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Autocrine enhancement of leukotriene synthesis by endogenous leukotriene B_4 and platelet-activating factor in human neutrophils

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1 Platelet-activating factor (PAF) and leukotriene B_4 (LTB_4), two potent lipid mediators synthesized by activated neutrophils, are known to stimulate several neutrophil functional responses. In this study, we have determined that endogenous LTB_4 and PAF exert autocrine effects on LT synthesis, as well as the underlying mechanism involved.

2 Pretreatment of neutrophils with either pertussis toxin (PT), or with receptor antagonists for LTB_4 and PAF, resulted in an inhibition of LT synthesis induced by calcium ionophore, A23187. This inhibition was most marked at submaximal (100– 300 nM) A23187 concentrations, whilst it was least at ionophore concentrations which induce maximal LT synthesis (1–3 μM). Thus newly-synthesized PAF and LTB_4 can enhance LT synthesis induced by A23187 under conditions where the LT-generating system is not fully activated.

3 In recombinant human (rh) granulocyte-macrophage colony-stimulating factor (GM-CSF)-primed neutrophils, LT synthesis in response to chemoattractants (fMet-Leu-Phe or rhC5a) was also significantly inhibited by the LTB_4 receptor antagonist, and to a lesser extent by PAF receptor antagonists.

4 Further investigation revealed that LTB_4 and/or PAF exert their effects on LT synthesis via an effect on arachidonic acid (AA) availability, as opposed to 5-lipoxygenase (5-LO) activation. Indeed, the receptor antagonists, as well as PT, inhibited LT synthesis and AA release to a similar extent, whereas 5-LO activation (assessed with an exogenous 5-LO substrate) was virtually unaffected under the same conditions. Accordingly, we showed that addition of exogenous LTB_4 could enhance AA availability in response to chemoattractant challenge in rhGM-CSF-primed cells, without significantly affecting the 5-LO activation status.

5 Our data show that newly-generated PAF and LTB_4 have the ability to positively feedback on LT synthesis by acting at the level of the phospholipase A_2/re-esterification component of the LT biosynthetic pathway in neutrophils. Such autocrine affects are likely to represent an important amplification step of LT synthesis, and may as such contribute to the rapid onset, as well as to the evolution, of inflammatory responses.

Keywords: Arachidonate 5-lipoxygenase; phospholipase A_2; leukotriene B_4 receptor; PAF receptor; guanine nucleotide-binding proteins; pertussis toxin; receptor antagonists

Introduction

Leukotriene B_4 (LTB_4) and platelet activating factor (PAF) are potent activators of various phagocyte responses, such as chemotaxis, aggregation, adherence, degranulation, and oxygen radical generation (O'Flaherty *et al.*, 1981; Lad *et al.*, 1985; Filep & Foldes-Filep, 1990; Hirafuji & Shinoda, 1991), and are thus considered as important lipid mediators of inflammation. Both LTB_4 and PAF are synthesized in large amounts upon activation of polymorphonuclear leukocytes (PMN) by calcium ionophores such as A23187, or by phagocytic stimuli such as zymosan or inflammatory microcrystals (Borgeat & Samuelsson, 1979; Chilton *et al.*, 1984; Poubelle *et al.*, 1987; 1989; Riches *et al.*, 1990). Receptor-dependent soluble stimuli such as N-formyl-methionyl-leucyl-phenylalanine (fMLP), C5a and interleukin-8, as well as LTB_4 and PAF themselves, also have the ability to induce PAF synthesis, a process that is amplified following PMN treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF) (Tessner *et al.*, 1989; Wirthmueller *et al.*, 1989; Gomez-Cambronero *et al.*, 1989; Bussolino *et al.*, 1992; McDonald *et al.*, 1993). Similarly, receptor-dependent agonists induce a significant production of LTB_4 in PMN primed with GM-CSF (Dahinden *et al.*, 1988; McColl *et al.*, 1991; McDonald *et al.*, 1993).

Previous studies have indicated that PAF and LTB_4 may act as autocrine activators of cell functions. For instance, it has been reported that the polymerisation of actin in response to calcium ionophores can be prevented following PMN treatment with pertussis toxin (PT) or the LTB_4 receptor antagonist, LY-223982, or (to a lesser extent) with PAF receptor antagonists (Shefeyk *et al.*, 1985; Downey *et al.*, 1990). It has also been shown that selective 5-lipoxygenase (5-LO) inhibitors profoundly inhibit degranulation in macrophages undergoing phagocytosis, whilst exogenous LTB_4 or LTC_4 enhance degranulation, suggesting an autocrine effect of LTs on these processes (Lew *et al.*, 1991). Similarly, a recent study showed that PAF receptor antagonists inhibit LT synthesis and superoxide anion generation in response to fMLP in rabbit PMN (Stewart & Harris, 1991a). With respect to LT synthesis in human PMN, it is known that exogenous PAF and LTB_4 both have the ability to stimulate LT synthesis (Lin *et al.*, 1982; McDonald *et al.*, 1992). Accordingly, we have recently shown that PAF and LTB_4 are potent activators of the human neutrophil 5-LO (McDonald *et al.*, 1991; 1992). Finally, we have also reported that endogenous LTB_4 partially accounts for several of the stimulatory effects of exogenous arachidonic acid (AA) on human PMN, including calcium mobilisation and LT synthesis (McColl *et al.*, 1989; Naccache *et al.*, 1989). Together,

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these observations suggest that endogenous PAF and/or LTB_4 may modulate LT synthesis in an autocrine fashion.

PAF and LTB_4 exert their effects on PMN responses after binding to specific cell-surface receptors. Once occupied, these receptors interact with PT-sensitive, guanine nucleotide-binding proteins (G proteins) to simulate phospholipases A_2, C and/or D, resulting in the generation of second messengers, which participate in triggering functional responses. Thus an autocrine effect of endogenous PAF abd LTB_4 may take place at any of these levels. That LT synthesis ultimately depends upon precursor availability however, makes phospholipase A_2 (PLA_2) a likely target for such autocrine effects. In addition, the neutrophil 5-LO may represent another potential target for autocrine modulation of LT synthesis by PAF and LTB_4, since both products are potent 5-LO activators (McDonald *et al.*, 1991; 1992). Evidence for autocrine effects could be obtained by blocking either the first step of the signal transduction cascade (i.e. the LTB_4 or PAF receptors), or the second step (i.e. the PT-sensitive G proteins). In the current study, we used both approaches to characterize the autocrine effects of PAF and LTB_4 on LT synthesis in human PMN.

