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Modulation of leukotriene release from human polymorphonuclear leucocytes by PMA and arachidonic acid

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SUMMARY

Stimulation of human neutrophils (PMN) with Ca ionophore A23187, opsonized zymosan and formyl-L-methionyl-L-leucyl-phenylalanine (FMLP) led to a time- and dose-dependent release of LTB_4, 20-OH-LTB_4, 20-COOH-LTB_4, 6-trans-LTB_4, 12-epi-6-trans LTB_4 and LTC_4, as detected by reverse-phase HPLC. Preincubation of the PMN suspension in the presence of Ca^{2+} and Mg^{2+} with phorbol-12-myristate-13-acetate (PMA) did not release leukotrienes by itself, but modulated the subsequent Ca ionophore-induced leukotriene release. The release of LTC_4, 20-OH-LTB_4 and 20-COOH-LTB_4 was significantly decreased. Lesser effects were observed for the release of LTB_4 and the non-enzymatic LTB_4 isomers. In contrast, opsonized zymosan and FMLP enhanced the release of LTB_4 and LTB_4-ω-oxidation products from cells pretreated with PMA. With arachidonic acid as prestimulus, the amounts of the LTB_4 isomers (6-trans-LTB_4 and 12-epi-6-trans-LTB_4) were enhanced significantly on subsequent stimulation with Ca ionophore. Prestimulation of lymphocytes, monocytes and basophilic granulocytes (LMB) with PMA had no significant effects on the ionophore-induced release of LTC_4 and LTB_4. PMN, but not LMB, suspensions prestimulated with PMA convert exogenously added LTC_4 to LTB_4 isomers and LTC_4 sulphoxide. Our data suggest that preincubation of human granulocytes with PMA modifies leukotriene release by activation or inhibition of different metabolic pathways for LTC_4 and LTB_4.

INTRODUCTION

The leukotrienes are lipoxygenase-derived arachidonic acid metabolites which are considered to be mediators of inflammation and allergy due to their pronounced biological activities (Bray, 1986). The biosynthesis of leukotrienes is initiated by oxygenation of arachidonic acid at the C-5 position via 5-lipoxgenase (Borgeat, Hamberg & Samuelsson, 1979), leading to 5-hydroperoxyeicosatetraenoic acid (5-HPETE). Subsequent reaction leads to the intermediate leukotriene A_4. Neutrophils with the enzyme leukotriene A_4 hydrolase catalyse the hydrolysis of leukotriene A_4 to the chemotactic and chemokinetic active dihydroxy acid leukotriene B_4 (Rådmark *et al.*, 1980, 1984). The hydrolysis can also occur non-enzymatically and then leads to the formation of two LTB_4 isomers 5 (S) 12 (R)-6-trans-LTB_4 and 5 (S) 12 (S)-6-trans-LTB_4 (Borgeat & Samuelsson, 1979). In human polymorphonuclear leucocytes, LTB_4 and its isomers are metabolized by omega oxidation to 20-OH-LTB_4 and 20-COOH-LTB_4 (Powell, 1984; Shak & Goldstein, 1984; Brom *et al.*, 1987). Alternatively, LTA_4 can conjugate with glutathione by a glutathione-S-transferase to produce leukotriene C_4. A step-by-step elimination of the amino acid residues by a γ-glutamyl transpeptidase and LTD_4 dipeptidase generates the leukotriene D_4 and E_4, respectively. In addition, the inactivation of peptidoleukotrienes LTC_4 and LTD_4 to LTB_4 isomers and the corresponding sulphoxides by hypochlorous acid (HOCl) is a result of the interaction of myeloperoxidase, hydrogen peroxidase derived from the respiratory burst and chloride ion (Lee *et al.*, 1983; Henderson & Klebanoff, 1983; Raulf, Stüning & König, 1986).

Granulocytes are capable of synthesizing as well as inactivating leukotrienes, thus interest has been focused on the biochemical mechanisms responsible for the induction and metabolism of leukotrienes. Synthesis of leukotrienes occurs as a result of arachidonic acid release from membrane phospholipids mediated by extracellular signals via receptor-linked transducing mechanisms. The key reaction is the hydrolysis of

Abbreviations: EDTA, ethylendiamine tetraacetate; 12-epi-6-trans-LTB_4, 5 (S), 12 (S)-dihydroxy-6, 8, 10, 14-(EEEZ)-eicosatetraenoic acid; FMLP, formyl-methionyl-leucyl-phenylalanine; LMB, lymphocytes, monocytes and basophils; LT, leukotriene; PBS, phosphate-buffered saline; PDBU, phorbol-12, 13-dibutyrate; PMA, phorbol-12-myristate-13-acetate; PMN, polymorphonuclear neutrophil leucocyte; RP-HPLC, reverse-phase high-performance liquid chromatography; 6-trans-LTB_4, 5 (S), 12 (R)-dihydroxy-6, 8, 10, 14-(EEEZ)-eicosatetraenoic acid.

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the phosphatidylinositol, 4, 5-bisphosphate into the two second messenger molecules, diacylglycerol (DAG) and inositol trisphosphate (IP_3) (Berridge & Irvine, 1984; Berridge, 1984; O'Flaherty, 1987). IP_3 is released into the cytoplasm and mobilizes calcium from the endoplasmic reticulum, the intracellular calcium stores. DAG activates the protein kinase *C*, a calcium- and phospholipid-dependent enzyme (Nishizuka, 1984). Activation of protein kinase *C* by DAG may be substituted by tumour-promoting phorbol esters, if intercalated into the cell membrane. The tumour-promoting phorbol esters bypass the phospholipase C step and directly activate protein kinase *C* without the release of calcium-mobilizing IP_3. Protein kinase *C* stimulation and increases in intracellular calcium can be obtained separately in cells by exposing them to activators of the kinase (e.g. PMA, mezerine) and ionophores (e.g. ionomycin, A23187), respectively (Badwey & Karnovsky, 1986).

