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Interactions of formylmethionyl-leucyl-phenylalanine, adenosine, and phosphodiesterase inhibitors in human monocytes

Effects on superoxide release, inositol phosphates and cAMP

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Cessation of the fMLF-induced burst of human monocyte superoxide release was associated with a rise in cAMP. This was not due to inhibition of phophodiesterase (PDE), the major form of which was the PDE IV isozyme. The action of burst inhibitors did not correlate with cAMP levels: Rolipram, a PDe IV inhibitor, increased cAMP 6-fold, with minimal effects on the burst; whereas theophylline increased cAMP less than 2-fold but decreased the burst to less than half. Although theophylline and the adenylate cyclase activator, adenosine, inhibited fMLF-induced superoxide release, they did not inhibit production of inositol phosphates. Thus, these studies on inhibition of superoxide release implicated neither cAMP nor inositol phosphates.

Formylmethionyl-leucyl-phenylalanine; Adenosine; cyclic AMP; Theophylline; Superoxide release; (Human monocyte)

1. INTRODUCTION

Addition of the chemotactic peptide, fMLF, to a suspension of human monocytes causes a burst of superoxide release, which ceases in approx. 3 min [1]. This is associated with a rise in cellular cAMP, which occurs within the first minute of the burst. The cause of the increase in cell cAMP has not been established. The two obvious possibilities are activation of adenylate cyclase or inhibition of cyclic nucleotide phosphodiesterase (PDE). Neutrophil adenylate cyclase is not activated by fMLF [2]. Adenosine, which activates adenylate cyclase via A_2 receptors, causes a synergistic rise in the cAMP content of fMLF-stimulated human monocytes [3]. This suggests that the fMLF-induced increase in cAMP may be via inhibition of PDE. In this report, we address the question directly by measuring PDE activity in extracts of human monocytes.

We also evaluated the possibility that a rise in cAMP leads to diminished superoxide release in monocytes. This is suggested by the adenosine data, since the synergistic rise in cAMP of fMLF-stimulated adenosine-treated monocytes is associated with depressed superoxide release. We first determined the inhibitor sensitivity of PDE extracted from human monocytes. The PDE inhibitors or the adenylate cyclase activator, adenosine, were then used to increase cellular cAMP in the presence and absence of fMLF. Cellular cAMP and superoxide release were measured to determine if there was a correlation between cAMP levels and inhibition of superoxide release.

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Abbreviations: fMLF, formylmethionyl-leucyl-phenylalanine; Gey-BSA, Gey's balanced salt solution containing 2% bovine plasma albumin; HBSS, Hank's balanced salt solution; PDE, phosphodiesterase; PIP_2, phosphatidylinositol 4,5-bisphosphate

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2. MATERIALS AND METHODS

2.1. *Reagents*

fMLF was from Peninsula Laboratories, Belmont, CA. Theophylline, type IV cytochrome *c* from horse heart, superoxide dismutase, cAMP, and *Ophiophagus hannah* venom were from Sigma, St. Louis, MO. AG 1-X8 resin was from Bio-Rad, Richmond, CA. HBSS, Gey-BSA and RPMI 1640 culture medium were prepared at the Frederick Cancer Research Facility. Fetal bovine serum, obtained from Sterile Systems, Logan, UT, was heated at 56°C for 30 min before use. [2,8-^{3}H]cAMP (spec. act. 36.1 Ci/mmol) and RIANEN cAMP ^{125}I RIA kits were obtained from New England Nuclear, Boston, MA. PCS scintillation fluid was from Amersham, Arlington Heights, IL. Rolipram was a gift from Schering AG, Berlin. CI-914 and CI-930 were gifts from Dr R. Weishaar, Warner-Lambert Co., Ann Arbor, MI. Ro 20-1724 was a gift from Dr P.F. Sorter, Hoffmann-La Roche Inc., Nutley, NJ.