Methods

Cell separation

Blood from healthy donors was collected by venepuncture using heparin as anticoagulant. PMN were purified as previously described (Boyum, 1968), with modifications (McDonald *et al.*, 1992), and were resuspended at a final concentration of 5×10^6 PMN ml^{-1}, in HBSS buffered with HEPES (1 mM final). The percentage of PMN in the cell suspensions used in this study exceeded 95%, and cell viability was greater than 98%, as determined by trypan blue exclusion.

Cell incubations

Cell suspensions were supplied with $CaCl_2$ and $MgCl_2$ (1.6 mM and 1.0 mM final concentrations, respectively), and warmed to 37°C for 10 min prior to incubation with substrate and/or stimulus. All agonists were dissolved in dimethyl sulphoxide (DMSO), except for recombinant human (rh)C5a, which was dissolved in PBS plus 0.01% bovine serum albumen (BSA), and added to 0.1 ml aliquots of the cell suspension. The final concentration of DMSO (maximum of 0.3%) consistently failed to stimulate any detectable LT synthesis. The stock solution of 15(S)-hydroperoxy-5,8,11,13 (Z,Z,Z,E)-eicosatetraenoic acid (15-HpETE) (3 mM in ethanol) was diluted 10 fold in 0.6 mM sodium carbonate solution (to form the sodium salt of the fatty acid), and 10 µl of the resulting sodium salt solution was added to 1.0 ml of the cell suspensions, to yield a final concentration of 3.0 µM. After the desired incubation time with the stimuli, samples were processed for reverse-phase high performance liquid chromatography (r.p.-h.p.l.c.) analysis, as described earlier (McDonald *et al.*, 1992). When AA release was determined, reactions were stopped by the addition of 1.0 ml ice-cold methanol containing 25 ng 5,6,8,9,11,12,14,15-octadeutero-AA (D_8-AA) as internal standard.

In the experiments involving PT, cell suspensions were pre-incubated with the toxin at a final concentration of 0.5 µg ml^{-1} for 3 h at 37°C. During this time, the cells were swirled in a rotary water bath (New Brunswick Scientific, Edison, New Jersey, U.S.A.). Calcium and magnesium were added 15 min prior to stimulation. Pretreatment of PMN with rhGM-CSF (1 nM, for 30 min at 37°C) was also performed in this manner. These conditions were chosen from the results of previous studies, for optimal priming (McColl *et al.*, 1991; McDonald *et al.*, 1993). In the experiments involving LTB_4 or PAF receptor antagonists, or MK-886, the compounds were added to the cells 5 min prior to stimulation. The receptor antagonists and MK-886 were dissolved in DMSO.

Analysis of lipoxygenase products by r.p.-h.p.l.c.

Analysis of lipoxygenase products was performed by r.p.-h.p.l.c. as described previously, using an on-line extraction procedure (Borgeat *et al.*, 1990). The lower limit of detection was 0.2 ng at 280 nm and 1 ng at 229 nm.

Analysis of free AA by l.c.-m.s.

Denatured samples were processed by r.p.-h.p.l.c. as described previously (Borgeat *et al.*, 1990). AA-containing fractions were collected, evaporated under reduced pressure in a Speed Vac (model SVC 100D, Savant Instruments Inc., Farmingdale, NY, U.S.A.) and redissolved into 100 µl of acetonitrile. AA was assayed by liquid chromatography-mass spectrometry (l.c.-m.s.), using a nebuliser-assisted electrospray (ion spray) interface coupled to a triple-quadrupole m.s. (API-III, PE Sciex, Thornhill, Ontario, Canada). Aliquots (6 µl) of the samples were injected into the electrospray interface via the 20-µl loop of a Rheodyne injector (model 9125, Rheodyne, Cotati, CA, U.S.A.) connected to a short column (2 × 30 mm, packed with 5 µm octadodecylsilyl silica particles), using acetonitrile:H_2O (87.5:12.5, v:v, containing 0.1% acetic acid) as solvent, at a flow rate of 150 µl min^{-1}. Samples were analysed in negative ion mode. The ions at m/z 303 and 311 (representing the carboxylate anions of AA and D_8-AA, respectively) were monitored. Quantitation was performed using a calibration curve generated by l.c.-m.s. analysis of mixtures of AA and D_8-AA standards.

Statistical analyses

Where mentioned, statistical significance was assessed by Student's paired *t* test (one-tailed).

Materials

Calcium ionophore A23187, fMLP, HEPES, soybean lipoxygenase and PT were obtained from the Sigma Chemical Company (St-Louis, Missouri, U.S.A.). HBSS was from GIBCO (Burlington, Ontario, Canada), and all solvents were h.p.l.c. grade from Anachemia (Montréal, Québec, Canada). 15-HpETE was synthesized and purified as previously described (McDonald *et al.*, 1992). LTB_4 and the LT synthesis inhibitor, MK-886 (3-[3-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]2,2-dimethylpropanoic acid), were generous gifts from Dr Robert N. Young of the Merck-Frosst Centre for Therapeutic Research (Pointe-Claire, Québec, Canada). The PAF receptor antagonists, L-659,989 ((±)-trans-2-(3-methoxy-5-methylsulphonyl-4-propoxyphenyl)-5-(3,4,5-trimethoxyphenyl)tetrahydrofuran) and BN 50730 (tetrahydro-4,7,8,10-methyl-1-(chloro-2-phenyl)-6-(methoxy-4-phenylthiocarbamoyl)-9-pyrido[4',3'-4,5]thieno[3,2-f]triazolo-1,2,4[4,3-α] diazepine-1,4), were respectively provided by Dr W.H. Parsons of Merck Sharp & Dohme (Rahway, New Jersey, U.S.A.), and by Dr Pierre Braquet (Laboratoires Henri-Beaufour, Le Plessis-Robinson, France). The LTB_4 receptor antagonist, LY-223982 ((E)-5-(3-carboxybenzoyl)-2-{[6-(4-methoxyphenyl)-5-henenyl]oxy}benzenepropanoic acid), was obtained from Dr M.H. Niedenthal of Lilly Research Laboratories (Indianapolis, Indiana, U.S.A.). rhGM-CSF was donated by The Genetics Institute (Boston, Massachusetts, U.S.A.), and rhC5a was kindly provided by Dr Henry Showell of the Pfizer Central Research Center (Groton, Connecticut, U.S.A.).