It was the purpose of the present investigation to analyse cells prestimulated with PMA and arachidonic acid with regard to the leukotriene release induced by various stimuli (Ca ionophore A23187, opsonized zymosan and FMLP). Evidence is presented that modulation of leukotriene induction and metabolism by activation of protein kinase *C* is dependent on the potency of the subsequent stimulus to initiate the arachidonic acid cascade.

MATERIALS AND METHODS

Material

Reagents used were from the following sources: Ficoll 400 was obtained from Pharmacia, Uppsala, Sweden; Macrodex 6% (w/v) was from Knoll, Ludwigshafen; sodium metrizoate solution 75% (w/v) from Nyegaard, Oslo, Norway; zymosan A, Ca ionophore, heparin, FMLP, PMA, phorbol-12, 13-dibutyrate (PDBU) and arachidonic acid were obtained from Sigma Chemical Co., Deisenhofen. [^{3}H]PDBU (specific activity 370–840 GBq/mmol) was from New England Nuclear, Dreieich. Synthetic leukotrienes C_4, D_4, E_4, B_4, 20-OH-LTB_4 and 20-COOH-LTB_4 were a generous gift from Dr J. Rokach (Merck, Frosst Pointe-Claire, Québec, Canada) and 6-trans-LTB_4 and 12-epi-6-trans-LTB_4 were from Dr P. Borgeat (L'Université Laval, Québec, Canada).

Buffer

The buffer used throughout all experiments consisted of 0·137 M NaCl, 0·008 M Na_2HPO_4, 0·0027 M KH_2PO_4 and 0·0027 M KCl, pH 7·4 (modified Dulbecco's PBS; referred to as PBS).

Preparation of the cells

Human polymorphonuclear leucocytes (PMNs) were obtained from 200 ml of heparinized blood (15 U/ml) of healthy donors separated on a Ficoll–metrizoate gradient followed by dextran-sedimentation (Böyum, 1968). In order to lyse the erythrocytes, the cells were resuspended in 0·85% NH_4Cl in 0·015 M Tris/HCl, pH 7·4, for 5 min and washed twice with PBS at low speed (300 *g*, 4°, 10 min; Heraeus Christ, Cryofuge 6–4, Osterode). This method led to more than 95% pure and intact PMNs. The cell fraction containing lymphocytes (88–93%), monocytes (7–12%) and basophilic granulocytes (1–2%) (called LMB) was also obtained by Ficoll–metrizoate separation of the blood. The PMN and LMB were resuspended to a final concentration of 2×10^7 cells/ml in PBS (unless stated otherwise).

Preparation of opsonized zymosan

Zymosan A (2 mg/experimental tube) was suspended in PBS (2 ml) and boiled for 5 min. PBS was added and the suspension centrifuged for 10 min at 4° at 1900 *g* (Heraeus Christ, Cryofuge 6–4). The pellet was washed twice with PBS, resuspended in human serum (10 mg/ml human serum) and incubated for a further 30 min at 37°. After centrifugation the pellet was washed with PBS and resuspended to a final concentration of 10 mg opsonized zymosan/ml in PBS.

Stimulation of PMN and LMB

PMNs (1×10^7/500 µl) were incubated in the presence of calcium (0·8 mM) and magnesium (0·8 mM) (unless stated otherwise) for 5 min, or for various times as indicated, at 37° with different concentrations of PMA or the same volume of PBS (50 µl). After preincubation, the stimulus (Ca ionophore A23187, opsonized zymosan or FMLP) or the same volume of PBS was added to the cell suspension, and incubation proceeded for an additional 10 min (unless stated otherwise). The reaction was terminated by centrifugation for 5 seconds at 9700 *g* (Eppendorf centrifuge 3200, Hamburg).

The same procedure was used for prestimulation with arachidonic acid (different concentrations, diluted in PBS). Samples receiving PBS instead of PMA or arachidonic acid served as controls in order to determine the modulatory effects of PMA or arachidonic acid for the subsequent stimulation. In several experiments cells were prestimulated for 5 min with PMA and incubation was terminated by centrifugation (5 seconds, 9700 *g*). Subsequently the cell pellets were resuspended, washed with PBS and stimulated with the Ca ionophore, opsonized zymosan or FMLP. This procedure did not affect the modulatory influence of PMA with regard to leukotriene release. In addition to PMNs the LMB cell fraction was prestimulated with PMA under the same conditions and treated as is described above.

The release of lactate dehydrogenase (LDH), as a marker enzyme for cell viability, was determined as previously described (König, Frickhofen & Tesch, 1978). The extracellular release of LDH was calculated as percentage of the total enzyme activity available after sonication of unstimulated cells (1×10^7/500 µl).

Metabolism of exogenously added LTC_4

PMNs or LMBs (1×10^7, 500 µl) were preincubated with different concentrations of PMA or PBS in the presence of calcium (0·8 mM) and magnesium (0·8 mM) for 5 min at 37°. After preincubation, LTC_4 (100 ng in 50 µl buffer) or the same volume of PBS without leukotriene was added to the cell suspension, and the incubation proceeded for an additional 15 min. Samples receiving PBS instead of LTC_4 served as control. The reaction was stopped by addition of 3 ml methanol : acetonitrile (50 : 50, v/v) and extracted as described above.

