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Response of Adenosine Cyclic 3′,5′-Monophosphate Level in Rabbit Neutrophils to the Chemotactic Peptide Formyl-Methionyl-Leucyl-Phenylalanine

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SUMMARY

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Addition of the synthetic chemotactic peptide, fMet-Leu-Phe, to rabbit neutrophils elevates the basal level of cyclic 3′,5′-AMP. Stimulation of the cyclic AMP level reaches a maximum at 1 min (60% increase over basal value at 5 nM fMet-Leu-Phe) and subsequently the cyclic AMP level returns to the basal value within 5 min. In the absence of extracellular Ca^{2+}, the cyclic AMP level increases minimally (<20%) upon exposure to fMet-Leu-Phe. The effect of fMet-Leu-Phe is detectable at 0.5 nM and increases at higher concentrations. Inhibition of the cyclic AMP-dependent phosphodiesterase activity by pretreatment with 1-methyl-3-isobutylxanthine enhances both the basal and stimulated cyclic AMP levels.

INTRODUCTION

The directional movement of neutrophils (PMN)[2] toward a chemical stimulus, chemotaxis, is the first in a myriad of events involved in the recognition and destruction of foreign microorganisms. The synthetic peptide fMet-Leu-Phe was recently shown to be an effective chemotactic stimulus inducing not only PMN migration but also lysosomal enzyme secretion in the presence of cytochalasin B (1–3). fMet-Leu-Phe interacts specifically with intact rabbit and human neutrophils as well as with plasma membranes isolated from rabbit neutrophils and its binding affinity is directly related to its biological activity (4, 5).

Exogenous cyclic nucleotides and agents that elevate intracellular cyclic nucleotide levels modulate the interrelated PMN functions of chemotaxis and phagocytosis and, in addition, lysosomal enzyme secretion (6–8). For example, extracellular cyclic 3′,5′-GMP and cholinergic amines which generally elevate cyclic GMP levels promote these neutrophil responses (9–13). Chemotaxis, phagocytosis and lysosomal enzyme secretion are inhibited, on the other hand, by cyclic 3′,5′-AMP, epinephrine, isoproterenol, cholera toxin, prostaglandins A_1 and E_1, as well as inhibitors of cyclic AMP-dependent phosphodiesterase, all of which are hormonal or pharmacological agents generally capable of raising intracellular cyclic AMP levels (7, 10, 12, 13–17). The measured intracellular levels of cyclic GMP

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[2] The abbreviations used are: PMN, neutrophils; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; BSA, bovine serum albumin; TCA, trichloroacetic acid; MIX, 1-methyl-3-isobutylxanthine; GMP-PNP, 5′-guanylylimidodiphosphate.

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in human neutrophils increase in response to both zymosan-induced phagocytosis (12) and the chemotactic synthetic peptide, fMet-Ala (18). This peptide is less effective than fMet-Leu-Phe but nonetheless chemotactic (3, 18). There is some question, however, concerning the response of the intracellular cyclic AMP levels in neutrophils to phagocytic or various chemotactic agents. The phagocytosis of latex particles by PMN results in a prompt, 3-fold increase in cyclic AMP in the first five minutes (19). In contrast, the phagocytosis of zymosan particles is not accompanied by a change in the cyclic AMP level (12). Neither chemotactic bacterial filtrates nor fMet-Ala (18) have been shown to elicit a change in the PMN cyclic AMP levels excepting the report of Anderson *et al.* (9) in which the cyclic AMP level in human neutrophils was elevated about 25% within the first five minutes of exposure to an *E. coli* endotoxin and subsequently returned to basal levels.

These conflicting data may result from the lack of a potent, well-defined chemotactic stimulus and from the choice of sampling times after addition of the stimulus (1, 20). The present experiments were undertaken to test the effect of fMet-Leu-Phe on neutrophil cyclic AMP levels and attention was focused on the first 5 min after addition of the peptide to the cells.