Results

Autocrine effects of endogenous LTB_4 and PAF on A23187-induced LT synthesis

In the course of previous studies, we have observed that PT pretreatment partially inhibited LT synthesis in PMN challenged with submaximal A23187 concentrations, suggesting that the effect of the ionophore may involve G protein-dependent events. Since PAF and LTB_4 are actively synthesized upon ionophore stimulation, we investigated the possibility that a feedback stimulation by these products may account, at least partially, for the inhibitory effect of PT. PMN were pre-incubated with PT or its diluent, or with a combination of the LTB_4 receptor antagonist, LY-223982, and of the PAF receptor antagonist, BN 50730, and stimulated with increasing concentrations of A23187. These concentrations of the receptor antagonists were used on the basis of their being found to inhibit by ≈80% the elevation in free intracellular calcium elicited by either 30 nM LTB_4 or PAF (data not shown). Cells exposed to A23187 alone required a minimal dose of 100 nM to allow the detection of 5-LO products derived from endogenous AA, with a maximal response being achieved at between 1 and 3 μM A23187 (Figure 1). In cells pretreated with either PT or with the LTB_4 and PAF receptor antagonists however, the formation of all AA-derived 5-LO products (i.e. LTB_4, its ω-oxidation products, and the LTA_4 non-enzymatic degradation products) was markedly inhibited in the 100–300 nM range, the inhibition being less evident at A23187 concentrations which induced a maximal response. A parallel effect was consistently observed between the inhibition by PT and that by both receptor antagonists, suggesting that newly synthesized PAF and/or LTB_4 contribute to ongoing LT synthesis induced by submaximal concentrations of A23187, and that they account for most of the G protein-mediated effects of A23187 on LT synthesis.

To determine the respective contributions of endogenous PAF and LTB_4 to LT synthesis in ionophore-challenged PMN, we investigated the effect of the PAF receptor antagonists, BN 50730 and L-659,989, and of the LTB_4 receptor antagonist, LY-223982, either individually or in combination, as well as the effect of PT. At concentrations of A23187 which elicit a nearly maximal LT synthesis (i.e. 1.0 μM), the receptor antagonists had a moderate inhibitory effect on LT synthesis (Figure 2a). In contrast, when a submaximal dose of A23187 was used (i.e. 200 nM), pretreatment with individual receptor antagonists yielded a profound inhibition of LT synthesis (Figure 2b). The combination of BN 50730 and LY-223982, as well as PT pretreatment, consistently led to a somewhat greater inhibition of LT synthesis.

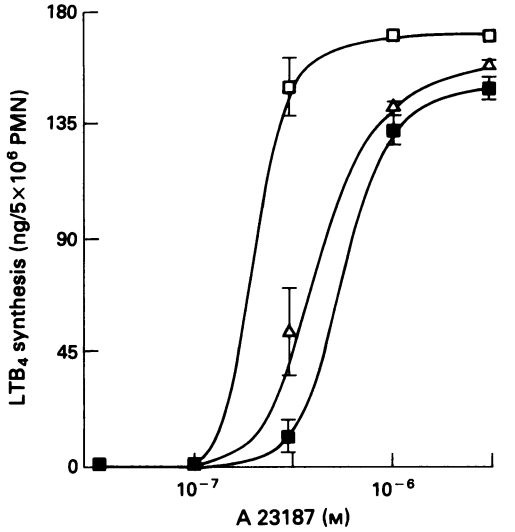

Figure 1 Concentration-dependence of A23187-elicited LTB_4 generation in human PMN, and the effect of pertussis toxin or of receptor blockade. PMN were stimulated (5 min, 37°C) with the indicated concentrations of A23187, following pre-incubation (3 h, 37°C) with 0.5 μg ml^{-1} PT (■), or with the PT diluent (□), or following pretreatment with a combination of 1.0 μM LY-223982 and 100 nM BN 50730 (▲). Samples were analysed by r.p.-h.p.l.c., as described in Methods. Mean ± s.e.mean of triplicate determinations from a single experiment representative of 4. The amounts of LTB_4 represent the sum of LTB_4 and of its ω-oxidation products, 20-hydroxy- and 20-carboxy-LTB_4. For abbreviations, see text.

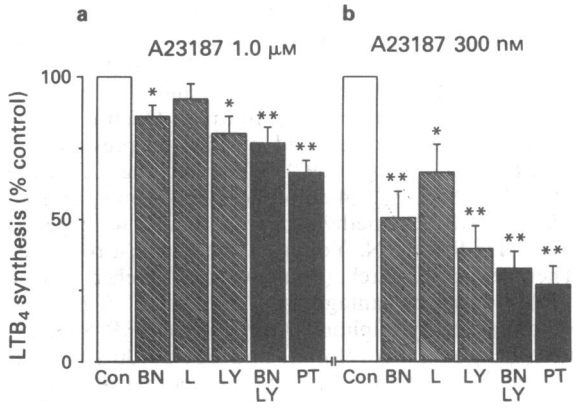

Figure 2 Effect of pertussis toxin, and of PAF and LTB_4 receptor antagonists, on A23187-induced LT synthesis in human PMN. PMN were stimulated (5 min, 37°C) with 1.0 μM (a) or 300 nM (b) A23187, in the presence or absence (5 min before stimulation) of 1.0 μM LY-223982 (LY), 100 nM BN 50730 (BN) or 100 nM L-659,989 (L), or following pre-treatment with PT (0.5 μg ml^{-1}, 3 h at 37°C). Samples were analysed by r.p.-h.p.l.c., as described in Methods. Mean ± s.e.mean of averaged triplicate determinations from at least 5 independent experiments. The 100% control (Con) values, representing the sum of LTB_4 and of its ω-oxidation products, averaged 162 ± 10 ng per 5 × 10^6 PMN (a, $n = 7$) and 123 ± 8 ng per 5 × 10^6 PMN (b, $n = 8$). *$P < 0.04$; **$P < 0.01$. For abbreviations, see text.