Analysis of leukotrienes

RP-HPLC. For analysis of leukotriene release the cell-free supernatants were deproteinized by addition of 2 ml of methanol/acetonitrile (50 : 50, v/v), overlayered with argon and frozen at −70° for 12 hr. After centrifugation at 1900 *g* for 10 min at 4°, the supernatants were evaporated to dryness by lyophilization (EF 4 Modulyo, Edwards-Kniese, Marburg.) The residues were dissolved in 600 µl of methanol/water (30 : 70, v/v), covered with

argon and left at −20° for 2 hr. Centrifugation was performed at 9700 *g* for 2 min at room temperature (Eppendorf Centrifuge 3200); the supernatants were then applied to HPLC. HPLC analysis was performed on reverse-phase columns (4·6 × 250 mm, packed with Nucleosil C18, 5 μm particles, Machery Nagel, Düren) with methanol:water:acetonitrile:phosphoric acid (59:33:8:0·03, adjusted to pH 5·1 with ammonia; the aqueous phase contained 0·05% EDTA) as effluent with a flow rate of 1 ml/min. The absorbance of the column effluent was monitored using a variable ultraviolet detector (LDC-Milton Roy/-Spectromonitor D) adjusted to 280 nm. The peak areas were integrated and calculated using the programme of Nelson Analytical Chromatography Software, AS-Analysensystem, Wuppertal. The recorded HPLC peaks were identified by calculating their α-factors as has been described previously (Köller *et al.*, 1985). For analysis of leukotriene release, LTB_4 served as a reference substance to calculate the α-factors of the other substances. The calculated α-factors were : 0·15–0·17 for 20-COOH LTB_4, 0·19–0·21 for 20-OH LTB_4, 0·65–0·67 for LTC_4, 0·77–0·79 for 6-trans-LTB_4 and 0·88–0·90 for 12-epi-6-trans-LTB_4 and LTD_4. The applied conditions were not suitable for the separation of LTB_4 and 5S, 12S-di-HETE. In some cases, with samples from different donors, it was determined that the contamination of LTB_4 with the double lipoxygenase product 5S, 12S,-di-HETE never exceeded 10–15% (data not presented). The quantification of identified leukotrienes was performed by area integration of the absorption peaks. With the extraction procedure, the recovery rates of leukotrienes from the cell suspension were 75–85% for the peptido-leukotrienes and 90–95% for LTB_4.

Phorbol 12, 13-dibutyrate-binding assay

Binding of [^{3}H]phorbol 12, 13-dibutyrate ([^{3}H]PDBU) to PMN was performed by a modification of the method described by Sando *et al.* (1981) for human lymphocytes.

Binding studies were carried out at 4°, in a total volume of 0·3 ml containing 200 μl stimulated or non-stimulated PMN (2×10^6), [^{3}H]PDBU (40 nM final concentration) and non-labelled 3 μM PDBU (50 μl) or the same volume of buffer for 1 hr, in 96-well filtration plates (containing Durapore membrane with a 5·0 μm pore size; Millipore, Eschborn). After the incubation, the bound [^{3}H]PDBU was separated from free ligands by rapid passage through the filters using a millititre vacuum holder (Millipore). The dried filters were then transferred to scintillation vials which contained 9 ml of Rotiszint 2200 (Roth, Karlsruhe) and counted in a LKB Liquid Scintillation Counter (1219 Spektral Rackbeta LKB, Turko, Finland).

All samples were carried out as triplicates with variation coefficients ranging from 7% to 10%. Total or non-specific binding is expressed as the mean of triplicate determinations carried out in the absence or presence of unlabelled PDBU, respectively. Specific binding was determined by subtracting the means of non-specific binding from the total value measured at the same concentration of [^{3}H]PDBU.

In order to determine the specific binding of PDBU with stimulated and non-stimulated PMNs, cells (1×10^7) in 1·2 ml buffer in the presence of calcium and magnesium were prestimulated for 5 min with various stimuli (PMA, arachidonic acid) or buffer prior to incubation with the Ca ionophore, FMLP, opsonized zymosan or buffer for 10 min. Stimulation was terminated by centrifugation for 5 seconds at 9·700 *g* (Eppendorf centrifuge 3200); the cell pellets were resuspended with 1·2 ml PBS. Values for total and non-specific binding were calculated as the means of triplicate determinations, thus one sixth (200 μl) of the cell suspension were obtained and assayed as described.

Statistical analyses

Data from different experiments with different donor cells were combined and reported as the mean ± SEM. The Student's *t*-test for independent means was used to provide a statistical analysis ($P > 0.05$ was considered as not significant).

RESULTS

Preincubation of human PMN with PMA and subsequent stimulation with different stimuli

Stimulation with Ca ionophore A23187. Human polymorphonuclear granulocytes (1×10^7) stimulated with the Ca ionophore A23187 (7·3 μM) for 10 min at 37° released different amounts of leukotrienes, as detected by RP-HPLC. The metabolites generated were identified as LTB_4 (122·9 ± 15·0 ng), 20-OH-LTB_4 (130 ± 17·2 ng), 20-COOH-LTB_4 (27·0 ± 3 ng), LTC_4 (41·3 ± 7·0 ng), 6-trans-LTB_4 (30·0 ± 8·1 ng) and 12-epi-6-trans-LTB_4 (28·6 ± 2·7 ng) (mean ± SEM of three independent experiments). Preincubation of human granulocytes with various concentrations of PMA or the same volume of PBS (as control) for 5 min in the presence of calcium (0·8 mM) and magnesium (0·8 mM), followed by incubation with 7·3 μM Ca ionophore for another 10 min at 37° changed the afore-mentioned pattern of leukotriene release. PMA alone, in the absence of any additional stimulus, was not sufficient to induce leukotriene release.

Figure 1 demonstrates the results of four separate experiments, in which cells were preincubated with PMA. Subsequent stimulation with the Ca ionophore A23187 showed a dose-dependent inhibition of LTC_4 release. With PMA concentrations higher than 1×10^{-8} M no LTC_4 release was detected. PMA concentrations less than 1×10^{-9} M exhibited no inhibitory effect; LTC_4 levels were the same as those obtained from cells which were preincubated with PBS. The release of 20-OH-LTB_4 and 20-COOH-LTB_4 was inhibited by PMA in a similar dose-dependent manner. In contrast, the release of LTB_4 and the non-enzymatically generated LTB_4-isomers 6-trans-LTB_4 and 12-epi-6-trans-LTB_4 (Fig. 1 shows 6-trans-LTB_4, for example) was not affected significantly when the cells had been preincubated with PMA. The inhibitory effect was also observed when the combined amounts of LTB_4 and LTB_4-ω-oxidation products were calculated, reflecting the result of *de novo* synthesis and metabolism. Inhibition was obtained when the cells were preincubated with PMA in a concentration range from 5×10^{-8}–1×10^{-6} M.

