Biochemical Pharmacology, Vol. 38, No. 15, pp. 2559–2563, 1989.
Printed in Great Britain.

0006-2952/89 \$3.00 + 0.00
Maxwell Pergamon Macmillan plc

SYNERGISM BETWEEN CHEMOTACTIC PEPTIDE AND PLATELET-ACTIVATING FACTOR IN STIMULATING THROMBOXANE B_2 AND LEUKOTRIENE B_4 BIOSYNTHESIS IN HUMAN NEUTROPHILS

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(*Received* 2 *August* 1988; *accepted* 13 *December* 1988)

Abstract—Formyl-Met-Leu-Phe (FMLP) and platelet-activating factor (PAF) were capable of stimulating thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB_4) syntheses in human neutrophils, albeit in a relatively poor degree. A combination of FMLP and PAF, however, was synergistic in stimulating TXB_2 and LTB_4 syntheses. Phorbol myristate acetate (PMA) appeared to attenuate PAF- but not FMLP-induced arachidonate metabolism. These results suggest that cooperative action of FMLP and PAF on arachidonate release and metabolism does exist and that PMA-mediated protein kinase C activation may regulate FMLP and PAF actions in a different manner.

Polymorphonuclear neutrophils release superoxide anion, granular enzymes and arachidonate metabolites of cyclooxygenase and lipoxygenase pathways when challenged with chemotactic peptide (*N*-Formyl-Met-Leu-Phe, FMLP) or platelet-activating factor (PAF) [1–4]. Although release of superoxide anion and granular enzymes may not be mediated by arachidonate metabolites [5], these fatty acids may modulate the superoxide generating ability and degranulating actions of FMLP and PAF since inhibitors of arachidonate metabolism, particularly of the lipoxygenase pathway, attenuate neutrophil responses [6, 7]. In fact, LTB_4 itself shows degranulating activity [8], and 5-hydroxyeicosatetraenoic acid (5-HETE) potentiates degranulating and superoxide generating responses to PAF and phorbol myristate acetate (PMA) [9–12]. A role for arachidonate metabolites, particularly lipoxygenase-derived products, in neutrophil functions appears to be evident.

Experiments *in vitro* have also shown that FMLP and phagocytic stimuli induce the release of PAF from human neutrophils [13, 14]. *In vivo*, at sites of bacterial invasion, it is therefore expected that neutrophils which respond to chemotactic molecules are also exposed to endogenous stimuli such as PAF. Studies have revealed that exogenous FMLP may work in concert with endogenously released PAF to exhibit synergism in the activation of neutrophils, as shown by an increase in respiratory burst [15]. Whether such a synergism between FMLP and PAF is also found in the production of arachidonate metabolites remains to be determined. Our results as described herein indicate that there is a marked synergism between FMLP and PAF in stimulating arachidonate release and metabolism in human neutrophils.

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MATERIALS AND METHODS

Materials. Formyl-Met-Leu-Phe (FMLP), platelet-activating factor (PAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphorylcholine), 4β-phorbol 12-myristate 13-acetate (PMA), bovine serum albumin (BSA) and Histopaque 1077 were obtained from the Sigma Chemical Co. (St Louis, MO). Thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB_4) were supplied by the Upjohn Co.

Preparation and incubation of neutrophils. Preparation of human neutrophils was carried out as described previously [16]. Briefly, 0.5 volume of 3% dextran–saline solution was added to human venous blood (30 ml) anticoagulated with 0.1 volume of 3.8% sodium citrate and left for 1 hr at room temperature. The leukocyte rich upper layer was removed and centrifuged at 400 *g* for 10 min. The pellet was resuspended in Gey's medium containing 2% BSA and treated with hypotonic solution for 20 sec at 4° followed by centrifugation at 400 *g* for 10 min. The cell pellet resuspended in 12 ml of Ca^{2+}, Mg^{2+}-free Hanks' buffer was overlayered onto 3 ml of Histopaque 1077 solution and centrifuged at 550 *g* for 30 min. The neutrophil pellet was washed and finally resuspended in Hanks' buffer. An aliquot (1 ml) of the neutrophil suspension (2×10^6 cells/ml) was used in various incubations. Incubation of neutrophils with FMLP, PAF, or FMLP plus PAF was carried out at 37° for 5 min at indicated concentrations of each agonist and was terminated by placing the sample in ice followed by centrifugation at 1900 *g* for 10 min at 4°. The supernatant fraction was removed and used for assays of TXB_2 and LTB_4.