2.2. *Superoxide production*

Blood monocytes were collected from normal human subjects, separated by elutriation, stored overnight at 4°C and incubated at 37°C for 3 h to regain maximal responsiveness [3]. Superoxide production was measured as superoxide dismutase-inhibitable reduction of cytochrome *c* by an end-point assay. Monocytes, 4×10^5 per assay, were preincubated for 10 min at 37°C in 1.5 ml polypropylene microcentrifuge tubes containing 0.9 ml of 0.11 mM cytochrome *c* in HBSS. Reference tubes contained 20 μl of 3 mg/ml superoxide dismutase. Superoxide generation was induced by addition of 10 μl of 10^{-4} M fMLF. After 7 min at 37°C, suspensions were cooled in ice for 3 min and then centrifuged in an Eppendorf microfuge. The 550 nm absorbance of supernatants was recorded. Theophylline and Rolipram were present throughout the incubations where indicated. Adenosine was added 2 min before fMLF.

2.3. *PDE assay*

Monocyte homogenates were obtained by osmotic shock, followed by extrusion of suspensions 3 times through a 30 gauge needle. For experiments on the effect of fMLF on PDE activity, incubations were stopped by rapidly freezing the cell suspensions and making the homogenates in 3 vols 50 mM β-glycerophosphate, 15 mM NaF and 1 mM phenylmethylsulfonyl fluoride. These procedures were shown microscopically to break all cells. cAMP PDE activity was assayed by a modification [4] of the method of Thompson and Appleman [5]. Each assay mixture contained cell homogenate, 40 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 3.75 mM 2-mercaptoethanol, 0.1 μM cAMP and 0.2 μCi [^{3}H]cAMP in a total volume of 100 μl. For determination of K_m values, total cAMP was varied at constant total radioactivity. Mixtures were incubated at 37°C for 30 min (5′-AMP production rate being constant over this period) and stopped by 3 min immersion in a boiling water bath. The 5′-AMP produced was converted to adenosine by incubation with 25 μl of 1 mg/ml *O. hannah* venom before separation from unreacted cAMP by elution from a 1 ml column of AG 1X8 (Cl^-) with 0.1 M $NaHCO_3$. Eluted radioactivity was measured in PCS scintillation fluid on a Beckman LS7800 scintillation counter.

2.4. *Inositol phosphate production*

Monocyte suspensions (5×10^7–1×10^8 cells per ml) were incubated at 37°C in Gey-BSA containing 50 μCi [^{3}H]inositol per ml. Cells were washed three times with Gey-BSA and resuspended in HBSS at 1×10^7 per ml; 200-μl aliquots were incubated with agents as described below. Incubations were stopped with 400 μl of 0.4% perchloric acid. After centrifugation of precipitated protein, supernatants were neutralized with KOH and $KHCO_3$. Inositol phosphates were fractionated on an AG 1X8 (formate) column [6] and radioactivity was measured as above.

3. RESULTS

The predominant form of monocyte PDE was the PDE IV isoenzyme (nomenclature of Reeves et al. [7]) based on inhibitor sensitivity and K_m for cAMP. Table 1 shows that the ability of the cell homogenate to hydrolyze 1 μM cAMP was potently inhibited by both Rolipram and Ro 20-1724 (specific inhibitors of PDE IV), but not by CI-914 or CI-930 (specific inhibitors of PDE III isoenzyme). Dose-response curves for both Rolipram and Ro 20-1724 were similar in shape to those of the non-specific inhibitors theophylline and isobutyl methyl xanthine, indicating that type IV was the predominant isoenzyme and there was no significant Rolipram- or Ro 20-1724-insensitive component. The K_m for cAMP was 2.9 μM.

To determine if fMLF affected PDE activity, PDE was prepared from fMLF-stimulated monocytes at several time points over a 10 min period, during which superoxide generation and a rise and fall in cAMP occur. To avoid proteolysis and alterations in phosphorylation state, cells were homogenized in phenylmethylsulfonyl fluoride, NaF and β-glycerophosphate. Fig.1 shows that fMLF did not alter PDE activity. The level remained at approx. 5 pmol/min per 10^6 cells.