In another series of experiments, the time-dependent effects of preincubation were investigated. Therefore, PMN (1×10^7) were incubated in the presence of calcium and magnesium with PMA ($1{\cdot}25 \times 10^{-7}$ M) or PBS (as control) for 5, 15 and 30 min at 37°; the incubation was then continued in the presence of the Ca ionophore A23187 (7·3 μM) for another 10 min at 37°. The results obtained by HPLC analysis from three independent experiments are shown in Fig. 2. A prolonged preincubation of cells with PMA resulted in decreasing amounts of LTC_4 and 20-

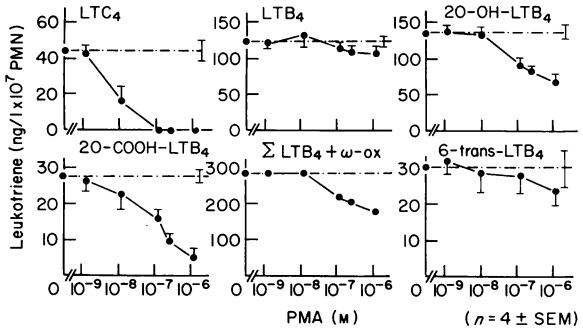

Figure 1. Preincubation of human granulocytes with various concentrations of PMA or PBS for 5 min followed by incubation with Ca ionophore for another 10 min at 37°. PMNs (1×10^7) were preincubated with various concentrations of PMA or PBS (served as control –·–·–) in the presence of calcium and magnesium for 5 min at 37°, followed by incubation with Ca-ionophore (7·3 μM) for another 10 min. Leukotriene release was measured by RP-HPLC. Each value represents the mean ± SEM of four independent experiments with different donor cells.

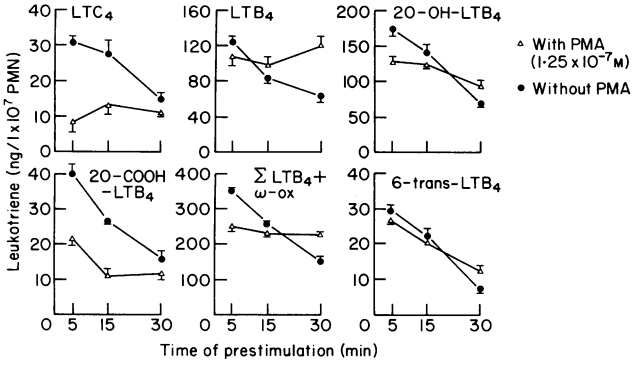

Figure 2. Preincubation of human granulocytes in the absence (●) or presence (△) of PMA ($1{\cdot}25 \times 10^{-7}$ M) for different times, followed by incubation with Ca ionophore (7·3 μM) for another 10 min at 37°. Leukotriene release was measured by RP-HPLC. Each value represents the mean ± SEM of three independent experiments.

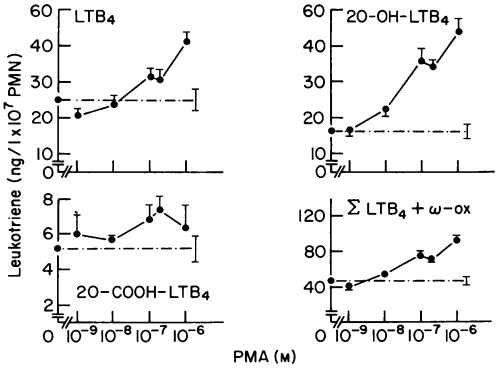

Figure 3. Preincubation of human granulocytes (1×10^7) with various concentrations of PMA for 5 min in the presence of calcium and magnesium, followed by incubation with opsonized zymosan (2 mg) for another 10 min at 37°. Prestimulation of PMNs with PBS instead of PMA served as control (–·–·–). Leukotriene release was determined by RP-HPLC. Each value represents the mean ± SEM of three independent experiments.

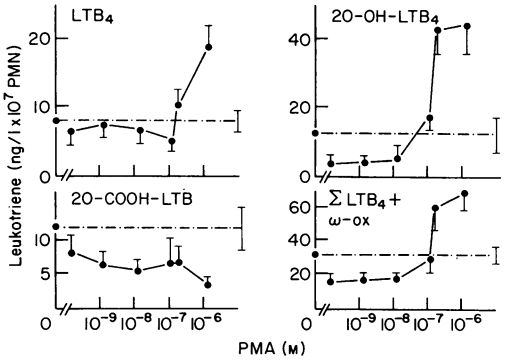

Figure 4. Preincubation of human granulocytes (1×10^7) with various concentrations of PMA in the presence of calcium and magnesium for 5 min at 37°, followed by stimulation with FMLP ($1{\cdot}8 \times 10^{-5}$ M) for another 10 min at 37°. Preincubation with PBS instead of PMA served as control (–·–·–). Each value represents the mean of leukotriene release from three independent experiments ($n = 3 \pm$ SEM).

COOH-LTB$_4$. With longer times of preincubation the effect of PMA was significantly lowered. In contrast, the amounts of LTB$_4$, 20-OH-LTB$_4$ and 6-trans-LTB$_4$ were enhanced when cells were prestimulated with PMA for 30 min.

The effects demonstrated in Figs 1 and 2 were obtained with concentrations of the Ca ionophore A23187 ranging from 1·0 to 7·3 μM. The temperature of prestimulation did not influence the PMA effects.

The release of lactate dehydrogenase (LDH) was always less than 7%, indicating that the cells remained intact during the incubation and stimulation period (data not shown).