METHODS

PMNs were obtained by collecting the neutrophil-rich peritoneal exudate from New Zealand albino rabbits injected 12–14 hours previously with 400 ml 0.1% glycogen in 0.9% NaCl. All of the cells used within an experiment were obtained from the same rabbit. The cell suspension was centrifuged at 600 × *g* for 5 min and the pelleted cells were washed twice with modified Hanks' balanced salt solution containing (concentrations expressed as mM): 125 NaCl, 4.9 KCl, 1.6 $CaCl_2$, 0.74 $MgSO_4$, 15 $NaHCO_3$, 0.64 Na_2HPO_4, 0.66 KH_2PO_4, 10 Hepes, pH 7.4. After the second wash, the cells were resuspended in Hanks' solution containing 0.1% glucose and 0.1% BSA at a density of 1 to 2 × 10^7 cells per ml. Cell number and population distribution were determined on a Coulter Counter Channelyzer and X-Y Recorder II (Coulter Electronics, Inc.). Greater than 90% PMN was obtained regularly from the peritoneal exudates. The value of 90% PMN represents the upper value of heterogeneity. Most preparations were better than 95% PMN. Aliquots of 1 ml of the washed cell suspension were added to 12 × 75 mm Falcon disposable plastic tubes #2052 (Becton, Dickinson and Company) and preincubated 40 to 60 min at 37°. MIX and/or fMet-Leu-Phe was added to the cell suspensions in a 10 μl volume of 100-fold concentrated stock and the samples were vortexed gently. At specified intervals 150 μl of ice cold 30% TCA was added quickly to the incubating cell suspensions and the samples were immediately and vigorously vortexed.

The denatured suspensions were frozen-thawed once (sonication for 30 sec at 45 Watts on a Sonifier Cell Disruptor Model W185, Ultrasonics, Inc., was found to be equivalent) and approximately 0.07 pmoles [^{3}H]-cyclic AMP of specific activity 37 Ci/mmole (New England Nuclear), was added to each sample for the determination of cyclic AMP recovery. Samples were vortexed, centrifuged at 2400 × *g* for 20 min and 1 ml of the resulting supernatant was applied to columns (1.7 × 5.0 cm) of AG 50W-X8, H+ form, analytical grade cation exchange resin, 200–400 mesh, (Bio-Rad Laboratories) equilibrated with distilled water. Elution of cyclic AMP essentially follows the procedure of Steiner (21).

The dried samples were reconstituted in 200 μl 0.05 M sodium acetate buffer, pH 6.2 and 50 μl aliquots were divided and prepared for liquid scintillation counting in a Searle Delta 300 (Searle Analytic Inc.) by the addition of 10 ml ACS (Amersham Corporation). Recovery of cyclic AMP was generally 85 to 95% and was determined for every sample. Aliquots of 50 to 75 μl were assayed for total cyclic AMP content by radioimmunoassay, which was performed according to the protocol for nonacetylated samples supplied in a double antibody precipitation kit (New England Nuclear). An effective measurement range of 0.5 to 10.0 pmoles cyclic AMP was routinely observed. Beef heart cyclic nucleotide phosphodiesterase was used to treat residues of control

and fMet-Leu-Phe treated cell suspensions to assess the nucleotide specificity of the immunoassayable material in the manner described by Steiner (21). Cyclic AMP levels are expressed as pmoles/10^7 cells.

Rabbit neutrophil membranes were prepared from a cell homogenate as described previously (22, 23). In this method the postnuclear fraction was obtained by centrifugation of the cell homogenate at 100 × *g* for 10 min. The supernatant was layered on a discontinuous gradient containing 3 ml of 30% (W/V) sucrose, 3 ml of 40% sucrose and 3 ml of 50% sucrose. All of the sucrose solutions contained 1 mM EDTA and 10 mM Tris-HCl buffer, pH 7.4. The tubes were placed in a SW40 rotor (Beckman Instruments, Inc.) and the gradients were centrifuged at 110,000 × g for 1 hr at 4°. Band I was formed between the suspending medium and 30% sucrose, band II between the 30% and 40% sucrose and band III between the 40% and 50% sucrose. The material designated as band II is referred to as plasma membrane and that designated as band III is the cytoplasmic membrane fraction (22, 23). Band II generally constitutes 30% of the total microsomal protein, i.e., 30% of 0.043 mg/10^7 cells or 12.9 μg Band II protein/10^7 cells. Adenylate cyclase activity was assayed by measuring the conversion of [α-^{32}P]-ATP into cyclic 3′,5′-AMP (24, 25). The final reaction mixture had a total volume of 100 μl and contained (expressed as mM): 25 Tris-HCl, pH 7.6, 5 $MgCl_2$, 1 cyclic AMP, 1 dithiothreitol, 0.1 ATP, [α-^{32}P]ATP (3 × 10^6 cpm, 10–30 Ci/mmol, New England Nuclear) 5 phosphocreatine and 50 units/ml creatine phosphokinase, type 1, from rabbit muscle. Band II membrane protein (300 μg or less per assay tube) was incubated for 5, 10 and 20 min. When added, the concentration of NaF was 8 mM and 5′-guanylylimidodiphosphate (GMP-PNP) was 0.1 mM. The adenylate cyclase assay is linear with time and protein under the conditions employed in this study. Recovery of cyclic AMP was determined by adding 5 × 10^3 cpm [^{3}H]-cyclic AMP to each assay tube prior to chromatography on AG50W-X8 and alumina, type WN-3, activity grade 1 (Sigma).