Table 1

Inhibitor sensitivity of monocyte PDE activity[a]

Inhibitor	EC_{50} (μM)
Theophylline	300
Isobutyl methyl xanthine	16
Rolipram	2.7
Ro 20-1724	7.2
CI-914	>200
CI-930	>200

[a] Substrate: 1 μM cAMP

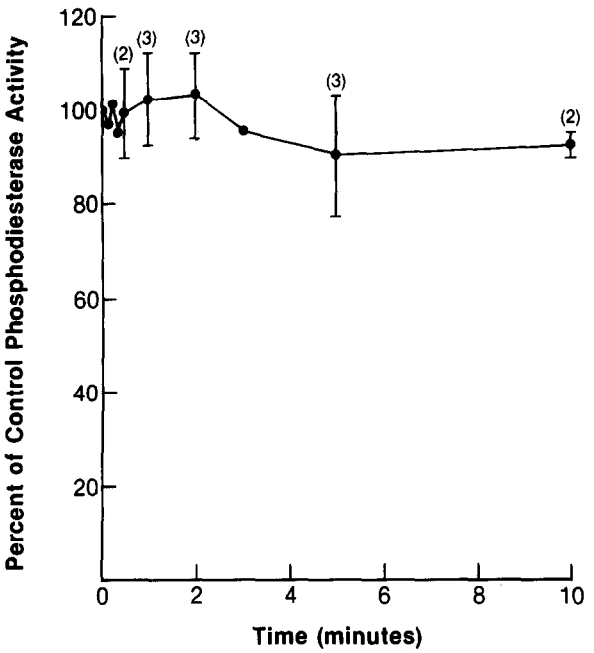

Fig.1. Effect of fMLF on the PDE activity of human monocytes. Cells were preincubated for 10 min before the addition of 10^{-6} M fMLF. PDE activity was normalized to percentage of appropriate control activity. PDE activity at zero time was 5.4 ± 1.4 pmol/min per 10^6 cells.

Table 2

Effects of PDE inhibition and adenylate cyclase activation on monocyte cAMP and superoxide production

Incubation conditions[a]	cAMP (pmol/10^6 cells)	Superoxide (% control)
Control	1.1 ± 0.2[b]	
+ fMLF	1.5 ± 0.2	(100)
1.5 mM theophylline	1.4 ± 0.2	
+ fMLF[c]	3.8 ± 0.6	38 ± 10
40 μM Rolipram	1.8 ± 0.2	
+ fMLF[c]	8.8 ± 2.5	81 ± 2
40 μM adenosine[d]	1.7[f]	
+ fMLF[e]	2.6[f]	45[g]

[a] Monocytes incubated for 10 min before addition of 10^{-6} M fMLF; incubation stopped at end of 10 min period or after a further 2 min in the presence of 10^{-6} M fMLF. Data are means ± SE for 3 experiments
[b] cAMP measured by radioimmunoassay [3]
[c] Theophylline or Rolipram present throughout the incubation
[d] Adenosine added 2 min before end of incubation
[e] Adenosine added 2 min before addition of fMLF
[f] Data from [3]
[g] Data from [1]

Effects of PDE inhibition and adenylate cyclase activation on cAMP generation in the absence and presence of fMLF were studied along with the production of superoxide. Concentrations of Rolipram (40 μM) and theophylline (1.5 mM) giving similar PDE inhibition in cell homogenates were chosen for study. Adenylate cyclase was activated via A_2 receptors by adenosine. Table 2 shows cAMP levels and amounts of superoxide generated in response to 10^{-6} M fMLF in the presence and absence of the above agents. No cor-