Stimulation with opsonized zymosan. Experiments were then carried out to analyse the effects of PMA prestimulation on the leukotriene release induced with opsonized zymosan. The amounts of leukotrienes released on stimulation with opsonized zymosan were lower (10–15%) than those obtained with Ca ionophore A23187. For this purpose, PMNs (1×10^7) were prestimulated with different concentrations of PMA or the same volume of PBS for 5 min at 37° in the presence of calcium and magnesium and the incubation continued in the presence of opsonized zymosan (2 mg) for another 10 min. The metabolites were analysed by HPLC; the results obtained from three experiments are demonstrated in Fig. 3. In contrast to the effects observed with Ca ionophore (7·3 μM) as stimulus, opsonized zymosan enhanced the release of LTB + ω-oxidation products from cells pretreated with PMA (1×10^{-6}–5×10^{-8} M). As the release of LTC$_4$ and non-enzmatically generated LTB$_4$ isomers was minute, no significant data were obtained when PMA was analysed with regard to these metabolites.

Incubation with FMLP. Further experiments were then performed to analyse the effects of PMA pretreatment on leukotriene release induced with FMLP. The level of leukotriene release after stimulation with FMLP ($1{\cdot}8 \times 10^{-5}$ M) was the same as observed by stimulation with opsonized zymosan. For this purpose, PMNs (1×10^7) were prestimulated with different concentrations of PMA or the same volume of PBS for 5 min at 37° in the presence of calcium and magnesium. The incubation was continued in the presence of FMLP ($1{\cdot}8 \times 10^{-5}$ M) for

Table 1. Prestimulation of PMNs

Stimuli	Leukotrienes (ng/1 × 10^7 PMN)						
	LTC_4	LTB_4	20-OH-LTB_4	20-COOH-LTB_4	$\Sigma LTB_4 + \omega$-oxid. products	6-tr.-LTB_4	12-epi-LTB_4
Opsonized zymosan (2 mg)							
Pretreatment							
PBS	0	5·4±2·5	5·5±0·6	3·6±0·5	14·5±1·2	0	0
AA 60·0 μM	0	30·1±5·0*	30·1±1·8*	17·3±3·2*	77·9±3·3*	5·1±1·3*	4·4±1·1*
AA 6·0 μM	0	12·9±2·4*	12·9±3·7*	6·1±1·3	31·9±2·5*	1·8±0·4*	0·9±0·3*
AA 0·6 μM	0	4·9±0·4	10·2±3·4	5·7±1·4	20·8±2·6	0	0
FMLP (18 μM)							
Pretreatment							
PBS	0	5·8±1·7	17·0±2·0	15·1±3·0	37·9±2·2	0	0
AA 60·0 μM	0	24·5±2·7*	49·9±1·8*	36·7±0·9*	111·1±1·8*	4·8±0·9*	1·3±0·5*
AA 6·0 μM	0	15·9±1·9*	32·6±4·2*	17·0±2·0	65·5±2·7*	2·9±0·7*	1·2±0·7*
AA 0·6 μM	0	4·3±2·7	19·2±2·9	12·9±3·2	36·4±2·9	0	0
Ca ionophore (7·3 μM)							
Pretreatment							
PBS	22·2±3·4	84·2±13·1	94·1±1·7	22·3±0·9	200·6±5·2	30·4±3·1	30·9±1·7
AA 60·0 μM	27·8±1·7	93·0±12·4	96·2±5·1	9·1±0·8*	198·3±6·1	66·3±5·9*	66·8±7·1*
AA 6·0 μM	25·8±4·2	86·4±10·1	96·1±1·8	15·8±1·0*	198·5±4·3	34·2±1·9	34·8±0·8
AA 0·6 μM	23·3±1·2	85·7±3·6	93·1±6·2	21·7±3·5	200·5±3·5	35·5±0·9	37·9±3·7
Ca ionophore (0·73 μM)							
Pretreatment							
PBS	16·1±2·4	61·3±4·0	111·3±3·8	22·0±0·6	194·6±2·8	20·3±1·8	17·5±0·8
AA 60·0 μM	11·8±5·3	97·3±7·1†	134·4±12·4	19·8±0·7	251·5±6·7*	47·8±3·2*	46·1±1·8*
AA 6·0 μM	18·4±4·2	73·1±11·6	128·4±6·1	23·7±1·9	225·2±6·5†	25·0±2·4	24·3±2·1
AA 0·6 μM	17·8±2·8	69·4±6·1	113·3±2·1	21·3±1·4	204·0±3·2	20·0±0·4	19·9±0·7
Ca ionophore (0·18 μM)							
Pretreatment							
PBS	5·4±2·5	7·1±2·4	44·6±3·7	14·9±1·5	66·6±2·9	4·8±0·2	2·5±0·7
AA 60·0 μM	7·0±1·5	14·1±0·1†	38·5±2·9	15·6±0·7	68·2±1·2	14·3±0·7*	13·7±0·9*
AA 6·0 μM	4·9±1·4	11·5±2·5	39·7±4·5	13·9±2·0	65·1±3·0	9·5±0·9*	8·9±0·3*
AA 0·6 μM	5·7±0·3	7·3±1·8	40·3±7·2	14·0±0·8	61·6±3·3	6·7±1·0	4·7±0·9

PMNs were prestimulated with various concentrations of arachidonic acid for 5 min at 37° followed by incubation with various stimuli (Ca ionophore, opsonized zymosan and FMLP) for an additional 10 min at 37°. Leukotriene release was measured by RP-HPLC. Each value represents the mean±SEM of three independent experiments performed with different donor cells. AA, arachidonic acid.

* $P<0·01$.

† $P<0·05$.

another 10 min. The results obtained by HPLC analysis from three experiments are demonstrated in Fig. 4.

