Studies on the activation of human neutrophil 5-lipoxygenase induced by natural agonists and Ca^{2+} ionophore A23187

Patrick P. McDONALD, Shaun R. McCOLL, Paul H. NACCACHE and Pierre BORGEAT*

Centre de recherche en Inflammation, Immunologie et Rhumatologie, Centre hospitalier de l'Université Laval, 2705 boul. Laurier, Sainte-Foy, Québec, Canada G1V 4G2

By using exogenous substrates, activation of human neutrophil 5-lipoxygenase can be investigated independently of the release of endogenous arachidonic acid. We have developed a sensitive assay to measure 5-LO activation which takes advantage of the 5-LO-mediated conversion of 15*S*-hydroperoxy-5,8,11,13(*Z,Z,Z,E*)-eicosatetraenoic acid (15-HpETE) into 5*S*,15*S*-dihydroxy-6,8,11,13(*E,Z,Z,E*)-eicosatetraenoic acid (5,15-DiHETE). When resting neutrophils were incubated with low micromolar concentrations of 15-HpETE, a minor dose- and time-dependent formation of 5,15-DiHETE was observed. In contrast, co-addition of 15-HpETE with Ca^{2+} ionophore A23187 or with the neutrophil agonists platelet-activating factor (PAF), fMetLeuPhe or complement component C5a resulted in a sizeable concentration-dependent synthesis of 5,15-DiHETE, while lyso-PAF and phorbol myristate acetate were without effect on 5,15-DiHETE formation from 15-HpETE. This stimulation of 5,15-DiHETE synthesis by A23187 or by natural agonists was effectively inhibited by MK-886, a compound that has recently been reported to inhibit the A23187-induced translocation of 5-LO to membrane structures. Furthermore, natural-agonist-induced activation of the 5-LO-mediated transformation of 15-HpETE was inhibited by pertussis toxin, indicating the involvement of a GTP-binding protein in the 5-LO activation process.

INTRODUCTION

Leukotriene B_4 (LTB_4), a product of the arachidonate 5-lipoxygenase (5-LO) pathway [1], is a potent activator of various cells of the immune system, and is thus regarded as an important mediator in inflammation. Indeed, LTB_4 is a stereospecific activator of neutrophil chemotaxis and aggregation [2], adherence [3] and degranulation [4], and stimulates other cells including eosinophils [5], monocytes [6] and lymphocytes [7]. In contrast with the well-characterized biological effects of 5-LO products, the mechanism of activation of the enzyme remains poorly understood. It is generally accepted that leukotriene synthesis in human neutrophils and monocytes requires the release of endogenous substrate and activation of 5-LO, both of which are Ca^{2+} -dependent processes [8–10]. Cellular levels of Ca^{2+} and lipid hydroperoxides appear to be the key elements required for the activation of 5-LO [9,11,12], while the enzymic activity is known to be further modulated by ATP and phosphatidylcholine [11,13]. In agreement with the role attributed to lipid hydroperoxides, studies have demonstrated the importance *in situ* of glutathione levels and possibly glutathione peroxidases [12,14]. More recently it has been shown that 5-LO activation is likely to involve a Ca^{2+} -dependent translocation of the enzyme to membrane structures [10,13]. Further insights have been provided by the development of novel inhibitors of leukotriene synthesis, such as the indole derivative, MK-886, which specifically binds to an 18 kDa membrane protein named FLAP (five-lipoxygenase-activating protein), thereby effectively inhibiting 5-LO translocation to the membrane and subsequent leukotriene synthesis [15–17]. Though

a direct interaction between FLAP and 5-LO has not yet been demonstrated, it is known that the presence of FLAP is required for leukotriene synthesis, and it has been proposed that this protein may act as a plasma membrane docking protein for 5-LO and/or be associated with the process whereby endogenous arachidonic acid (AA) is made available to the 5-LO [16].

