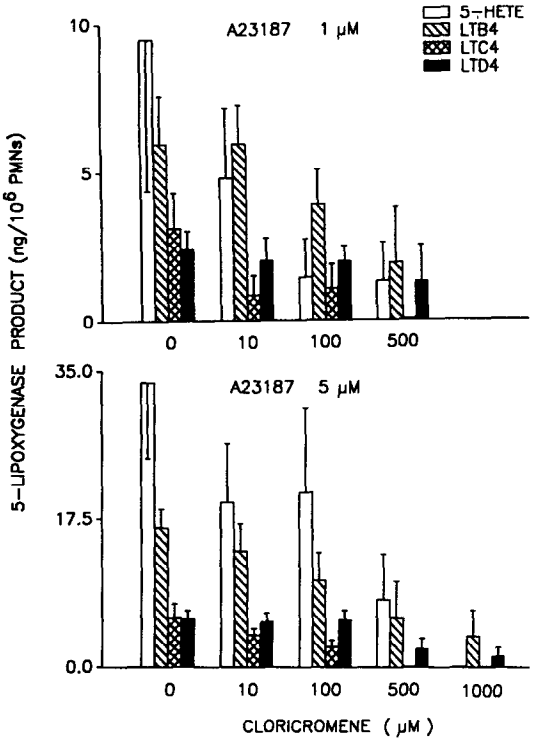

Fig. 3. Formation of 5-lipoxygenase products in A23187-stimulated human PMNs as assessed by HPLC. Cloricromene inhibits dose dependently the formation of all the products of the metabolic cascade. Columns represent means ± SEM of five experiments, at least.

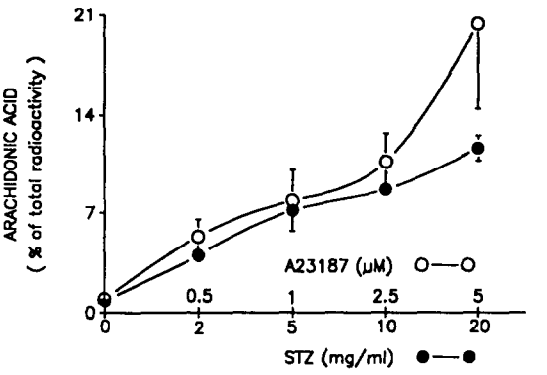

Fig. 4. Release of tritiated AA from prelabelled human PMNs stimulated with increasing doses of A23187 or STZ. Each point represents the mean ± SEM of six experiments.

a single peak of radioactivity was observed eluting at a RRT (1.63) similar to that of standard unlabelled LTB_4 (1.58) (data not shown).

In isolated purified human PMNs, both A23187 and STZ induced a dose-dependent production of LTB_4 (Fig. 2); STZ was a much weaker stimulus for LTB_4 synthesis under these experimental conditions, as already reported [21, 26]. Cloricromene, preincubated with isolated PMNs for 10 min, produced a dose-dependent inhibition of the release of LTB_4 induced by either stimuli. However, the inhibitory activity was particularly evident when STZ was used as an inducer ($IC_{50} = 2$ and 2.5×10^{-5} M against stimulation with STZ 10 and 25 mg/mL, respectively); higher concentrations of cloricromene were required upon stimulation with A23187 ($IC_{50} = 1.5$ and 2×10^{-4} M against stimulation with A23187 2 and 10 μM, respectively) (Fig. 2).

A statistically significant inverse correlation was present between the amounts of LTB_4 released by PMNs stimulated with either A23187 2 and 10 μM ($r = -0.64$, $N = 33$, $P < 0.001$ and $r = -0.54$, $N = 28$, $P < 0.01$) or with STZ 10 and 25 mg/mL ($r = -0.59$; $N = 24$; $P < 0.01$ and $r = -0.62$, $N = 23$, $P < 0.01$) and cloricromene concentration.

Effect of cloricromene on the formation of 5-lipoxygenase products in stimulated PMNs

LTB_4, LTC_4, LTD_4 and 5-HETE levels in the supernatant of A23187-stimulated PMNs were measured by HPLC. The supernatants of resting, unstimulated PMNs did not contain detectable amounts of LTs and hydroxyacids. The addition of A23187 to the cells induced the release of LTs and 5-HETE detected as peaks with an elution time and UV absorbance profile similar to those of authentic LT standards. Increasing the A23187 concentration from 1 to 5 μM led to a rise in all 5-lipoxygenase products, in particular LTB_4 and 5-HETE (Fig. 3). Preincubation of isolated PMNs with cloricromene caused a dose-dependent inhibition of the production of all the identified 5-lipoxygenase products; the inhibitory effect was weaker when the stimulus used was stronger (e.g. IC_{50} for 5-HETE = 3.5×10^{-5} M vs A23187 1 μM and 1.2×10^{-4} M vs A23187 5 μM) (Fig. 3).

Preincubation of PMNs with cloricromene (500 μM) did not affect their vitality (93 ± 0.4%; N = 6).