Prestimulation of PMNs with PMA (1×10^{-6}–$2·5\times10^{-7}$ M) enhanced the subsequent LTB_4 and 20-OH-LTB_4 release induced by stimulation with FMLP. Lower doses of PMA had no significant effects on the subsequent LTB_4 and 20-OH-LTB_4 release. The release of 20-COOH-LTB_4 decreased after PMA prestimulation (1×10^{-6}–1×10^{-10} M). This effect was also observed when cells were stimulated with Ca ionophore.

Preincubation of human PMN with arachidonic acid and subsequent stimulation with different stimuli

McPhail, Clayton & Snyderman (1984) described that arachidoniate and other unsatured long-chain fatty acids activate the protein kinase *C* from human neutrophils. For this purpose, PMNs were pretreated in the presence of calcium and magnesium with various doses of arachidonic acid or PBS (as control) for 5 min at 37°. Subsequently, the various stimuli (e.g. Ca ionophore, opsonized zymosan and FMLP) were added and the leukotriene release were analysed. Table 1 shows the results of three separate experiments. Three major effects were obtained: (i) Priming with arachidonic acid at concentrations from 60 μM to 6·0 μM increased the release of all leukotrienes induced by subsequent stimulation with FMLP or opsonized zymosan ($P<0·01$). (ii) With Ca ionophore (7·3–0·18 μM) as a stimulus, the release of the non-enzymatically generated LTB_4-isomers (6-trans-LTB_4 and 12-epi-6-trans-LTB_4) were significantly

Table 2. Modulation of the [^{3}H]PDBU binding sites on PMNs depending on the conditions of activation

		% specific binding of [^{3}H]PDBU
Control	PMN+PBS	100·0±2·4
PMN+PBS	+Zx (2 mg)	50·3±5·5*
PMN+AA 60·0 μM	+Zx (2 mg)	64·3±7·6*
PMN+AA 6·0 μM	+Zx (2 mg)	59·7±10·6*
PMN+PBS	+FMLP (18 μM)	96·7±14·7
PMN+AA 60·0 μM	+FMLP (18 μM)	93·3±27·0
PMN+AA 6·0 μM	+FMLP (18 μM)	67·1±7·2*
PMN+PBS	+Ca ionophore 7·3 μM	91·5±10·4
PMN+AA 60·0 μM	+Ca ionophore 7·3 μM	57·0±1·7*
PMN+AA 6·0 μM	+Ca ionophore 7·3 μM	73·0±11·6
PMN+PBS	+Ca ionophore 0·73 μM	91·0±6·7
PMN+AA 60·0 μM	+Ca ionophore 0·73 μM	64·6±13·4†
PMN+AA 6·0 μM	+Ca ionophore 0·73 μM	67·2±3·7*
PMN+PBS	+Ca ionophore 0·18 μM	62·8±8·0*
PMN+AA 60·0 μM	+Ca ionophore 0·18 μM	72·3±9·2†
PMN+AA 6·0 μM	+Ca ionophore 0·18 μM	94·2±18·7
PMN+AA 60·0 μM	+PBS	115·3±11·2
PMN+AA 6·0 μM	+PBS	101·7±9·2

The values demonstrate the percentage of [^{3}H]PDBU binding determined from the samples without addition of any stimuli (served as 100% value). Each value represents the mean ± SEM of five independent determinations with different donor cells.

Zx, opsonized zymosan.

* $P<0.01$.

† $P<0.05$.

enhanced ($P<0.01$). With FMLP or opsonized zymosan, these LTB_4 isomers were only detected in the presence of arachidonic acid. (iii) Priming with arachidonic acid at concentrations of 60 μM and 6 μM significantly ($P<0.01$) decreased the release of 20-COOH-LTB_4 induced by subsequent stimulation with Ca ionophore (7·3 μM).

Modulation of phorbol-dibutyrate binding for PMNs

Experiments were then directed to clarify whether the activation of protein kinase *C* is responsible for the modulation of the leukotriene release. For this purpose, the amount of binding sites for phorbol-dibutyrate (PDBU) on PMNs was determined. As became apparent by the Scatchard analysis, $2{\cdot}2\times10^5$–$5{\cdot}3\times10^5$ binding sites per cell were counted as total receptor number (values from three different experiments). The Kd (dissociation constant) varied between 27 nM and 54 nM (range determined by three experiments). Addition of PMA at different concentrations to 2×10^6 cells, showed that a PMA concentration of 12·6±7·6 ng (66·5±40 nM) ($n=3\pm$SEM) inhibited 50% of the total receptor number (EC_{50}). Table 2 summarizes the effects of the various stimuli, as obtained after different conditions of prestimulation. Our data show that the addition of

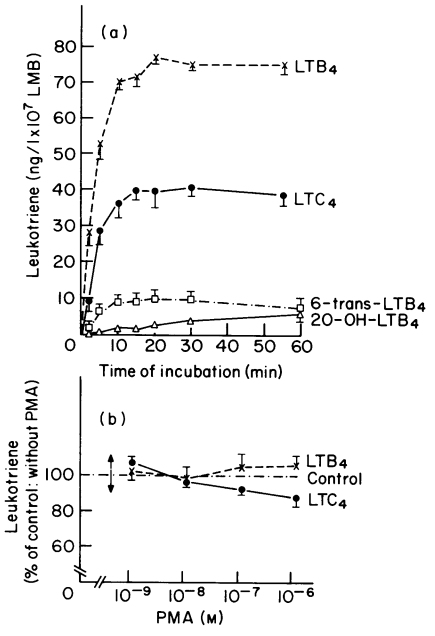

Figure 5. (a) Time-dependent release of LTB_4, LTC_4, 6-trans-LTB_4 and 20-OH-LTB_4 from the cell population consisting of lymphocytes, monocytes and basophilic granulocytes (LMB) on stimulation with Ca ionophore (7·3 μM) in the presence of calcium and magnesium. Leukotriene release was analysed by RP-HPLC. Each value represents the mean ± SEM of three independent experiments performed with different donor cells. (b) Effects of PMA prestimulation on the release of leukotrienes from LMBs. The LMB population (1×10^7) was prestimulated in the presence of calcium and magnesium with various concentrations of PMA or PBS for 5 min at 37° and the stimulation continued for another 10 min in the presence of Ca ionophore (7·3 μM). Leukotriene release was measured by RP-HPLC; the values for LTB_4 and LTC_4 release in the absence of PMA (PBS control –·–·–) were expressed as 100%. The values for the amount of LTB_4 and LTC_4 in the presence of PMA were calculated as a percentage of the 100% control value. The data represents the mean ± SEM of three independent experiments.