Leukotriene synthesis can be induced by different stimuli, including the Ca^{2+} ionophore A23187 [1], receptor-dependent soluble agonists such as platelet-activating factor (PAF) [18,19], fMet-Leu-Phe [20,21], complement fragment C5a [20] and neutrophil-activating factor (NAF)/interleukin-8 [22], as well as by insoluble stimuli such as zymosan [23] or urate and pyrophosphate crystals [24]. Worthy of mention is the fact that all of these agents stimulate the mobilization of intracellular Ca^{2+} in human neutrophils [25–29], in agreement with the central role attributed to Ca^{2+} in the activation of the enzyme. As activation of both 5-LO and phospholipase A_2 (PLA_2) are required for the onset of leukotriene synthesis, either process might constitute a limiting step. Recent studies have indicated that AA availability is indeed the limiting factor for leukotriene synthesis [19–22,30].

Given the involvement of metabolites of the 5-LO pathway in host defence mechanisms and in various pathophysiological conditions, the assessment of the 5-LO activation status in phagocytes, as well as a better understanding of the mechanisms of activation, appear to be of major importance. In the present study we have used the 5-LO product 15-hydroperoxyeicosatetraenoic acid (15-HpETE) as an exogenous substrate to specifically investigate the activation of human neutrophil 5-LO independently of endogenous substrate release. 15-HpETE has

Abbreviations used: LTB_4 , leukotriene B_4 ; AA, arachidonic acid; Mead acid, 5,8,11-(all-*cis*)-eicosatrienoic acid; PAF, platelet-activating factor (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine); lyso-PAF, 1-*O*-hexadecyl-2-hydroxy-*sn*-glycero-3-phosphocholine; fMetLeuPhe, formyl-methionyl-leucyl-phenylalanine; 15-HpETE, 15*S*-hydroperoxy-5,8,11,13(*Z,Z,Z,E*)-eicosatetraenoic acid; 5-HpETE, 5*S*-hydroperoxy-6,8,11,14(*E,Z,Z,Z*)-eicosatetraenoic acid; 15-HETE, 15*S*-hydroxy-5,8,11,13(*Z,Z,Z,E*)-eicosatetraenoic acid; 5-HETE, 5*S*-hydroxy-6,8,11,14(*E,Z,Z,Z*)-eicosatetraenoic acid; 5,15-DiHETE, 5*S*,15*S*-dihydroxy-6,8,11,13(*E,Z,Z,E*)-eicosatetraenoic acid; MK-886, 3-[1-(*p*-chlorobenzyl)-5-isopropyl-3-*r*-butylthioindol-2-yl]-2,2'-dimethylpropanoic acid; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulphoxide; 5-LO, arachidonate 5-lipoxygenase; PLA_2 , phospholipase A_2 ; PKC, protein kinase C; RP-h.p.l.c., reversed-phase h.p.l.c.; SP-h.p.l.c., straight-phase h.p.l.c.; PMN, polymorphonuclear leucocytes; FLAP, 5-lipoxygenase-activating protein; HBSS, Hanks' balanced salt solution; NAF, neutrophil-activating factor.

* To whom correspondence should be addressed.

previously been shown to be an excellent 5-LO substrate [9], being converted to 5,15-dihydroxyicosatetraenoic acid (5,15-DiHETE), a stable product that is easily detected by reverse-phase (RP)-h.p.l.c. We report herein that neutrophil agonists induce a dose-dependent activation of 5-LO, which is inhibited by MK-886 and which is mediated through pertussis toxin-sensitive G-proteins.

EXPERIMENTAL

Materials

Reagents were obtained as follows: calcium ionophore A23187, fMetLeuPhe, PAF, Hepes, cholera toxin and AA from Sigma Chemical Company, St. Louis, MO, U.S.A.; Mead acid from Cayman Chemicals, Ann Arbor, MI, U.S.A.; 5,15-DiHETE, from Oxford Biomedical Research Inc., Oxford, MI, U.S.A.; fura-2/AM from Molecular Probes Inc., Eugene, OR, U.S.A.; pertussis toxin from List Biological Laboratories Inc., Campbell, CA, U.S.A.; Hank's balanced salt solution (HBSS) from GIBCO, Burlington, Ontario, Canada. All solvents were h.p.l.c.-grade from Anachemia, Montréal, Québec, Canada. 15-HpETE and 15-hydroxyicosatetraenoic acid (15-HETE) were synthesized using soybean 15-lipoxygenase as described for 13-hydroperoxyoctadecadienoic acid [31], using AA as substrate. AA was purified on a silica column prior to enzymic synthesis, and 15-HpETE was purified by straight-phase (SP)-h.p.l.c. It must be stressed that it is of critical importance to purify AA and 15-HpETE freshly before use, because of the natural instability of these products. LTB₄ and MK-886 were generously provided by Dr. Robert N. Young, Merck-Frosst Centre for Therapeutic Research, Pointe-Claire, Québec, Canada; rhC5a was donated by Dr. Henry Showell of the Pfizer Central Research Center, Groton, CT, U.S.A.