The chemotactic factor fMet-Leu-Phe was obtained through the coutesty of H. J. Showell and E. L. Becker from Dr. R. F. Freer, Medical College of Virginia. The MIX was obtained from Aldrich Chemical Company, Inc. Glucose, glycogen, BSA, beef heart adenosine 3′,5′-cyclic nucleotide phosphodiesterase, phosphocreatine and creatine phosphokinase were obtained from Sigma Chemical Company. Salts and TCA were obtained from J. T. Baker Chemical Company and were analytical reagent grade or better.

RESULTS AND DISCUSSION

The basal level of cyclic AMP in rabbit neutrophils and the effect of the synthetic chemotactic peptide fMet-Leu-Phe (5 nM) on this value were studied. In these experiments, the cells were exposed to fMet-Leu-Phe for various lengths of time (0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 5 min) before the reaction was stopped with TCA. The results are summarized in Figure 1, which indicates that this concentration of fMet-Leu-Phe produces a significant increase (>60%) in the basal level of cyclic AMP. The stimulation of the cyclic AMP level is observed within the first 15 sec of fMet-Leu-Phe addition, reaches a maximum at 1 min, and returns to the basal value within the next 4 min. Such a time course signifies that the chemotactic factor elicits a transient, short-lived stimulation of the cyclic AMP level.

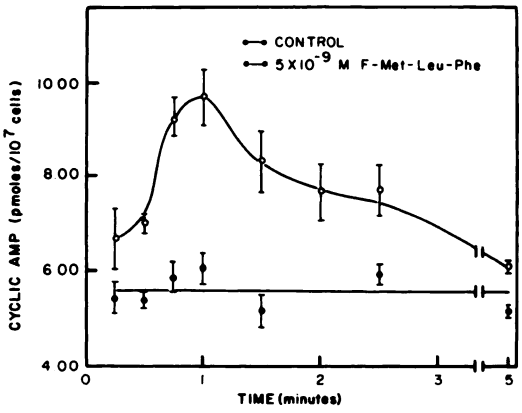

FIG. 1. *Time course of the basal* (●——●) *and [fMet-Leu-Phe]-stimulated* (○——○) *cyclic AMP levels in rabbit neutrophils*

Data are expressed as pmoles/10^7 cells and represent the mean ± S.E.M. of 6 experiments.

In addition, this time course is similar to those observed for other modes of PMN activation by fMet-Leu-Phe, e.g., lysosomal enzyme release with cytochalasin B, and increased cation transport (1, 20). It is unlikely that the observed stimulation is due to elevation of the cyclic AMP levels within the contaminating mononuclear cells, e.g., macrophages. These constitute 5–10% of the cell population and an average increase of 4.1 pmoles/10^7 total cells following treatment with fMet-Leu-Phe would, in such a case, represent an increase of 40 to 80 pmol/10^7 contaminating cells, values considered extraordinary in light of current literature (27). Most previous studies of the response of neutrophil cyclic AMP levels to chemotactic stimuli employed sampling times greater than 2 min after addition of the stimulus, thereby accounting for the lack of detection of a response. Interestingly, the phagocytosis of latex particles by human neutrophils has been shown very recently to be accompanied by elevation of the cyclic AMP level in a manner strikingly similar to that reported here (28). It should be noted that this stimulation is also very rapid and transient. Earlier investigations of the cyclic AMP levels in response to phagocytosis employed sampling times much too late to observe this phenomenon.

The basis for the transient nature of the effect cannot be deduced from these studies. There is a loss of fMet-Leu-Phe activity in the extracellular medium presumably due, in large part, to the breakdown of the peptide by the peptidase of the neutrophil (29). However the time course of the breakdown process is significantly different from that of the cyclic AMP response described here. Aswanikumar and co-workers (29) have found that only 30% of the chemotactic factor was degraded in 30 min. Furthermore, it has been shown that the extracellular media from cells incubated with 1 nM fMet-Leu-Phe is capable of stimulating lysosomal enzyme release when transferred to fresh cells (30). Showell *et al.* (30) have also shown that even when the addition of $^{45}Ca^{2+}$ or $^{22}Na^{+}$ tracer was delayed up to five minutes after the addition of fMet-Leu-Phe to the neutrophils, there was no loss in the known ability of the peptide to increase the influx of Ca^{2+} and Na^{+}. In any case, to verify that simple loss of fMet-Leu-Phe activity due to extracellular degradation cannot totally account for the transient nature of the effect, we carried out experiments identical to that shown in Figure 1 except that the fMet-Leu-Phe concentration was 0.1 μM. We found that the time course of cyclic AMP stimulation was very similar to that observed when the chemotactic factor concentration was 5 nM.