Uptake and release of [^{3}H]AA in isolated PMNs

The cellular uptake of [^{3}H]AA was 29.03 ± 3.14% (N = 9) after 60 min of incubation. The effect of stimulation with A23187 or STZ on [^{3}H]AA release is shown in Fig. 4. Incubation of human PMNs with increasing concentrations of A23187 (0.5–5 μM) caused a dose-dependent release of [^{3}H]AA. Maximal release was obtained with 5 μM A23187 (20.5 ± 6.0% of total radioactivity) (Fig. 4). STZ (2–20 mg/mL) dose-dependently increased the release of [^{3}H]AA. Maximal liberation of free [^{3}H]AA attained (11.7 ± 0.9% of total radioactivity) was lower than the maximal release provoked by A23187 (Fig. 4). Preincubation of neutrophils with cloricromene (10–500 μM) dose-dependently reduced the release of [^{3}H]AA induced by A23187 or STZ. Cloricromene appeared to be more effective against STZ stimulation ($IC_{50} = 1.3 \times 10^{-4}$ M) than against A23187 ($IC_{50} = 3.2 \times 10^{-4}$ M) (Fig. 5). By comparison, mepacrine (500 μM), an inhibitor of PLA_2 [29], caused inhibition of 75.3 ± 0.9% and 85.7 ± 3.9% in A23187- and STZ-stimulated neutrophils, respectively (data not shown).

The stimulation of [^{3}H]AA-labelled leucocytes with A23187 caused a loss of radioactivity primarily

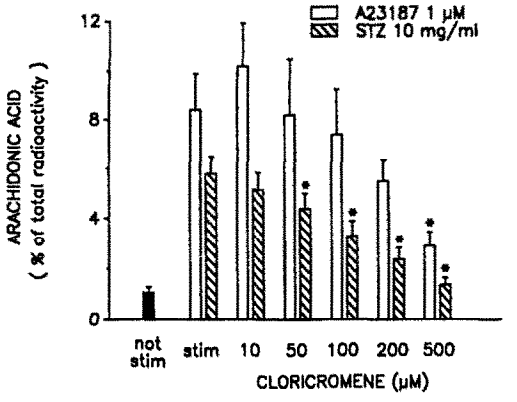

Fig. 5. Effect of cloricromene on the release of tritiated AA in prelabelled human PMNs stimulated with A23187 or STZ. Data represent means ± SEM of six experiments, at least. Asterisks indicate a statistically significant difference as compared with control ($P < 0.05$, at least).

from phosphatidyl inositol (PI) and, to a lesser extent, from phosphatidyl choline (PC), in agreement with previous reports [3, 26]. Cloricromene reduced in parallel the release of arachidonate and the loss of radioactivity from PI without modifying the PC changes (Table 1).

A statistically significant inverse correlation was evident between the concentration of cloricromene and the amounts of [^{3}H]AA released by prelabelled PMNs stimulated with either A23187 1 μM ($r = -0.54$; $N = 35$; $P < 0.001$) or with STZ 10 mg/mL ($r = -0.70$; $N = 35$; $P < 0.0005$). In addition, a statistically significant direct correlation was present between the amounts of [^{3}H]AA released by prelabelled PMNs, in the presence and absence of cloricromene, and the amounts of LTB_4 measured by RIA, in leucocytes stimulated with STZ 10 mg/mL ($r = 0.54$; $N = 16$; $P < 0.05$).

The supernatant of [^{3}H]AA-labelled PMNs stimulated with A23187 1 μM was analysed by HPLC. The major peak of radioactivity (0.98% of total lipid radioactivity) was observed at 9 min (peak I, RRT = 0.49), while smaller peaks were evident at 24 min (peak II, RRT = 1.58, 0.52% of total lipid radioactivity) and at 78 min (peak III, RRT = 5, 0.41% of total lipid radioactivity). Based on the relative retention times peak II and III were identified as LTB_4 and 5-HETE, respectively. Peak I contained prostanoids and thromboxane B_2 based on their relative retention times. Preincubation with cloricromene (100 μM) reduced the radioactivity of peak I (−49%), peak II (−27%) and peak III (−64%) (data not shown). However, when [^{3}H]AA-labelled PMNs were stimulated with STZ 10 mg/mL and the supernatant subjected to HPLC, only a minimal amount of radioactivity was recovered with the solvent eluted at the relative retention times of LTB_4, 5-HETE and prostanoids (0.37% of total lipid radioactivity, cumulatively). This indicates that with this stimulus most of the released AA was left unmetabolized (data not shown).

DISCUSSION

Our data demonstrate that cloricromene inhibits LT production by stimulated human PMNs. A dose-dependent inhibitory activity of cloricromene on the synthesis of LTB_4 was evident using isolated, purified PMNs as well as stimulated whole blood. This finding suggests that plasma protein binding or cellular uptake of cloricromene [30] does not affect its action and shows that the drug is active on PMNs suspended in a physiological environment. Indeed, the use of stimulated whole blood for the measurement of drug effects on LT synthesis is a simple and effective system [21, 23, 24, 31] and allows the detection of occasional discrepancies in the inhibitory activity of drugs when tested in a purified system as compared with complex systems [32].