Cell separation

Venous blood was collected from healthy donors and mixed with sodium heparin (15 USP units/ml, final concentration) as an anticoagulant. Following dextran sedimentation of erythrocytes, neutrophils were purified by centrifugation on Ficoll-Paque cushions (Pharmacia, Dorval, Québec, Canada). The erythrocytes remaining in the final pellet were removed by hypotonic lysis with water (30 s), and the cells were resuspended in HBSS buffered with Hepes (10 mM, pH 7.4) and supplemented with Ca²⁺ (1.6 mM) at a final concentration of 5×10^6 polymorphonuclear leucocytes (PMN)/ml (unless otherwise stated). The entire cell separation was carried out at room temperature. The percentage of neutrophils in the cell suspensions used in this study exceeded 97%, and cell viability was greater than 98%, as determined by Trypan Blue exclusion. The platelet/leucocyte ratio varied from 2 to 6.

Cell incubations

Incubation conditions were as follows. Cell suspensions were warmed to 37 °C for 5 min prior to incubation with substrate and/or stimulus. All agonists, including exogenous AA, were dissolved in dimethyl sulphoxide (DMSO) (except for rhC5a, which was dissolved in phosphate-buffered saline) and added to 1.0 ml aliquots of the cell suspension; the final concentration of DMSO (max. 0.4%) consistently failed to stimulate any detectable leukotriene synthesis. 15-HpETE and 15-HETE stock solutions (in ethanol) were diluted 10-fold in a Na₂CO₃ solution containing the amount of base necessary for stoichiometric neutralization of the fatty acids (resulting in a solution of pH 6–7); 10 µl of this sodium salt solution was added to 1.0 ml

of the cell suspensions. After the desired incubation time with the stimuli, the cells were inactivated by the addition of 1.0 ml of an ice-cold mixture of methanol/acetonitrile (1:1, v/v) containing internal standards (12.5 ng of prostaglandin B₂ and 19-hydroxyprostaglandin B₂/ml). The samples were then stored at –20 °C until analysed by RP-h.p.l.c. In the experiments involving pertussis toxin and cholera toxin, the cell suspensions were pre-incubated with either toxin at a final concentration of 0.5 µg/ml for 3 h at 37 °C. During this time the cells were gently swirled in a rotary water bath (New Brunswick Scientific, Edison, NJ, U.S.A.). In the experiments involving MK-886, the drug was added to the cells 1 min before stimulation.

Measurement of intracellular free Ca²⁺

Intracellular free Ca²⁺ levels were monitored using the fluorescent probe fura-2 [32]. Briefly, PMN suspensions (1×10^7 cells/ml) were incubated with 1 µM-fura-2/AM for 30 min at 37 °C. The cells were then washed free of extracellular probe, resuspended at 5×10^6 cells/ml and allowed to re-equilibrate for 10 min at 37 °C. They were then transferred to the thermostatically controlled cuvette compartment (37 °C) of a spectrofluorimeter (SLM 8000 C; SLM Aminco, Urbana, IL, U.S.A.) and the fluorescence was monitored (excitation and emission wavelengths of 340 and 510 nm respectively). The intracellular Ca²⁺ concentrations were calculated according to Tsien *et al.* [33].