Table 1 Effect of pertussis toxin or of PAF and LTB_4 receptor antagonists on A23187-elicited 5-LO activation under conditions where neither PAF nor LTs are synthesized in detectable amounts

Compound	*5-LO activation* (% of control)	*Number of experiments*	P
BN 50730 100 nM	104.2 ± 6.6	3	0.296
L-659,989 100 nM	103.7 ± 4.1	3	0.234
LY-223982 1.0 μM	98.2 ± 4.3	5	0.347
BN + LY	101.7 ± 9.1	5	0.429
PT 0.5 μg ml^{-1}	92.7 ± 9.4	7	0.233

PMN suspensions were pretreated with the receptor antagonists (5 min, 37°C), or with PT (3 h, 37°C) and subsequently incubated for 10 min at 37°C with 3.0 μM 15-HpETE, either alone or with 30 nM A23187. The conversion of 15-HpETE into 5,15-DiHETE (reflecting 5-LO activation) was then determined by r.p.-h.p.l.c. analysis, as described in Methods. Comparisons of 5-LO activity (expressed as % of control) have been calculated after subtracting the 5,15-DiHETE levels resulting from exposure of PMN to 15-HpETE only (± antagonists or PT). Values are the mean ± s.e.mean from the indicated number of experiments, each performed in triplicate. The 100% control values averaged 21 ± 1 ng 5,15-DiHETE per 5 × 10^6 PMN for A23187 30 nM ($n = 7$). BN + LY, simultaneous exposure to 100 nM BN 50730 and 1.0 μM LY-223982. For abbreviations, see text.

To rule out possible nonspecific effects of the antagonists or of PT on 5-LO activity, we investigated whether these compounds would affect 5-LO activation induced by a low concentration (30 nM) of A23187, which allows 5-LO activation without triggering a detectable LTB_4 or PAF synthesis. For this purpose, we used exogenous 15-HpETE, which is converted into 5(S),15(S)-dihydroxy-6,8,11,13 (E,Z,Z,E)-eicosatetraenoic acid (5,15-DiHETE) by the 5-LO. We have shown previously that this approach allows the determination of 5-LO activation independently of endogenous substrate release (McDonald *et al.*, 1991). PMN were therefore incubated with 15-HpETE and/or 30 nM A23187, following treatment with the receptor antagonists or PT (Table 1). A23187 alone did not elicit the formation of 5-LO products or of PAF in detectable amounts (data not shown). Under all conditions tested, no significant inhibition of the A23187-induced transformation of 15-HpETE into 5,15-DiHETE (reflecting 5-LO activation) was observed. Similarly, the 5,15-DiHETE synthesis resulting from exposure of the cells to 15-HpETE alone was unaffected by prior treatment with the antagonists or with PT (data not shown).

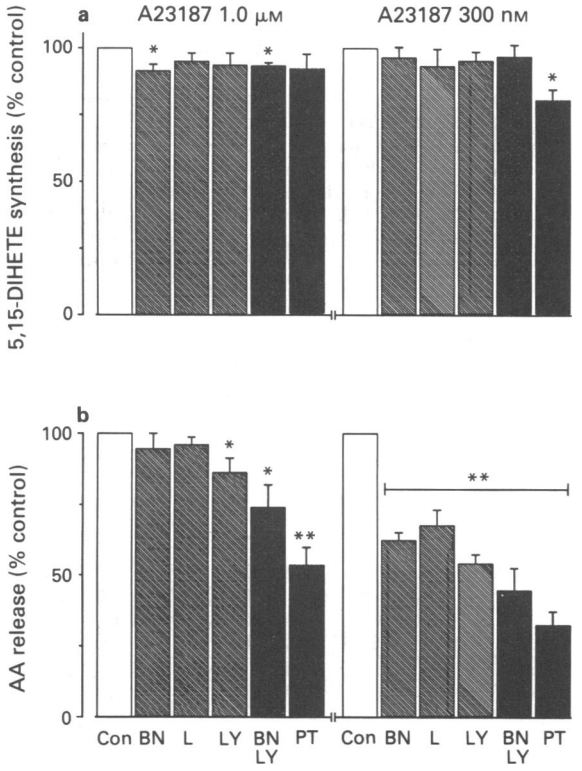

Figure 3 Effect of pertussis toxin, and of PAF and LTB_4 receptor antagonists, on A23187-induced 5-LO activation and AA release in human PMN. Incubation conditions are as in Figure 2. (a) Ionophore-induced 5-LO activation (as determined by the 5-LO-mediated conversion of exogenous 15-HpETE into 5,15-DiHETE). Samples were analysed by r.p.-h.p.l.c., as described in Methods. The given percentages have been calculated after subtraction of the 5,15-DiHETE synthesis resulting from exposures of PMN to 15-HpETE only (± receptor antagonists or PT). Mean ± s.e.mean of averaged triplicate determinations from at least 3 independent experiments. The 100% control (Con) values averaged 512 ± 33 ng 5,15-DiHETE per 5×10^6 PMN (left panel, $n = 3$) and 270 ± 31 ng per 5×10^6 PMN (right panel, $n = 5$). (b) Ionophore-induced AA release (2.5 min stimulations). Samples were analysed by l.c.-m.s., as described in Methods. Mean ± s.e.mean of averaged triplicate determinations from 5 independent experiments. The 100% control values averaged 203 ± 27 ng AA per 5×10^6 PMN (left panel, $n = 5$) and 94 ± 3 ng per 5×10^6 PMN (right panel, $n = 5$). BN, BN 50730; L, L-659,989; LY, LY-223982. $^*P < 0.04$; $^{**}P < 0.01$. For abbreviations, see text.

Effect of endogenous LTB_4 and PAF on A23187-induced 5-LO activation and AA release

Our previous observation, that both PAF and LTB_4 are potent 5-LO activators (McDonald *et al.*, 1991; 1992), led us to determine whether the amplification of A23187-induced LT synthesis by endogenous LTB_4 and PAF could take place at the 5-LO level. PMN were pretreated with PT or the receptor antagonists (individually or in combination), and then stimulated with 300 nM or 1.0 μM A23187, along with 15-HpETE. Figure 3a shows that regardless of the ionophore concentration used, PAF and/or LTB_4 receptor antagonists had no significant effect on A23187-induced conversion of 15-HpETE into 5,15-DiHETE in all but two cases, where a minor inhibition was noted, whilst PT had a moderate inhibitory effect at 300 nM A23187.

We next examined whether A23187-induced AA release would be affected upon pretreatment of the cells with the receptor antagonists or PT. In these experiments, PMN were stimulated with either 1.0 μM or 300 nM A23187. At the higher ionophore concentration, the PAF receptor antagonists failed to alter AA release significantly, whereas LY-223982 and PT exerted a moderate inhibition (Figure 3b, left panel). In contrast, all compounds potently inhibited AA release induced by 300 nM A23187 (Figure 3b, right panel).