The stimulatory effect of fMet-Leu-Phe on the level of cyclic AMP in rabbit neutrophils can be due to a stimulation of adenylate cyclase activity or an inhibition of the cyclic AMP-dependent phosphodiesterase activity. The adenylate cyclase activity of a plasma membrane-enriched fraction from rabbit neutrophil microsomes was assayed in the presence and absence of chemotactic peptide and the results for 1 nM fMet-Leu-Phe are presented in Table 1. Neither the basal activity nor the activity with NaF or GMP-PNP was significantly altered by fMet-Leu-Phe; higher concentrations of fMet-Leu-Phe yielded equivalent results. These data indicate that interaction between fMet-Leu-Phe and adenylate cyclase does not occur under the conditions of the assay. The possibilities remain that, given the proper environment, interaction between the peptide and the cyclase via receptor-enzyme coupling can be demonstrated or indirect stimulation of the cyclase via an additional intermediate pro-

TABLE 1

Adenylate cyclase activity of rabbit neutrophil membranes

Condition	Basal	$^{+}$NaF	+GMP-PNP
	(pmole/mg protein, min)		
Control	0.20 ± 0.04 (14)[a]	2.2 ± 0.6 (4)	10 ± 3 (4)
+fMet-Leu-Phe (1 nM)	0.18 ± 0.03 (8)	1.9 ± 0.8 (2)	9 ± 2 (4)

[a] Means are given with the standard error of the mean. The number in parenthesis refers to the total number of experiments.

vides the basis for the observed phenomenon. A primary candidate for such a mediatory role is cyclic GMP. We have attempted to measure the cyclic GMP levels in rabbit peritoneal neutrophils and we have found that the basal level, both in the presence and absence of 1-methyl-3-isobutylxanthine (MIX), as well as the levels in the presence of fMet-Leu-Phe, are not detectable with a radioimmunoassay kit comparable to that employed in the measurement of cyclic AMP.

We have also investigated the effect of MIX on both the basal level of cyclic AMP and the stimulatory action of fMet-Leu-Phe. In these studies the cells were exposed to MIX (0.1 mM) for 5 min before the experiment was initiated and then exposed to fMet-Leu-Phe (5 nM) for one minute before the reaction was stopped with TCA. This concentration of MIX, together with the preincubation period, appears to inhibit maximally the MIX-sensitive phosphodiesterase activity. Elevation of the basal cyclic AMP level is evident after 25 min at 37° in the presence of 0.1 mM MIX and is equivalent to the increased level observed with 1.0 mM MIX (31, 32) or 8 mM theophylline (33). The results are summarized in Table 2. MIX increases both basal cyclic AMP levels and levels determined in the presence of fMet-Leu-Phe. In light of the lack of a demonstrated stimulation of adenylate cyclase by fMet-Leu-Phe, there is a possibility that the increased cyclic AMP level results from an inhibition of cyclic AMP-dependent phosphodiesterase activity, albeit a MIX-insensitive activity. The effect of MIX on the time course of the stimulatory effect of fMet-Leu-Phe was investigated. Again, the cells were exposed to MIX (0.1 mM) for 5 min before the experiments were initiated. The results presented in Figure 2 indicate that MIX changes the magnitude and the characteristics of the cyclic AMP response. MIX amplifies the effect on the cyclic AMP level and shortens somewhat the time of maximum response. In addition, the MIX prevented the cyclic AMP levels from returning to normal in the allotted 5 min, thus indicating the presence of an active, MIX-sensitive phosphodiesterase that participates in the breakdown of

TABLE 2

Effect of the chemotactic peptide on cyclic AMP level in rabbit neutrophils

Experimental Condition	Cyclic AMP level in pmoles/10^7 cells	
	Control	F-Met-Leu-Phe (5 nM)[a]
Basal	6.4 ± 0.4 (69)[c]	10.5 ± 0.6 (39)
+MIX (0.1 mM)[b]	9.7 ± 0.7 (24)	15.6 ± 1.0 (21)

[a] Determined 1 min after fMet-Leu-Phe addition.