RP-h.p.l.c. analysis of lipoxygenase products

RP-h.p.l.c. analysis of lipoxygenase products was performed as described previously, using an on-line extraction procedure [34]. Briefly, the denatured cell suspensions were centrifuged at 500 g (4 °C, 10 min) to remove precipitated material, and the supernatants were directly injected on to a Resolve C₁₈ Radial Pak column (5 mm × 100 mm, 5 µm particles, Waters Millipore) protected by Guard-Pak Resolve C₁₈ and silica cartridges (Waters Millipore). The various lipoxygenase products were eluted at 1.5 ml/min using gradients of organic solvents and a change of pH. Elution was monitored using fixed-wavelength u.v. photometers at 229 and 280 nm; quantification was performed by comparing peak heights of each compound with those of calibrated standards, after correction for recovery using the internal standard prostaglandin B₂. The lower limit of detection was 0.5 ng at 280 nm and 1 ng at 229 nm.

RESULTS AND DISCUSSION

Dose-dependent effect of A23187 on the transformation of endogenous and exogenous substrates in neutrophils

To demonstrate that 5-LO activation could be investigated as a distinct process from leukotriene synthesis (which depends on both 5-LO activation and endogenous AA mobilization), we characterized the formation of 5,15-DiHETE from exogenous 15-HpETE under conditions where the 5-LO was activated by the Ca²⁺ ionophore A23187. Neutrophils were incubated for 5 min with increasing concentrations of A23187, alone or together with 3.0 µM-15-HpETE. By itself, the ionophore (up to 300 nM) did not induce any detectable synthesis of 5,15-DiHETE (results not shown). Conversion of 15-HpETE to 5,15-DiHETE induced by A23187 was dose-dependent, required a minimal concentration of 3 nM, and a maximal response was reached using between 0.1 and 1.0 µM-A23187 (Fig. 1). By comparison, cells exposed to A23187 alone required a minimal dose of 30 nM to enable the detection of 5-LO products derived from endogenous AA, and a maximal response was achieved using between 0.3 and 1.0 µM-A23187. These results indicate that, in

Fig. 1. Dose-dependent effect of Ca^{2+} ionophore A23187 on the transformation of endogenous and exogenous substrate in intact neutrophils

PMN ($5 \times 10^6/\text{ml}$) were incubated for 5 min at 37°C with increasing concentrations of ionophore, either alone or with $3.0 \mu\text{M}$ -15-HpETE. ▲, 5,15-DiHETE synthesis by PMN incubated with ionophore and 15-HpETE; the small amounts of 5,15-DiHETE resulting from exposure to 15-HpETE alone ($6.8 \pm 1.5 \text{ ng}/5 \times 10^6 \text{ PMN}$) have been subtracted, so as to only depict the effect of the ionophore. △, 5-LO product synthesis (comprising LTB_4 , its all-*trans* isomers and ω -oxidation products, and 5-HETE) by PMN incubated with ionophore only. This experiment is representative of six; each data point is the mean $\pm$ S.E.M. of triplicate incubations.

Table 1. Effect of various eicosanoids on the intracellular free Ca^{2+} concentration in human neutrophils

PMN were loaded with the fluorescent Ca^{2+} probe fura-2/AM as described in the Experimental section and exposed to the eicosanoids at the indicated concentrations. The basal unstimulated level of cytoplasmic free Ca^{2+} $[\text{Ca}^{2+}]_i$ was $83 \pm 8 \text{ nM}$ ($n = 6$). Values are means $\pm$ S.E.M. of single determinations in the indicated number of experiments.

Stimulus	$\Delta[\text{Ca}^{2+}]_i$	<i>n</i>
3 μM -15-HpETE	25 ± 6	5
9 μM -15-HpETE	38 ± 3	4
3 μM -15-HETE	19 ± 3	3
9 μM -15-HETE	27 ± 1	2
3.3 μM -AA	66 ± 19	5
10 μM -AA	100 ± 27	4
3.3 μM -Mead acid	73 ± 36	3
10 μM -Mead acid	150 ± 29	3
10 nM- LTB_4	377 ± 72	6
3 μM -5,15-DiHETE	129 ± 23	3

the presence of an alternative exogenous substrate, 5-LO activation can be quantitatively evaluated in intact neutrophils under conditions where LT synthesis from endogenous AA is not detectable by RP-h.p.l.c. Therefore these data demonstrate that 5-LO activation can be investigated independently of PLA_2 activation, and lend further support to the concept that endogenous substrate availability is the limiting factor for leukotriene synthesis, as suggested in numerous studies [19–22,30].