Table 3. Metabolism of exogenously added LTC_4 to LTB_4 isomers and LTC_4 sulphoxide by PMA-stimulated PMNs and LMBs

Cell population (1×10^7)	PMA concentration	% LTC_4 metabolized
PMN	$1{\cdot}25\times10^{-6}$ M	74·2±5·2
PMN	$2{\cdot}50\times10^{-7}$ M	53·4±7·8
PMN	$1{\cdot}25\times10^{-7}$ M	34·5±10·5
LMB	$1{\cdot}25\times10^{-6}$ M	0
LMB	$2{\cdot}50\times10^{-7}$ M	0
LMB	$1{\cdot}25\times10^{-7}$ M	0

PMNs or LMBs (1×10^7) were preincubated with different concentrations of PMA in the presence of calcium and magnesium for 5 min at 37°. After prestimulation, 100 ng LTC_4 were added to the cell suspension and the incubation was continued for further 15 min. The metabolites were analysed by RP-HPLC. The sum of all identified leukotrienes is expressed as 100%. The data represent the metabolization of three independent experiments ($n=3\pm$SEM).

opsonized zymosan decreased the binding of PDBU by 50% compared with the unstimulated-cell control. Prestimulation with arachidonic acid had no significant effect on the reduction of binding sites after stimulation with opsonized zymosan. Prestimulation with arachidonic acid decreased the binding sites of PDBU after stimulation with FMLP (by arachidonic acid concentration of 6·0 μM), Ca ionophore (7·3 μM) (by arachidonic acid concentration of 60 μM) and Ca ionophore (0·73 μM) (by arachidonic acid concentration of 60 and 6 μM). The addition of Ca ionophore (0·18 μM) in the presence and absence of arachidonic acid (60 μM) decreased the binding sites of PDBU. In contrast, no significant effects on the binding of PDBU were observed for cells stimulated with FMLP or the Ca ionophore.

Release of leukotrienes from LMBs and modulation by PMA

In addition to human neutrophils, human LMBs were also analysed. Upon stimulation with Ca ionophore (7·3 μM) leukotrienes were released in a time-dependent fashion that differed from the release of PMN (Fig. 5a represents the mean of three experiments). LTB_4 was released after 1 min as major product and reached a maximum after 20 min of incubation. No significant decrease in LTB_4 levels was observed, even with longer times of incubation. LTC_4 reached maximal levels after 20 min of incubation and showed no significant variations even after incubation periods up to 60 min. Only minute amounts of non-enzymatic LTB_4 isomers and LTB_4-ω-oxidation products—20-OH-LTB_4—were detected. Experiments were then performed to analyse the effect of prestimulation with PMA. For this purpose, LMBs (1×10^7) were prestimulated in the presence of calcium and magnesium with PMA (1×10^{-6}–1×10^{-10} M) or PBS (100% control) for 5 min at 37° and the stimulation was continued for another 10 min in the presence of the Ca ionophore (7·3 μM). As is apparent (Fig. 5b), neither LTB_4 nor LTC_4 release was significantly affected by prestimulation with PMA.

Metabolism of exogenously added LTC_4 by PMA-preincubated PMN and LMB

In order to determine the metabolism of exogenously added LTC_4, PMNs or LMBs (1×10^7) were preincubated with PMA (different concentration) or PBS in the presence of calcium and magnesium for 5 min at 37°. After preincubation, LTC_4 (100 ng) or the aliquot of buffer was added and the incubation proceeded for an additional 15 min at 37°. Table 3 demonstrates the results of three independent experiments. PMA-pretreated PMNs metabolized exogenously added LTC_4 into 6-trans-LTB_4, 12-epi-6-trans-LTB_4 and a product which is more polar than LTC_4, the LTC_4 sulphoxide. The pattern of metabolism depended on the concentration of PMA and was not detected in non-stimulated PMNs. In LMBs stimulated with PMA, no LTC_4 metabolism to the two isomers of LTB_4 was observed. In several experiments 7–10% of the added LTC_4 was metabolized to LTE_4 via LTD_4.

DISCUSSION

Our data demonstrate that prestimulation of granulocytes with various concentrations of PMA modulates the subsequent leukotriene release induced by optimal Ca ionophore concentrations (1–7·3 μM). The effects of prestimulation with PMA differ with regard to the amounts of the various metabolites. As became apparent, the amounts of LTC_4, 20-OH-LTB_4 and 20-COOH-LTB_4 released after stimulation with the Ca ionophore were significantly decreased, whereas the release of LTB_4 and the non-enzymatic LTB_4 isomers were hardly affected after prestimulation with PMA. In contrast to the experiments with the Ca ionophore, PMA-prestimulated cells showed an enhanced release of LTB_4 and LTB_4-ω-oxidation products after subsequent stimulation with opsonized zymosan. Our experiments also emphasize that stimulation with FMLP-induced LTB_4, 20-OH-LTB_4 and 20-COOH-LTB_4. This release is enhanced once the cells are prestimulated with PMA. Among the leukotriene metabolites, 20-COOH-LTB_4 release was inhibited. It is well established that the various stimuli interact by different membrane biochemical events with the neutrophils. Ca ionophore induces calcium influx, opsonized zymosan interacts with the C3b and Fc receptors for binding and signal transduction. The chemotactic peptide FMLP interacts with specific binding sites for FMLP. In this case PMA prestimulation and the resulting activation of protein kinase *C* modulate the subsequent biochemical signals induced by the various stimuli. In the case of opsonized zymosan one may suggest that PMA enhances C3b-receptor expression, thus facilitating phagocytosis (O'Shea *et al.*, 1985). The enhanced C3b-receptor binding sites lead to an increased attachment between opsonized zymosan and receptor, resulting in an enhanced generation of leukotrienes. Interaction of neutrophils with PMA is accompanied with a down-regulation of the FMLP receptors (Anderson *et al.*, 1987), which proceeds via an activation of the cells. One therefore may suggest that these cells, although they dispose of less receptors for FMLP, are nonetheless in a higher state of activation.