Autocrine effects of endogenous LTB_4 and PAF on LT synthesis induced by chemoattractants in rhGM-CSF-primed PMN

Having shown that endogenous LTB_4 and PAF enhance A23187-induced LT synthesis in an autocrine fashion, we determined to what extent these neutrophil products may contribute to LT synthesis elicited under physiologically-relevant conditions. PMN were pretreated with rhGM-CSF or its diluent, and during the last 5 min of pre-incubation, the cells were exposed to the PAF and LTB_4 receptor antagonists, individually or in combination. Cells were then stimulated with either fMLP or rhC5a, and LT synthesis was measured. The effect of PT pretreatment was not investigated in these experiments, as it would have prevented PMN

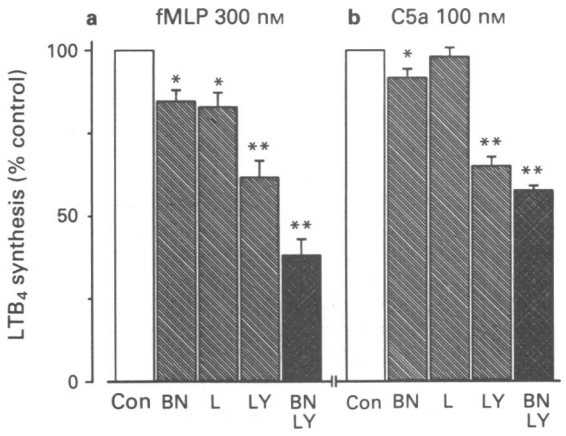

Figure 4 Effect of PAF and LTB_4 receptor antagonists on LT synthesis induced by fMLP (a) or rhC5a (b) in rhGM-CSF-primed PMN. PMN were pretreated with 1 nM rhGM-CSF or its diluent (30 min, 37°C) and stimulated for 10 min at 37°C with 300 nM fMLP or 100 nM rhC5a, in the presence or absence of 1.0 μM LT-223982 (LY), 100 nM BN 50730 (BN) or 100 nM L-659,989 (L) in the last 5 min of pre-incubation. Samples were analysed by r.p.-h.p.l.c., as described in Methods. Mean ± s.e.mean of averaged triplicate determinations from at least 4 independent experiments. The 100% control values (Con), representing the sum of LTB_4 and of its ω-oxidation products, averaged 2.1 ± 0.2 ng per 5×10^6 PMN (fMLP, $n = 10$) and 1.6 ± 0.2 ng per 5×10^6 PMN (C5a, $n = 5$). $^*P < 0.05$; $^{**}P < 0.003$. For abbreviations, see text.

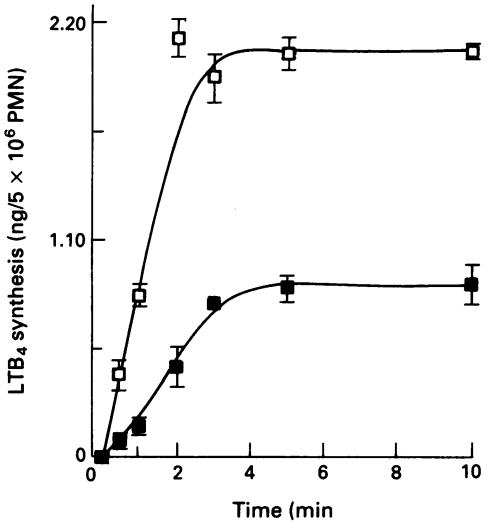

Figure 5 Effect of PAF and LTB_4 receptor antagonists on the time course of chemoattractant-induced LT synthesis in rhGM-CSF-primed PMN. PMN were pretreated with 1 nM rhGM-CSF or its diluent (30 min, 37°C) and stimulated with 300 nM fMLP for varying lengths of time, in the presence (■) or absence (□) of 1.0 μM LY-223982 and 100 nM BN 50730 in the last 5 min of pre-incubation. Samples were analysed by r.p.-h.p.l.c., as described in Methods. Mean ± s.e.mean of triplicate determinations from a single experiment representative of 4. The amounts of LTB_4 represent the sum of LTB_4 and of its ω-oxidation products. For abbreviations, see text.

stimulation by fMLP and C5a. Figure 4 shows that the PAF receptor antagonists exerted a significant but moderate inhibition of LT synthesis. In contrast, the LTB_4 receptor antagonist was found to inhibit potently LT synthesis, whilst the combination of both types of receptor antagonists yielded the highest degree of inhibition.

We next examined the effect of receptor antagonist pretreatment on the kinetics of chemoattractant-elicited LT synthesis in rhGM-CSF-primed cells. PMN were pretreated with rhGM-CSF, with or without a combination of LY-223982 and BN 50730 in the last 5 min of pre-incubation, and were stimulated with fMLP for increasing lengths of time. In the absence of the receptor antagonists, LTB_4 synthesis was detectable by 30 s, and reached a plateau between 2 and 5 min (Figure 5), depending on the donor. In the presence of the receptor antagonists, the pattern of synthesis was similar, with the exception that the initial rate of LT synthesis was lower, resulting in markedly decreased total amounts of LTB_4 at later time points.

To rule out possible nonspecific effects of the receptor antagonists on PMN stimulation by the chemoattractants, in particular at the level of 5-LO activation, we assessed the effect of these compounds on the 5-LO-mediated conversion of 15-HpETE into 5,15-DiHETE, under conditions where LT synthesis is not detected, i.e. in the absence of GM-CSF. PMN were incubated with 15-HpETE and/or 30 nM of fMLP, rhC5a, LTB_4 or PAF, following treatment with individual receptor antagonists (Table 2). By themselves, the agonists did not elicit the formation of 5-LO products from endogenous AA in detectable amounts (data not shown). Except for the expected blockade of the effect of LTB_4 by LY-223982, no significant inhibition of LTB_4-, rhC5a- or fMLP-elicited 5-LO activation by any antagonist was observed. In the case of PAF-induced 5-LO activation however, the LTB_4 receptor antagonist, LY-223982, was found to antagonize the response significantly, albeit less potently than the PAF receptor antagonists (see discussion).