Fig. 2. Substrate-concentration-dependence and kinetics of the 5-LO-catalysed transformation of 15-HpETE into 5,15-DiHETE

Neutrophils ($5 \times 10^6/\text{ml}$) were incubated (A) for 5 min at 37°C with increasing concentrations of 15-HpETE, either by itself or with 10 nM-A23187; or (b) for various lengths of time at 37°C with $3.0 \mu\text{M}$ -15-HpETE, by itself or with 10 nM-A23187. △, 15-HpETE alone; ▲, 15-HpETE and ionophore. Each experiment is representative of 10; each data point is the mean $\pm$ S.E.M. of triplicate incubations.

Selection of an exogenous substrate to investigate 5-LO activation

We next examined the individual potential of four different compounds to serve as exogenous substrates for 5-LO under conditions where neutrophils are stimulated with 10 nM-ionophore, which by itself does not stimulate the synthesis of 5-LO products derived from endogenous AA. As depicted in Fig. 1, cells stimulated with 10 nM-ionophore showed marked 5,15-DiHETE synthesis when supplied with $3.0 \mu\text{M}$ -15-HpETE. Under the same conditions the use of 15-HETE or Mead acid (up to $9.0 \mu\text{M}$) consistently failed to enable the detection by RP-h.p.l.c. of their respective 5-LO products, while $3.3 \mu\text{M}$ -exogenous AA led to the formation of small amounts of 15-HETE, LTB_4 , its ω -oxidation metabolites and 6-*trans*- LTB_4 isomers (results not shown).

We also examined the relative abilities of AA and 15-HpETE to stimulate the mobilization of intracellular Ca^{2+} . The conceptual basis for such experiments stemmed from the fact that all.

Fig. 3. Concentration-dependence of 5-LO activation induced by natural agonists

PMN (5×10^6 /ml) were incubated for 15 min at 37 °C with 3.0 μ M-15-HpETE, with increasing concentrations of (a) PAF-acether (■) or rhC5a (□); and (b) fMetLeuPhe. The small amounts of 5,15-DiHETE resulting from exposure to 15-HpETE alone (a, 19.1 ± 1.0 ng/5 × 10⁶ PMN; b, 15.6 ± 0.9 ng/5 × 10⁶ PMN) have been subtracted, so as to only depict the effect of the agonists. Experiments depicted are representative of four; each data point is the mean $\pm$ S.E.M. of triplicate incubations.

Table 2. Effect of pertussis toxin (PT) or cholera toxin (CT) pre-treatment on agonist-induced 5-LO activation

PMN (5×10^6 /ml) were pre-treated with the toxins (0.5 μ g/ml for 3 h at 37 °C) and incubated for 15 min at 37 °C with 3.0 μ M-15-HpETE, either alone or with 5-LO agonists at the indicated concentrations. The percentage inhibitions have been calculated after subtracting the 5,15-DiHETE synthesis resulting from exposure to 15-HpETE only ($\pm$ toxin). Values are means $\pm$ S.E.M. of averaged triplicate determinations in the indicated numbers of experiments.

Stimulus	Inhibition (%)		n
	PT	CT	
100 nM-PAF	57 $\pm$ 7	20 $\pm$ 2	6
100 nM-fMetLeuPhe	84 $\pm$ 5	18 $\pm$ 2	5
100 nM-rhC5a	77 $\pm$ 8	3 $\pm$ 3	4
100 nM-LTB ₄	74 $\pm$ 8	9 $\pm$ 2	7
10 nM-A23187	7 $\pm$ 9	17 $\pm$ 3	7