Separation by HPLC of AA metabolites in stimulated human PMNs demonstrated that cloricromene was able to suppress the formation of both LTB_4 and cysteinyl LTs as well as the formation of 5-HETE. Thus, cloricromene acts at an early stage of the AA/5-lipoxygenase metabolic cascade, possibly at the level of the enzyme 5-lipoxygenase

Table 1. Modifications induced by stimulation of [^{3}H]AA-labelled PMNs with A23187 in the radioactivity associated with AA and with various phospholipid fractions, and effects of the preincubation with cloricromene

	AA	PA	PI	PC	PE	PS	CP	EP
Resting	2.2 ± 0.1	4.2 ± 0.6	70.1 ± 2.0	24.6 ± 2.3	6.4 ± 0.5	3.2 ± 0.2	2.9 ± 0.2	14.0 ± 1.5
A23187 (1 μM)	25.6 ± 1.4*	3.8 ± 0.3	37.8 ± 4.4 *	17.2 ± 2.5*	4.9 ± 2.1	2.7 ± 0.2	2.7 ± 0.3	13.5 ± 2.2
A23187 (1 μM) + cloricromene (200 μM)	12.9 ± 1.4*†	4.6 ± 0.7	52.2 ± 5.4*†	17.0 ± 1.5*	4.6 ± 0.4	2.8 ± 0.2	2.5 ± 0.21	13.7 ± 1.7

Data represent means ± SEM of 12 samples from four different experiments; nCi/10^7 PMNs.
* Indicates a significant difference as compared with the resting value ($P < 0.02$, at least).
† Indicates a significant difference as compared with A23187 1 μM-stimulated ($P < 0.02$, at least).
Abbreviations: PA = phosphatidic acid; PE = phosphatidyl ethanolamine; PS = phosphatidyl serine; CP = choline plasmalogens; EP = ethanolamine plasmalogens.

itself or at the level of the release of the substrate AA. In order to define the level of the action of cloricromene we studied the release of [^{3}H]AA from membrane phospholipids in prelabelled PMNs. Cloricromene was able to reduce dose-dependently [^{3}H]AA release induced by both the calcium ionophore A23187 and by STZ. This suggests that the inhibitory effect of cloricromene is exerted at the level of the release of AA from membrane phospholipids. Indeed, cloricromene was able to reduce the loss of radioactivity from PI and it has been reported previously that the bulk of AA in human neutrophils comes from PI [3, 26]. PI is degraded principally by a PLA_2 in human leucocytes [3, 5]; thus, this enzyme may be the target of cloricromene. It remains to be established whether this inhibition is the result of a direct suppression of the enzyme or if it is the consequence of an interference with the mechanisms regulating the activation of PLA_2, as happens in platelets [20]. Under all the experimental conditions adopted the inhibitory effect of cloricromene appeared to be inversely related to the strength of the stimulus; in addition, its activity was higher against STZ stimulation. It has been reported that the inhibitory effect of cloricromene on platelet aggregation is reduced when the strength of the stimulus employed is increased [33]. Moreover, while the agonist A23187 induces LT formation by transporting extracellular Ca^{2+} into the cell, thus massively activating PLA_2 and 5-lipoxygenase [34], STZ interacts with the C3bi receptor on the neutrophil surface thus activating in a more physiological manner arachidonate release and metabolism [35]. The greater inhibition exerted by cloricromene on STZ-induced LT production suggests that the drug interferes with receptor-linked mechanisms regulating PLA_2 activation rather than with PLA_2 itself.

The preferential inhibition by cloricromene of AA release and metabolism induced in PMNs by STZ may modify metabolic interactions between cells: upon STZ stimulation a large amount of the AA released remains unmetabolized and may serve as a substrate for the cyclooxygenase or lipoxygenase enzymes of other cells [26]; in this regard, it is interesting to note that cloricromene is especially active in suppressing platelet function in mixtures of leucocytes and platelets [36].

The suggestion that cloricromene interferes with PLA_2 activity in human PMNs appears to be further strengthened by preliminary findings indicating its ability to inhibit the synthesis of platelet-activating factor [37], in which the enzyme PLA_2 is involved [38].

Possibly the inhibitory effect of cloricromene on some leucocyte functions [19] could be due to its suppressive action on LT synthesis and/or PLA_2 activation. Leucocytes participate in the processes leading to thrombogenesis [13–16] and drugs inhibiting LT synthesis have been found to exert an antithrombotic activity in some animal models [15, 39]. It is tempting to speculate that some of the antithrombotic and vasodilatory activities of cloricromene [17, 18] may be the consequence of its effect on LT synthesis by PMNs. Indeed, the concentrations of cloricromene that we showed to inhibit LT synthesis *in vitro* were, under the most favourable conditions, in the range of those found in dog plasma after the administration of 0.5 mg/kg of the drug intravenously [17, 19]. Moreover, suppression of AA release and of PLA_2 activity can lead to reduced leucocyte activation independent of eicosanoid synthesis [11, 12]. The protective effects of cloricromene in some animal shock models [40] might also be the consequence of the suppression of leucocyte PLA_2 activity [41].

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