Table 2 Effect of PAF and LTB_4 receptor antagonists on chemoattractant-elicited 5-LO activation under conditions where no detectable LT synthesis occurs

Conditions	*5-LO activation* (% of control)	*Number of experiments*	P
fMLP 30 nM + BN	99.3 ± 5.4	3	0.456
fMLP 30 nM + L	94.1 ± 2.6	3	0.078
fMLP 30 nM + LY	101.7 ± 10.7	4	0.441
C5a 30 nM + BN	101.5 ± 1.7	3	0.244
C5a 30 nM + L	96.9 ± 4.3	3	0.275
C5a 30 nM + LY	108.3 ± 6.6	3	0.170
LTB_4 30 nM + BN	98.7 ± 7.3	3	0.439
LTB_4 30 nM + L	97.7 ± 4.2	3	0.316
LTB_4 30 nM + LY	23.9 ± 2.6	4	0.001
PAF 30 nM + BN	7.6 ± 8.7	3	0.005
PAF 30 nM + L	21.1 ± 6.8	3	0.005
PAF 30 nM + LY	41.7 ± 3.7	3	0.002

PMN suspensions were pretreated with the receptor antagonists (5 min, 37°C), and subsequently incubated for 10 min at 37°C with 3.0 μM 15-HpETE, either alone or with the 5-LO agonists at the indicated concentrations. The conversion of 15-HpETE into 5,15-DiHETE (reflecting 5-LO activation) was then determined by r.p.-h.p.l.c. analysis, as described in Methods. Comparisons of 5-LO activity (expressed as % of control) have been calculated after subtracting the 5,15-DiHETE levels resulting from exposure of PMN to 15-HpETE only (± antagonists). Values are the mean ± s.e.mean from the indicated number of experiments, each performed in triplicate. The 100% control values, representing 5,15-DiHETE synthesis per 5×10^6 PMN, averaged 29 ± 1 ng (fMLP, $n = 4$), 35 ± 3 ng (C5a, $n = 4$), 33 ± 4 ng (LTB_4, $n = 4$), and 40 ± 2 ng (PAF, $n = 5$). BN, 100 nM BN 50730; L, 100 nM L-659,989; LY, 1.0 μM LY-223982. For abbreviations, see text.

Table 3 Effect of exogenous LTB_4 on AA release and 5-LO activation resulting from fMLP stimulation of rhGM-CSF-primed PMN

Added LTB_4	*AA release*[1] (% of control)	P	*5-LO activation*[2] (% of control)	P
10 nM	107.7 ± 4.4	0.077	–	–
100 nM	129.2 ± 7.1	0.007	101.4 ± 3.5	0.360

[1]PMN were pretreated with 1 nM rhGM-CSF or its diluent (30 min, 37°C) and stimulated for 2.5 min at 37°C with 300 nM fMLP, either alone or with exogenous LTB_4, in the presence of 100 nM MK-886 (added in the last 5 min of pre-incubation). Free AA levels were determined by l.c.-m.s., as described in Methods. Mean ± s.e.mean of averaged triplicate determinations from 5 independent experiments. [2]PMN were pretreated with 1 nM rhGM-CSF or its diluent (30 min, 37°C) and stimulated for 10 min at 37°C with 300 nM fMLP, either alone or with exogenous LTB_4, in the presence of 3.0 μM 15-HpETE. The conversion of 15-HpETE into 5,15-DiHETE (reflecting 5-LO activation) was then determined by r.p.-h.p.l.c. analysis, as described in Methods. The % control have been calculated after subtraction of the 5,15-DiHETE synthesis resulting from exposure of PMN to 15-HpETE only. Mean ± s.e. mean of averaged triplicate determinations from 3 independent experiments. For abbreviations, see text.

Effect of endogenous LTB_4 and PAF on chemoattractant-induced 5-LO activation and AA release in rhGM-CSF-primed PMN

We next investigated whether the autocrine amplification of chemoattractant-induced LT synthesis by endogenous LTB_4 or PAF occurred at the 5-LO level, or at the level of AA

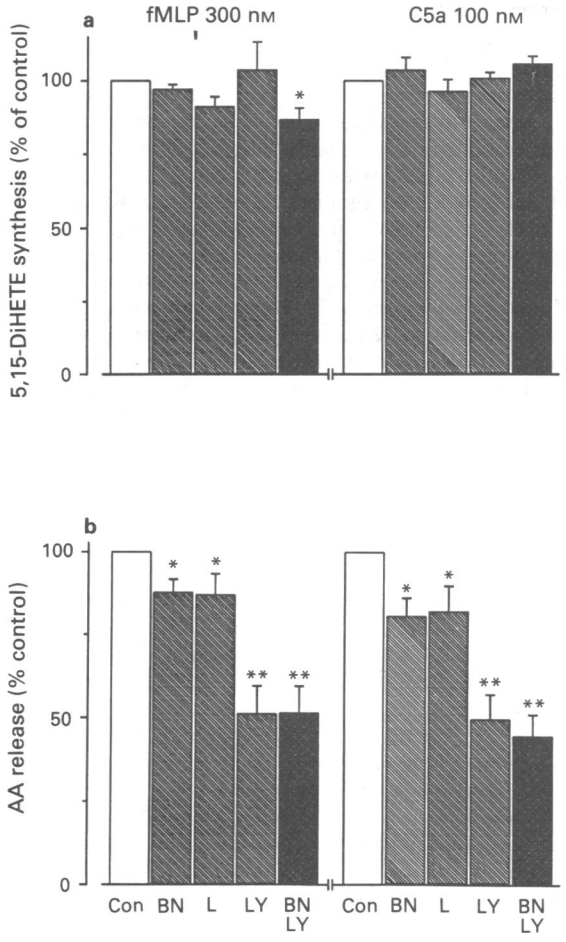

Figure 6 Effect of PAF and LTB_4 receptor antagonists on 5-LO activation and AA release induced by chemoattractants in rhGM-CSF-primed PMN. Incubation conditions are as in Figure 4. (a) Chemoattractant-induced 5-LO activation (as determined by the 5-LO-mediated conversion of exogenous 15-HpETE into 5,15-DiHETE). Samples were analysed by r.p.-h.p.l.c., as described in Methods. The given percentages have been calculated after subtraction of the 5,15-DiHETE synthesis resulting from exposure of PMN to 15-HpETE only ($\pm$ receptor antagonists). Mean $\pm$ s.e.mean of averaged triplicate determinations from at least 3 independent experiments. The 100% control values (Con) averaged 98 ± 9 ng, 5,15-DiHETE per 5×10^6 PMN (fMLP, $n = 6$) and 94 ± 11 ng per 5×10^6 PMN (C5a, $n = 4$). (b) Chemoattractant-induced AA release (2.5 min stimulations). Samples were analysed by l.c.-m.s., as described in Methods. Mean $\pm$ s.e.mean of averaged triplicate determinations from at least 4 independent experiments. The 100% control values (Con) averaged 24 ± 2 ng AA per 5×10^6 PMN (fMLP, $n = 5$) and 17 ± 2 ng per 5×10^6 PMN (C5a, $n = 4$). BN, BN 50730; L, L-659,989; LY, LY-223982. $^*P < 0.05$; $^{**}P < 0.003$. For abbreviations, see text.

availability. PMN were pretreated with rhGM-CSF, in the presence or absence of the receptor antagonists in the last 5 min of pre-incubation, and then stimulated with either fMLP or rhC5a, along with 15-HpETE. Figure 6a shows that chemoattractant-elicited 5-LO activation was slightly inhibited only in the case of fMLP-treated cells exposed to the combined antagonists for LTB_4 and PAF. In a second series of experiments, PMN were pre-incubated and stimulated as described above, and AA release was determined. As observed for LT synthesis under the same conditions, the PAF receptor antagonists were markedly less potent than the LTB_4 receptor antagonist in inhibiting AA release (Figure 6b).