known activators of 5-LO (including its natural substrate, AA) mobilize intracellular Ca^{2+} . Though a conclusive link has yet to be established between intracellular Ca^{2+} mobilization and leukotriene synthesis, Ca^{2+} has been shown to enhance 5-LO activity [11,35], as well as to promote the translocation of 5-LO to the cell membrane [10,13]. Consequently, the use of a substrate that causes an increase in intracellular Ca^{2+} to examine 5-LO activation by other agonists would entail inherent difficulties in interpretation. Neutrophils pre-loaded with the fluorescent Ca^{2+} probe fura-2 were incubated with the aforementioned fatty acids. As can be seen in Table 1, exogenous AA mobilized intracellular Ca^{2+} to a large extent, whereas 15-HpETE (up to 9.0 μ M) only had a modest effect. Results obtained with 15-HETE and Mead acid are also shown for comparison. Finally, since PMN incubated with an agonist and either 15-HpETE or AA as exogenous substrate synthesize 5,15-DiHETE or LTB₄ respectively, we also compared the relative abilities of exogenous LTB₄ and 5,15-DiHETE to mobilize intracellular Ca^{2+} . Table 1 clearly shows that both compounds are capable of causing intracellular Ca^{2+} levels to rise, but it is equally evident that 5,15-DiHETE is far weaker than LTB₄ in this respect. In agreement with our

observations, it has been recently reported that 8 μ M-5,15-DiHETE is without effect on the formation of 5-LO metabolites [36].

The Ca^{2+} -mobilizing properties of AA and of its major product (LTB₄) described above, together with the results of previous studies demonstrating that AA behaves in a similar manner to soluble neutrophil agonists and that it activates the 5-LO [37,38], led us to reject it as an exogenous substrate with which to assess 5-LO activation. We also rejected Mead acid and 15-HETE on the basis that they were found to be poor substrates for 5-LO. In fact, among the exogenous substrates tested, 15-HpETE was the only compound that was both a good 5-LO substrate and did not cause a marked increase in intracellular Ca^{2+} levels. These considerations led us to use 15-HpETE as an exogenous substrate in order to assess the 5-LO activation status.

Concentration-dependence and kinetics of the conversion of 15-HpETE into 5,15-DiHETE by human neutrophil 5-LO

We investigated the effect of substrate concentration on the 5-LO-dependent formation of 5,15-DiHETE. PMN were exposed to increasing concentrations (up to 9.0 μ M) of 15-HpETE, alone or in the presence of 10 nM Ca^{2+} ionophore A23187 for 5 min. A minor, concentration-dependent, synthesis of 5,15-DiHETE occurred when neutrophils were incubated with 15-HpETE alone (Fig. 2a); however, the co-addition of 10 nM-ionophore greatly enhanced 5,15-DiHETE formation. To determine the kinetics of 15-HpETE transformation into 5,15-DiHETE, neutrophils were incubated with 3.0 μ M-15-HpETE, either alone or with 10 nM-A23187, for increasing lengths of time (up to 15 min). When neutrophils were incubated with 15-HpETE alone, a rapid increase in 5,15-DiHETE synthesis occurred, which reached a plateau at about 3 min (Fig. 2b). Co-addition of 10 nM-ionophore led to greatly enhanced overall synthesis of 5,15-DiHETE; the pattern of synthesis, though amplified, was similar to that observed with 15-HpETE alone.

Concentration-dependence of the conversion of 15-HpETE into 5,15-DiHETE induced by neutrophil agonists

To investigate the effects of natural agonists on 5-LO activation, neutrophils were incubated for 15 min with increasing concentrations of PAF, the formylated peptide fMetLeuPhe or the complement fragment C5a, along with 3.0 μ M-15-HpETE. In

Fig. 4. Inhibition of agonist-induced 5-LO activation by MK-886

PMN (5×10^6 /ml) were pre-treated with increasing concentrations of MK-886 (1 min at 37°C) and subsequently incubated with $3.0 \mu\text{M}$ -15-HpETE, either alone (a, Δ) or with 100 nM-PAF-acether (a, $\blacktriangle$), 100 nM-fMetLeuPhe (b), 100 nM-rhC5a (c) or 20 nM-A23187 (d). Except in (a), the small 5,15-DiHETE levels resulting from exposure to 15-HpETE alone ($\pm$ MK-886) have been subtracted, so as to only depict the effect of the agonists. Each experiment is representative of several others (see Table 3 for the respective numbers of experiments); each data point is the mean $\pm$ S.E.M. of triplicate incubations.