Radioimmunoassays of TXB_2 and LTB_4. Levels

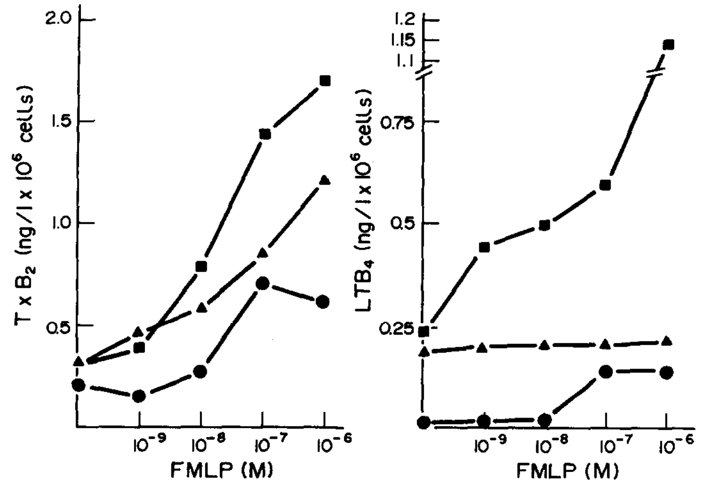

Fig. 1. Effects of FMLP, FMLP plus PAF, and FMLP plus PMA on TXB_2 and LTB_4 syntheses in human neutrophils. Neutrophil suspension was incubated with increasing concentrations of FMLP in the absence (—●—) and presence of 1 μM PAF (—■—) or 1 μM PMA (—▲—) as described in Materials and Methods. The amounts of TXB_2 and LTB_4 were determined by the respective radioimmunoassay. Values are the average of two determinations. The data are representative of two separate experiments with qualitatively similar results.

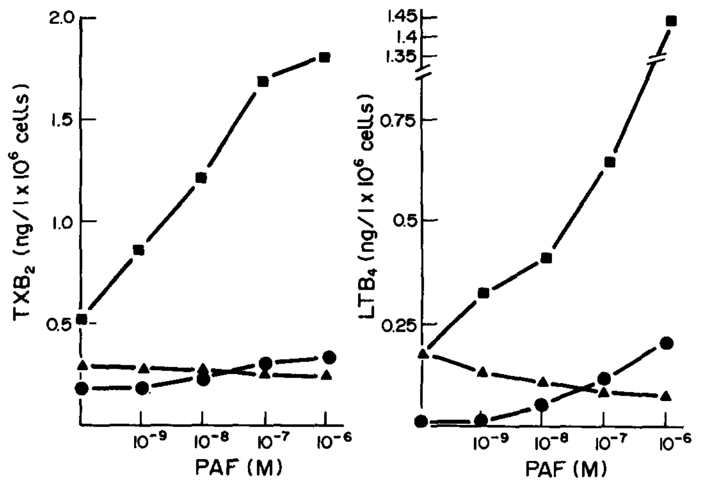

Fig. 2. Effects of PAF, PAF plus FMLP, and PAF plus PMA on TXB_2 and LTB_4 syntheses in human neutrophils. Neutrophil suspension was incubated with increasing concentrations of PAF in the absence (—●—) and presence of 1 μM FMLP (—■—) or 1 μM PMA (—▲—) as described in Materials and Methods. The amounts of TXB_2 and LTB_4 were determined by the respective radioimmunoassay. Values are the average of two determinations. The data are representative of two separate experiments with qualitatively similar results.

of TXB_2 and LTB_4 were determined by specific radioimmunoassays as described previously [17, 18]. Sensitivity for TXB_2 and LTB_4 assays was 5 and 10 pg/tube, respectively.