Effect of exogenous LTB_4 on chemoattractant-induced AA release and 5-LO activation in rhGM-CSF-primed PMN

Finally, we examined whether the autocrine amplification by LTB_4 of chemoattractant-induced AA release could be mimicked by use of exogenous LTB_4. PMN primed with rhGM-CSF were stimulated with fMLP, in the presence or absence of added LTB_4, and AA release was determined. To ensure that changes in AA availability would exclude the participation of endogenous LTB_4, these experiments were performed in the presence of the LT synthesis inhibitor, MK-886. The combined presence of fMLP and of 100 nM LTB_4 resulted in significantly increased free AA levels, relative to cells stimulated with fMLP only (Table 3). In the same experiments, we also examined the effect of exogenous LTB_4 on 5-LO activation under the same conditions, using 15-HpETE as exogenous substrate. No significant effect of exogenous LTB_4 could be demonstrated (Table 3).

Discussion

In recent years, it has been proposed that since PAF and LTB_4 are both neutrophil products and activators, these lipid mediators may exert autocrine effects on neutrophil functions (O'Flaherty, 1985; Stewart *et al.*, 1990; Stewart & Harris, 1991a; Lew *et al.*, 1991). In particular, we recently reported that endogenous LTB_4 accounts, at least partially, for the induction of calcium mobilisation and LT synthesis by exogenous AA in human PMN (McColl *et al.*, 1989; Naccache *et al.*, 1989). We now report that newly-generated LTB_4 and PAF both have the ability to stimulate LT synthesis, and that this autocrine amplification of LT synthesis occurs primarily at the level of AA availability.

Evidence for an autocrine effect of LTB_4 and PAF on LT synthesis was first obtained under conditions where both compounds are synthesized in large amounts, i.e. upon A23187 challenge of PMN. The LTB_4 receptor antagonist, LY-223982 (a dicarboxylic benzophenone derivative), and the structurally unrelated PAF receptor antagonists, BN 50730 (a triazolodiazepine derivative) and L-659,989 (a tetrahydrofuran derivative), as well as PT, all markedly inhibited LT synthesis in PMN stimulated by 100–300 nM A23187. This indicated that endogenously-generated LTB_4 and/or PAF could substantially amplify LT synthesis at submaximal A23187 concentrations, after binding to their cell-surface receptors, in a process involving PT-sensitive G proteins. In comparison, PT or the receptor antagonists had but a moderate effect on LT synthesis induced by micromolar A23187 concentrations, suggesting that newly synthesized PAF and LTB_4 cannot further stimulate the LT-generating system once it is strongly activated. Consistent with this conclusion is the fact that no greater inhibition was observed when cells were exposed to 10 fold higher concentrations of the receptor antagonists (data not shown). In a second series of experiments, we examined the effect of the PAF and LTB_4 receptor antagonists on LT synthesis under physiologically-relevant stimulatory conditions, using chemoattractants in GM-CSF-primed PMN. Under these conditions, the total quantities of LTB_4 being generated (and accordingly, the amount of AA being released) were much smaller than those yielded in ionophore-challenged neutrophils. Given the known requirement for calcium of both 5-LO and PLA_2 activities, this difference may reflect the fact that chemoattractants (even following GM-CSF treatment of PMN) induce calcium transients of relatively small amplitude, in comparison to the larger and sustained increase in intracellular calcium that occurs following A23187 stimulation of neutrophils. In GM-CSF-treated PMN stimulated by chemotactic agonists, it was also found that both endogenous LTB_4 and PAF can modulate LT synthesis, with endogenous LTB_4 being implicated to a greater extent than PAF. This

contrasts again with the results obtained with PMN stimulated by submaximal concentrations of A23187, where PAF and LTB_4 appeared equally effective in enhancing LT synthesis. Such a difference could be related to the fate of endogenous PAF, depending upon the type of cell stimulation. Indeed, it was reported that most of the PAF generated upon A23187 challenge remains intracellular, whereas newly-synthesized PAF is mostly released upon activation of GM-CSF-primed PMN by chemoattractants (Riches *et al.*, 1990; DeNichilo *et al.*, 1991; Stewart & Harris, 1991b). In light of many reports which suggest that the PAF receptors are located intracellularly (Hwang & Lam, 1991; Rogers *et al.*, 1991), the intracellular retention of endogenous PAF occurring in A23187-stimulated PMN could facilitate its interaction with its receptors, thereby providing a basis for the autocrine effects of PAF on LT synthesis under such conditions.

LT synthesis principally relies on the activity of two key enzymes, 5-LO and PLA_2. By investigating the effect of the combined receptor antagonists on the time course of LT synthesis in respect to fMLP, it was shown that endogenous LTB_4 and PAF modulate LT synthesis by increasing the initial synthesis rate, which results in greater synthesis levels overall. Similar observations were made on PMN undergoing LT synthesis in response to A23187 (data not shown). This suggested a stimulatory effect on the enzymatic activity of either PLA_2 or 5-LO, or both. We therefore examined which step may be modulated by endogenous PAF and LTB_4. It was found that prior treatment with receptor antagonists or PT had but a slight effect on 5-LO activation under the same stimulatory conditions where they inhibited LT synthesis, be it in response to A23187, or to chemoattractants. These observations indicate that the autocrine amplification of LT synthesis by LTB_4 and PAF probably does not occur at the level of the 5-LO. In contrast, the receptor antagonists and PT inhibited AA availability to a similar extent as they affected LT synthesis under the same stimulatory conditions. Thus endogenous LTB_4 and PAF amplify LT synthesis mainly through enhanced AA availability, implying that they act by stimulating PLA_2 activation and/or by inhibiting the re-esterification of released AA, thereby enhancing its availability for conversion into LTs. That exogenous LTB_4 enhanced the AA availability resulting from fMLP stimulation of GM-CSF-primed PMN, while 5-LO activation remained unaffected in the same experiments, further supports this view. Consistent with our conclusion, it was recently reported that 5-LO activation is nearly maximal under stimulatory conditions which constitute a threshold for PLA_2 activity in human PMN (Schatz-Munding *et al.*, 1991).