addition, we examined a possible effect of two other compounds, the PAF precursor (and metabolite) lyso-PAF and the protein kinase C (PKC) agonist phorbol 12-myristate 13-acetate (PMA). In most experiments, the agonists investigated (up to 100 nM) induced a weak synthesis of 5-LO products from endogenous AA, although in amounts undetectable by RP-h.p.l.c. procedures (results not shown). In the presence of 15-HpETE, however, significant 5,15-DiHETE synthesis was observed with as little as 1 nM (or less) of the neutrophil agonists, which increased in a concentration-dependent manner (Fig. 3). In the case of C5a a maximal response was typically obtained at about 30 nM, a concentration reported to achieve saturation of C5a-binding sites [39], whereas the response to PAF or fMetLeuPhe typically started plateauing at the highest concentration used (100 nM). Similar results were obtained when neutrophils were stimulated by the AA-derived 5-LO metabolite LTB₄ (results not shown). In marked contrast with PAF, lyso-PAF (up to $1.0 \mu\text{M}$) had no significant effect on the formation of 5,15-DiHETE from 15-HpETE (results not shown), in agreement with its general lack of biological activity towards neutrophils. In a similar way, PMA (up to 320 nM) failed to stimulate any significant formation of 5,15-DiHETE (results not shown), which suggests that PKC does not contribute, at least directly, to the activation of 5-LO. These results confirm that stimulation of neutrophils by soluble

agonists leads to 5-LO activation, as suggested by numerous studies [19–21,38].

Effect of pertussis toxin and cholera toxin on the agonist-induced activation of 5-LO

It has been shown that pertussis-toxin-sensitive G-proteins are involved in the transmembrane signalling process whereby PAF, fMetLeuPhe, LTB₄ and C5a exert their various effects on neutrophil functions [28,40–42]. We therefore examined whether the activation of 5-LO induced by these physiological agonists was also mediated through G-proteins. Neutrophil suspensions were pre-incubated with either pertussis toxin or cholera toxin, and then exposed to $3.0 \mu\text{M}$ -15-HpETE alone, or together with 100 nM-PAF, -fMetLeuPhe, -rhC5a, -LTB₄ or 10 nM-A23187 for 15 min (Table 2). Pre-incubation with cholera toxin failed to inhibit the agonist-induced conversion of 15-HpETE into 5,15-DiHETE. In contrast, pre-incubation with pertussis toxin strongly inhibited physiological agonist-induced 5,15-DiHETE formation from 15-HpETE. Worthy of mention is the observation that pertussis toxin inhibition was consistently less marked in the case of PAF, in agreement with numerous observations that some of the effects of PAF are not mediated by pertussis-toxin-sensitive G-proteins [29,43,44]. Moreover, it was observed that pertussis toxin was without effect on the stimulation

Table 3. Inhibition of agonist-induced 5-LO activation by 100 nM-MK-886

PMN (5×10^6 /ml) were pre-treated with 100 nM-MK-886 (or diluent) and subsequently incubated for 15 min at 37 °C with 3.0 μ M-15-HpETE, either alone or with 5-LO agonists at the indicated concentrations. The percentage inhibitions have been calculated after having subtracted the 5,15-DiHETE synthesis resulting from exposure to 15-HpETE only ($\pm$ MK-886). Values are means $\pm$ S.E.M. of averaged triplicate determinations in the indicated numbers of experiments.

Stimulus	Inhibition (%)	n
100 nM-PAF	74 $\pm$ 3	11
100 nM-fMetLeuPhe	63 $\pm$ 3	5
100 nM-rhC5a	60 $\pm$ 10	2
100 nM-LTB ₄	71 $\pm$ 7	4
20 nM-A23187	84 $\pm$ 1	4

of 5-LO by 10 nM-A23187, suggesting that, under these experimental conditions, stimulation by ionophore proceeds differently from that induced by receptor-dependent agonists. Such results clearly demonstrate the involvement of pertussis-toxin-sensitive G-proteins in the sequence of events that lead to 5-LO activation induced by physiological agonists, and further suggest the existence of a common sequence of events in the stimulation of 5-LO and in other neutrophil functions.