RESULTS

Chemotactic peptide, FMLP, stimulated human neutrophils to release TXB_2 and LTB_4 as shown in Fig. 1. Maximal stimulation was achieved at 10^{-7} M. Similarly, PAF also stimulated neutrophils dose responsively to release TXB_2 and LTB_4 (Fig. 2). Both FMLP and PAF appeared to be weak agonists compared to A-23187 [4 5]. However, addition of 10^{-6} M PAF to increasing concentrations of FMLP resulted in a synergistic stimulation of TXB_2 and LTB_4 synthesis (Fig. 1). Similarly, addition of 10^{-6} M FMLP to increasing concentrations of PAF also enhanced markedly TXB_2 and LTB_4 synthesis (Fig. 2). Enhancement of eicosanoid synthesis by one agonist over the other could be seen at concentrations as low as 10^{-9} M. Another agonist, PMA, which also stimulates neutrophil degranulation and respiratory burst, showed an additive effect on

Table 1. Interaction among three agonists of human neutrophils in stimulating TXB_2 and LTB_4 biosyntheses

Agonist	PAF		FMLP		PMA	
	TXB_2 (ng/10^6 cells)	LTB_4	TXB_2 (ng/10^6 cells)	LTB_4	TXB_2 (ng/10^6 cells)	LTB_4
None	0.30 ± 0.01	0.23 ± 0.01	0.59 ± 0.04	0.15 ± 0.01	0.30 ± 0.01	0.18 ± 0.01
PMA	0.24 ± 0.01	0.07 ± 0.01	1.16 ± 0.08	0.22 ± 0.01		
FMLP	1.73 ± 0.04	1.28 ± 0.09				

Neutrophil suspension was incubated with PAF (1 μM), FMLP (1 μM), PMA (1 μM) or a combination of two of the above agonists for 5 min as described in Materials and Methods.

The amounts of TXB_2 and LTB_4 released were determined by the respective radioimmunoassay. Values are means ± SEM (N = 3).

FMLP-induced TXB_2 and LTB_4 syntheses but exhibited inhibition of PAF-induced eicosanoid synthesis. A summary of the interaction among the three agonists is shown in Table 1. A synergism between FMLP and PAF in stimulating TXB_2 and LTB_4 syntheses and an attenuation of PAF- but not FMLP-induced arachidonate metabolism by PMA were clearly observed.

DISCUSSION

Both FMLP and PAF are known to stimulate degranulation and respiratory burst in the neutrophil [1–4]. A combination of FMLP and PAF or LTB_4 has been shown to exhibit synergism in respiratory burst [15]. It has been suggested that PAF may prime the cells for the subsequent respiratory response to FMLP and vice versa [12]. Our results on FMLP and PAF induced arachidonate metabolism also indicate that one agonist can potentiate the other in stimulating TXB_2 and LTB_4 syntheses in neutrophils. Either FMLP or PAF alone has been shown previously to induce arachidonate release [4, 19]. Synergistic stimulation of TXB_2 and LTB_4 syntheses was evidently the consequence of activation of arachidonate release induced by a combination of both agonists. Accordingly, not only LTB_4 synthesis was increased, but 5-HETE production from the same pathway should be enhanced. Although there is some concern that TXB_2 synthesis may be due, in part, to contaminated platelets, this is considered not likely for two reasons. First, FMLP is not an agonist for platelets. Consequently, TXB_2 synthesis induced by FMLP should be derived from neutrophils. Second, although PAF is a potent agonist for platelets, it induced TXB_2 synthesis only to a small extent in our preparation. Therefore, synergistic stimulation of TXB_2 synthesis by a combination of PAF and FMLP is due primarily to neutrophils.