Preliminary experiments with H7 [1-5 (isoquinoline-sulphonyl-2-methylpiperazine-dicloride)], an inhibitor of protein kinase *C*, demonstrated that the preincubation with PMA in the presence of H7 ($2{\cdot}4 \times 10^{-7}$ M and $2{\cdot}4 \times 10^{-8}$ M) diminished the increase of leukotriene release induced by the subsequent stimulation with opsonized zymosan (data not shown). These data indicate that the modulatory effect of PMA on leukotriene release induced by opsonized zymosan is linked to protein kinase *C* activation.

Cells activated with optimal Ca ionophore concentrations reveal, after prestimulation with PMA, a modulation of LTC_4 and LTB_4 metabolism. We have demonstrated previously that stimulation of PMNs with PMA leads to the activation of LTC_4 metabolism, thus leading to LTB_4 isomers (6-trans LTB_4 and 12-epi-6-trans-LTB_4) and a more polar product, the LTC_4 sulphoxide. Furthermore, it was shown, with the exogenously added LTB_4, that the ω-oxidation products and, especially, the appearance of 20-COOH-LTB_4 decreased when PMNs were stimulated with PMA (J. Brom, W. Schönfeld and W. König, manuscript submitted for publication). Thus, it appears likely that prestimulation of cells with PMA affects leukotriene induction by either enhancing LTC_4 and/or inhibiting LTB_4 metabolism.

In contrast to human neutrophils, different results were obtained with human LMBs. PMA prestimulation neither affected LTB_4 nor LTC_4 release significantly. One has to emphasize that the LMBs are not able to metabolize LTB_4 via

ω-oxidation (only minute 20-OH-LTB_4 could be detected). Furthermore, addition of exogenous LTC_4 to LMBs prestimulated with PMA showed no metabolization to LTB_4 isomers and LTC_4 sulphoxide. Experiments with exogenously added L-serine (20 mM) (data not shown), a scavenger for HOCl, to PMA-prestimulated PMN clearly demonstrated an increase in the amount of LTC_4 release after stimulation with the Ca ionophore. These results suggest that the oxidative response induced by PMA leads to a decrease in LTC_4 release.

In our experiments optimal Ca ionophore concentrations decreased the release of LTC_4, 20-OH-LTB_4 and 20-COOH-LTB_4 when the cells were preactivated with PMA. In contrast, suboptimal concentrations of the Ca ionophore acted synergistically with PMA when leukotriene release was studied (McColl, Hurst & Cleland, 1986; Liles, Meier & Henderson, 1987). Similar results were obtained when mouse peritoneal macrophages (Tripp, Mahoney & Needleman, 1985; Wey & Baxter, 1986) were stimulated with suboptimal concentrations of the Ca ionophore. A synergistic effect was obtained for LTC_4 synthesis, but no effects were obtained with regard to the cyclo-oxygenase pathway.

Haines *et al.* (1987) described that PMA (200 ng/1×10^7 PMN) had no synergistic effect with opsonized zymosan when LTB_4 was analysed. It has to be considered, however, that Haines *et al.* (1987) did not describe the LTB_4-ω-oxidation products in their experiments. In fact, the generation of ω-oxidation products was only observed by them in the presence of exogenous arachidonic acid (50 μM).

Our data clearly show that the addition of arachidonic acid above 0·6 μM up to 60 μM enhanced LTB_4 and 20-OH-LTB_4 release after subsequent stimulation with FMLP and opsonized zymosan. An increase in the release of LTB_4 isomers was observed. It has been shown that arachidonic acid and other unsaturated long-chain fatty acids activate protein kinase *C* in human granulocytes (McPhail *et al.*, 1984). Thus, it appears that the enhanced release of LTB_4 and ω-oxidation products occurs with stimuli which induce a suboptimal release of leukotrienes, such as FMLP and opsonized zymosan. Our data clearly demonstrate the decreased amounts of 20-COOH-LTB_4 in the presence of arachidonic acid (60 μM and 6 μM) after stimulation of the cells with the Ca ionophore (7·3 μM). The same effect was demonstrated when the cells were prestimulated with PMA. These data suggest that the addition of exogenous arachidonic acid influences the leukotriene release and metabolism not solely by an enhanced substrate concentration for the lipoxygenase.

The data thus provide evidence that the release process for leukotrienes is significantly modulated by the characteristics of the prestimulatory events. It appears that protein kinase *C* plays a major role. Indeed, preincubation of neutrophils with opsonized zymosan and Ca ionophore (0·18 μM) reduced the binding sites for PDBU. This inhibition of binding also occurred when cells had been preincubated with arachidonic acid. French *et al.* (1987) have described a conversion of the low to the high affinity state of the phorbol ester receptor after prestimulation with Ca ionophore. In addition one has to consider that the stimulation of cells with opsonized zymosan or prestimulation with arachidonic acid and subsequent stimulation with Ca ionophore lead to a down-regulation of receptors for phorbol ester and thus to an activation of protein kinase *C*.

Thus, evidence is provided that leukotriene generation and metabolization is dependent on the PMA-induced activation of protein kinase *C*. The resulting effects vary with regard to the subsequent stimulus and concentration, time of incubation, as well as the concentration of preactivation with PMA, which were used for the prestimulation of the granulocytes.

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