Inhibition of agonist-induced 5-LO activation by MK-886

MK-886 has recently been described as a unique inhibitor of leukotriene synthesis in A23187-stimulated neutrophils, in that it acts via the inhibition of 5-LO translocation to the plasma membrane [15]. In addition, we have recently shown that MK-886 also inhibits leukotriene synthesis induced by PAF [45], indicating that natural agonists may also promote 5-LO translocation to membrane structures, although direct evidence for 5-LO translocation by receptor-mediated agonists has not yet been obtained. It must be emphasized that MK-886 does not interfere with 5-LO activity in cell-free preparations, and that its effects are observed only in intact cells; thus its mechanism of action appears to rely essentially on the prevention of 5-LO association to FLAP within the cell membrane [15,16]. We therefore undertook to determine whether pre-incubation of PMN with MK-886 would result in the inhibition of 5-LO activation induced by natural neutrophil agonists, as determined by the 15-HpETE assay. Neutrophils were pre-treated with increasing concentrations of MK-886 (up to 300 nM) and then incubated for 15 min with 3.0 μ M-15-HpETE, either alone or together with 100 nM-PAF, fMetLeuPhe, C5a or 20 nM-A23187. Fig. 4 shows that 100 nM-MK-886 strongly inhibited 5,15-DiHETE synthesis induced by PAF, fMetLeuPhe, C5a or A23187; similar results were obtained when 100 nM-LTB₄ was used as stimulus (results not shown). In addition, we found that 100 nM-MK-886 partially inhibited the small amounts of 5,15-DiHETE generated from 15-HpETE alone (Fig. 4a); such an inhibition was consistently observed throughout our experiments, and amounted to an average of 35.8 ± 4.3 % ($n = 12$). Table 3 summarizes the results of the numerous experiments performed with the neutrophil agonists.

The observation that MK-886 dose-dependently inhibits 5,15-DiHETE synthesis induced by natural agonists strongly suggests that 5,15-DiHETE formation from 15-HpETE in activated neutrophils reflects activation of 5-LO by these compounds in a process that is likely to involve translocation of the enzyme to membrane structures. It is noteworthy, however, that MK-886

did not cause a complete inhibition of natural-agonist- and ionophore-induced conversion of 15-HpETE into 5,15-DiHETE. This contrasts with previous studies in which pre-treatment with 100 nM-MK-886 totally abolished 5-LO product synthesis from AA (endogenous or exogenous) induced by PAF or A23187 [45]; it is most likely that the different nature and properties of the two substrates (i.e. 15-HpETE and AA) account for this discrepancy in the effects of MK-886. We further propose that the enzymic activity not inhibited by MK-886 in our experiments involving 15-HpETE reflects the activity of the cytosolic 5-LO. Indeed, we have observed previously that pre-treatment with the drug does not significantly alter the Ca²⁺-mobilizing properties of the 5-LO agonists under study [45]. In other words, the simultaneous presence of elevated Ca²⁺ levels and lipid hydroperoxides (in the form of 15-HpETE) in the cytosol may allow some catalytic activity by the 5-LO, as such conditions fulfil the major requirements for 5-LO activation. Finally, the moderate inhibition by MK-886 of the small amounts of 5,15-DiHETE generated from 15-HpETE alone is likely to reflect the presence of basal levels of active 5-LO in unstimulated neutrophils. Whether such basal levels of active enzyme are physiological in resting cells, or somehow result from experimental manipulations, is difficult to assess.

In summary, the data presented in the present paper demonstrate that the use of 15-HpETE as an exogenous substrate represents a useful approach with which to assess the neutrophil 5-LO activation status independently of endogenous AA availability. We have demonstrated that A23187, PAF, fMetLeuPhe and C5a (as well as LTB₄) produce a dose-dependent activation of 5-LO. It has also been shown that activation of 5-LO by natural agonists is mediated by pertussis-toxin-sensitive G-proteins, and our experiments with MK-886 have provided evidence that 5-LO activation induced by natural agonists is likely to involve translocation of the enzyme to the membrane. Finally, our results also lend strong support to the concept that, in neutrophils exposed to soluble inflammatory agents, leukotriene synthesis is ultimately regulated by substrate availability [20,30].

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