The exact mechanism whereby endogenous PAF and LTB_4 enhance AA availability remains to be determined. On the one hand, it is known that AA release is associated with the calcium-dependent translocation of cytosolic PLA_2 to cell membranes in human PMN, macrophages and U937 cells (Diez & Mong, 1990; Goldman *et al.*, 1992; Smith & Waite, 1992). However, the mobilization of intracellular calcium induced by 100 nM ionomycin (a non-fluorescent calcium ionophore used instead of A23187 in the fura-2 calcium mobilization assay) is only marginally affected by PAF and LTB_4 receptor antagonists in PMN (P.P.McD., unpublished data), whereas the latter potently inhibit LT synthesis under the same conditions. Thus it does not appear likely that endogenous PAF and LTB_4 could increase cytosolic PLA_2 translocation to an extent sufficient to account for their modulatory effects on LT synthesis. On the other hand, it has been recently reported that phosphorylation of the PLA_2 by the microtubule-associated protein kinase or MAPK (a serine-threonine protein kinase) positively correlates with its enzymatic activity (Wijkander & Sundler, 1992; Xing & Mattera, 1992; Nemenoff *et al.*, 1993). Given the known ability of PAF and LTB_4 to induce tyrosine phosphorylation of several PMN proteins (Dryden *et al.*, 1992), and in particular that of the neutrophil MAPK (Gomez-Cambronero *et al.*, 1992), resulting in its activation, it is possible that endogenous PAF and LTB_4 could enhance LT synthesis through increased PLA_2 phosphorylation.

In investigating cell responses, conclusions based upon the use of pharmacological tools must take into consideration the generally limited specificity of these compounds. Hence, numerous control experiments were performed in the current study to rule out the possibility that nonspecific effects of PT or of the receptor antagonists could account for their inhibition of LT synthesis. First, we examined the effect of the compounds on 5-LO activation in response to 30 nM A23187 – an ionophore concentration that does not elicit a detectable synthesis of LTB_4 or PAF. Under these conditions, where autocrine effects of PAF and LTB_4 are unlikely to occur, neither the receptor antagonists nor PT significantly affected the 5-LO-mediated conversion of 15-HpETE into 5,15-DiHETE. Therefore, the inhibition of LT synthesis by these compounds seen at higher ionophore concentrations (100–300 nM) is not likely to reflect nonspecific inhibitory effects of the antagonists or of PT at the level of the 5-LO. Similarly, to ensure that the inhibition by the receptor antagonists of chemoattractant-induced LT synthesis was not due to their interfering with GM-CSF priming, we compared the effect of adding the antagonists either before GM-CSF, or in the last 5 min of pre-incubation with the cytokine. That no significant difference was noted (data not shown) is consistent with a recent report, which excluded the participation of endogenous LTB_4 or PAF in the priming of LT synthesis by GM-CSF (Stewart *et al.*, 1991). Additionally, to ascertain that the inhibition of chemoattractant-induced LT synthesis by the receptor antagonists did not reflect a possible interference with the fMLP or C5a receptors, we assessed the effect of these compounds on 5-LO activation induced by 30 nM fMLP or rhC5a in the absence of GM-CSF, i.e. under conditions where LT synthesis is not detected. Since no significant inhibition of the 5-LO-mediated conversion of 15-HpETE into 5,15-DiHETE was observed, it can be concluded that the receptor antagonists exert their inhibitory effects on LT synthesis neither through nonspecific blockade of C5a or fMLP receptors, nor at the level of the post-receptor events which lead to 5-LO activation. Similarly, the PAF receptor antagonists did not affect 5-LO activation in response to 30 nM LTB_4, thus further confirming their specificity. In contrast, 5-LO activation induced by 30 nM PAF was significantly antagonized by the LTB_4 receptor antagonist LY-223982, albeit to a lesser extent than when 10 fold lower concentrations of PAF receptor antagonists were used. The mobilization of intracellular calcium in response to PAF was also similarly inhibited by LY-223982 (data not shown). This is in agreement with a recent study, which reported that concentrations of LY-223982 similar to those used in the current study antagonized PMN chemotaxis towards PAF (Boyd & Jackson, 1988). Thus the inhibitory properties of LY-223982 on LT synthesis may partially reflect a contribution of PAF to LT synthesis. This is of little consequence insofar as the results obtained with chemoattractants in GM-CSF-primed PMN are concerned, since the PAF antagonists inhibited LT synthesis only slightly. The autocrine effects observed under such conditions therefore remain mostly attributable to endogenous LTB_4. In the case of A23187 stimulation, it also remains likely that the inhibitory effects of LY-223982 principally reflect the blockade of LTB_4 receptors, since the BN 50730 plus LY-223982 combination achieved a higher degree of inhibition than that seen with LY-223982 alone. Finally, it must be stated that the receptor antagonists and/or PT could possibly exert nonspecific effects at the level of PLA_2 activation. Nevertheless, the minor inhibition exerted by the antagonists or PT on LT synthesis induced by 3 μM A23187 (Figure 1) suggests that eventual nonspecific effects at the level of AA availability be equally minor. That 10 fold higher concentrations of the receptor antagonists failed to induce a significantly greater inhibition of LT synthesis under the same conditions, further supports this assertion.

In summary, the preceding findings show that in activated PMN, the autocrine enhancement of ongoing LT synthesis by endogenous PAF and/or LTB_4 represents an important amplification mechanism of LT synthesis, regardless of the type of stimulus used. Consistent with this assertion is that both PAF and LTB_4 receptor antagonists inhibit LT synthesis in response to particulate PMN agonists, namely zymosan and serum-opsonized zymosan (P.P.McD., unpublished data). Therefore, the modulatory effects of endogenous PAF and LTB_4 described herein are likely to be a general feature of LT synthesis regulation in PMN. That such autocrine effects take place at the level of AA availability raises the possibility that PAF synthesis may also be subject to autocrine stimulation by newly-generated LTB_4 and PAF, should PLA_2 be the actual target. Accordingly, it was recently reported that the PAF receptor antagonist, WEB 2086, inhibits the formation of PAF in response to fMLP (Müller & Nigam, 1992). In a broader context, the autocrine stimulation by LTB_4 and PAF of ongoing PMN responses may represent a general characteristic of neutrophil activation.

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