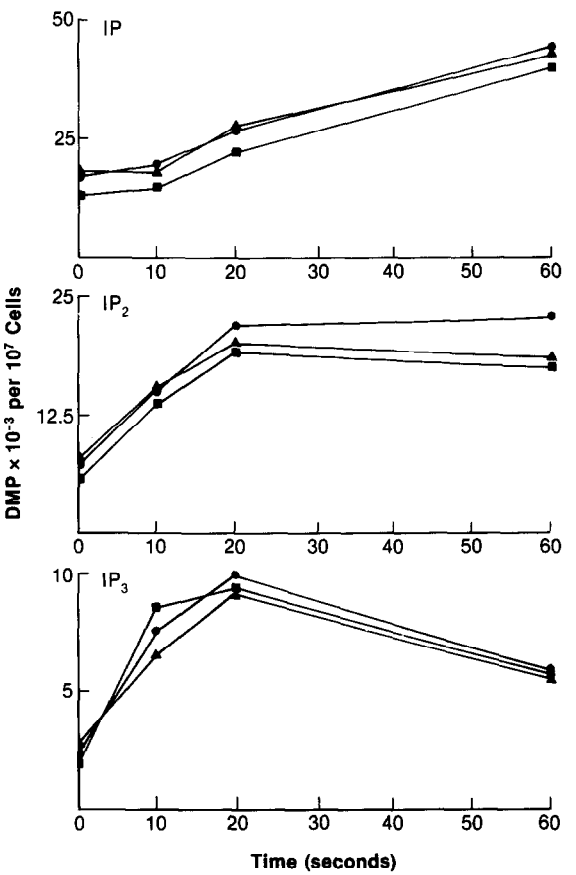

Fig.2. Effect of adenosine and theophylline on the fMLF-stimulated production of inositol phosphates in human monocytes. IP, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate. (●) fMLF alone; (■) fMLF plus 1.5 mM theophylline, which was present throughout the preincubation period; (▲) fMLF plus 40 μM adenosine, which was added 2 min before fMLF. Cells were preincubated for 10 min before the addition of 10^{-6} M fMLF. Values are the means from 3 or 4 experiments. For clarity, error bars are omitted; there were no significant differences between values at any time point.

relation was seen between the amount of monocyte superoxide production and cAMP level.

Adenosine and theophylline cause a significant inhibition of fMLF-induced superoxide production. In neutrophils, superoxide production is associated with stimulation of PIP_2 hydrolysis and production of inositol phosphates [8]. Fig.2 shows that fMLF stimulates monocyte PIP_2 hydrolysis, with a peak inositol trisphosphate production at approx. 20 s after stimulation. However, despite inhibition of superoxide release by adenosine or theophylline, neither showed any significant effect on the time course of PIP_2 hydrolysis.

4. DISCUSSION

We have shown by the use of isoenzyme specific inhibitors that monocytes contain PDE IV. This extends the report of Thompson et al. [9] who found that monocytes contain an isoenzyme with a K_m of 1.3 μM cAMP and no cGMP hydrolytic activity, which are characteristic of PDE IV [9]. Grady and Thomas [10] have reported a similar PDE activity in human neutrophils which increased in response to stimulation of the cells with fMLF. This was a surprising observation in view of the fact that fMLF leads to an increae in cAMP levels in neutrophils [2,11] which it has been suggested may be mediated by PDE inhibition [2]. We also suggested that the rise in cAMP in monocytes could be due to inhibition of the PDE in response to fMLF [3]. However, in the current study we did not detect any change in the PDE activity of monocytes over a 10 min period after fMLF stimulation (fig.1) despite a significant rise in cAMP. Furthermore, the increase in cAMP is dramatically potentiated by the PDE inhibitor, Rolipram (table 2), indicating that PDE inhibition is not the mechanism by which fMLF induces a rise in cAMP. It is therefore probable that fMLF increases the rate of synthesis of cAMP, although this is not a direct effect on the enzyme [2].

Theophylline, a methylxanthine-derived PDE inhibitor, is also a potent inhibitor of superoxide generation in monocytes. However the rises in cAMP induced by theophylline or adenosine are small compared with that produced by the non-methylxanthine, PDE IV specific inhibitor, Rolipram, which shows only a small inhibition of superoxide production. We therefore conclude that both adenosine and theophylline, which is structurally closely related to adenosine, inhibit superoxide generation in monocytes by a mechanism that is independent of their ability to increase cellular cAMP. Cronstein et al. reached a similar conclusion for neutrophils [12].

Neither adenosine nor theophylline altered production of inositol phosphates induced by fMLF (fig.2). This suggests that inhibition of superoxide production by these agents occurs at a site distal to the initial receptor-mediated activation of phospholipase C. Kato et al. [13] found a small inhibition of fMLF-induced inositol phosphate production by PGE_2 through a cAMP-dependent mechanism, although the major effect was on the enzymes involved in the synthesis of PIP_2.

Our data leave two problems unanswered. First, the mechanism by which fMLF causes an increase in the monocyte cAMP is unknown. It does not appear to involve either an inhibition of the PDE or a direct activation of adenylate cyclase. It is probable that the fMLF alters the internal environment of the cell in such a way as to cause an indirect activation of the cyclase, possibly involving Ca^{2+} [2,11]. Second, the mechanism of inhibition of superoxide generation by adenosine and its analogues, including the methylxanthine PDE inhibitors, is also unknown. It does appear to be certain, both in monocytes (this report) and neutrophils [12], that the inhibitory effect of adenosine or its analogues on superoxide production is not mediated by cAMP.

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