How one agonist can prime the neutrophils and facilitate the activation by another agonist is not clear. Both FMLP and PAF have been shown to activate phospholipase C, catalyzing the hydrolysis of phosphatidylinositol-4,5-bisphosphate to diacylglycerol and inositol-1,4,5-triphosphate [20, 21], and to stimulate arachidonate release and metabolism as shown in this and other studies [5, 22]. Diacylglycerol can activate protein kinase C [23], whereas inositol-1,4,5-triphosphate can mobilize the intracellular store of Ca^{2+} [24]. Several laboratories have reported that suboptimal amounts of an activator of protein kinase C, such as PMA, and a Ca^{2+} ionophore, such as A-23187, show marked synergism in activating the respiratory burst [25, 26] and degranulation [27] as well as in stimulating arachidonate release [28, 29] when added to cells together. Subsequently, it was demonstrated, in neutrophils, that the Ca^{2+} ionophore could be replaced by 5-HETE [11]. In fact, it was also shown that 5-HETE stimulates neutrophils to elavate cytosolic Ca^{2+} and enhances the action of a diacylglycerol in stimulating the cells to translocate protein kinase C from cytosol to membranes [30]. Therefore, the initial surge of Ca^{2+} elevation and protein kinase C activation induced by FMLP and PAF may be amplified further by the released 5-HETE. As a release of arachidonate from the membrane phospholipids by either the phospholipase A_2 [19] or the phospholipase C-lipases [31, 32] pathways is a Ca^{2+}-dependent process, more arachidonate will be released as a consequence of amplified Ca^{2+} mobilization. Furthermore, protein kinase C activation has been shown to inhibit arachidonate reincorporation into phospholipids, resulting in more arachidonate being available for further oxygenation [33]. The overall effect of agonist stimulation of neutrophils is reflected in the enhanced synthesis of thromboxane and leukotrienes. The fact that FMLP may potentiate the action of PAF in stimulating arachidonate release and vice versa indicates that the cooperative action of these two agonists leading to amplified responses of arachidonate release does exist.

PMA has been shown to stimulate respiratory burst [11, 12] and granular enzyme release [10] but not arachidonate release [28, 29]. PMA is also known to potentiate FMLP-induced respiratory burst [34] and to inhibit PAF-stimulated enzyme secretion [35]. Its effect on FMLP and PAF induced arachidonate release and metabolism is not clear. We have found that simultaneous addition of PMA and FMLP or PAF showed different responses in stimulating TXB_2 and LTB_4 syntheses. PMA appears to attenuate PAF- but not FMLP-induced eicosanoid synthesis. As PAF and FMLP may activate a similar signal transduction system, as described above, PMA may impair receptor binding of PAF and/or PAF receptor–G-protein coupling by protein kinase C mediated phosphorylation. The fact that PMA increases

FMLP-induced arachidonate metabolism may be due to PMA-mediated inhibition of arachidonate reincorporation into phospholipid which results in increased availability of arachidonate for further metabolism into oxygenated catabolites [33].

Synergism between FMLP and PAF in stimulating arachidonate metabolism can be of potential pathophysiological significance. Upon bacterial infection, the first signal to reach the neutrophils is most likely that of chemotactic peptides of bacterial origin. Once these neutrophils migrate into the tissues and respond to chemotactic stimulation, PAF and LTB_4 are liberated [13]. PAF not only may reinforce FMLP in inducing microbicidal activity of the neutrophils but also may potentiate FMLP in stimulating more synthesis of LTB_4 which acts as a very potent chemotactic agent to recruit more neutrophils to the site of infection. Such an upregulation through products of responding cells appears to be a valuable mechanism to maximize the targeted action of the neutrophils.

Acknowledgements—This work was supported, in part, by NIH Grant HL-32727. We are indebted to Ms Jean Cavenee for typing the manuscript.

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