[b] Cells incubated with MIX for 5 min at 37° before initiation of the experiments.

[c] Means are given with the standard error of the mean. The number in parentheses refers to the total number of experiments. Each value of the cyclic AMP level determined in the presence of fMet-Leu-Phe is significantly different ($p < 0.001$) from its respective control.

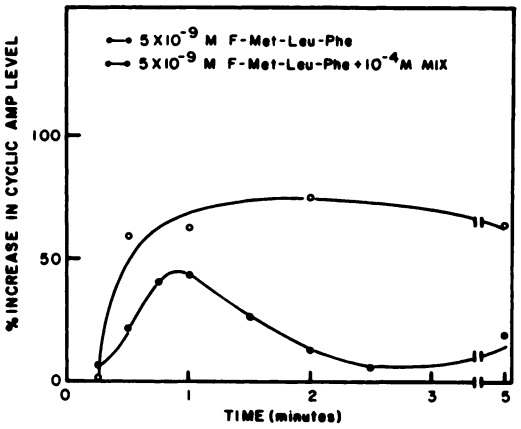

FIG. 2. *A time course of the stimulated cyclic AMP levels with fMet-Leu-Phe both in the absence* (●——●) *and the presence* (○——○) *of MIX (0.1 mM)*

Cells were incubated with MIX for 5 min prior to the addition of fMet-Leu-Phe. Data are expressed as the percent increase over control (without fMet-Leu-Phe) levels and represent the mean of 6 experiments. In the case of the experiments which deal with MIX the control contains 0.1 mM MIX. The fMET-Leu-Phe stimulation is slightly less than normal in these sets of experiments.

the fMet-Leu-Phe-stimulated cyclic AMP pool. These data imply that the phosphodiesterase activity is not inhibited in the presence of peptide alone but rather it is inhibited only after MIX is added. Here again, the rapid fall in the cyclic AMP level after stimulation in the absence of MIX cannot be totally due to degradation of the peptide because the cyclic AMP level re-

mains elevated in the presence of MIX.

The value of the basal level of cyclic AMP in rabbit neutrophils is within the range of values previously reported for both rabbit and human neutrophils (9, 12–14, 18, 19, 31–33), though it should be kept in mind that the published values range from 1.4 to 90 pmoles/10^7 cells. One possible explanation for the widespread discrepancies could be differences in the state of the ionic gradients across the cell membrane as the result of various preparative manipulations before the experiment is carried out. There is a great deal of evidence that suggests an interplay between intracellular calcium and cyclic nucleotides in a variety of systems (34). The level of free intracellular calcium can be affected, in turn, by ionic gradients across the cell membrane and particularly by the intracellular Na^+ concentration. To restore the Na^+ and K^+ gradients across the cell membrane, it is important for neutrophils to be preincubated with the proper ionic environment at 37° for at least 15 min before any experimental manipulation. Keeping in mind the possibility that Ca^{2+} may be involved in the fMet-Leu-Phe activation of the cyclic AMP levels, we have investigated the effect of removal of calcium from the extracellular medium on the stimulatory action of fMet-Leu-Phe. In these experiments cells were incubated at 37° for 40 min in Hanks' balanced salt solution containing 1.6 mM Ca^{2+}. At the end of the incubation period, the suspension was divided into centrifuge tubes, spun quickly at room temperature, and the resulting supernatant was removed from each tube. To one tube an equivalent volume of Hanks' buffer without Ca^{2+} was added and to the second tube an equivalent volume of complete Hanks' buffer was added. The experiment was then initiated by the addition of fMet-Leu-Phe. These manipulations were completed within three minutes, which means that the cells were exposed to the medium without Ca^{2+} for a maximum period of 3 min before the experiment was started. This time is too short for a drastic change in intracellular ionic composition to occur (20). The results are given in Table 3. The stimulatory effect at 5 nM fMet-Leu-Phe on the level of cyclic AMP is significantly decreased when the extracellular Ca^{2+} concentration is reduced to less than 10 μM from the 1.6 mM normally present in Hanks' solution. The percent increase in the level of cyclic AMP due to the presence of MIX is not significantly affected by the removal of calcium from the outside medium. It is interesting to point out that an observed increase in the cyclic AMP level as a result of the phagocytosis of latex particles by human neutrophils (referred to earlier) was also dependent on the presence of calcium in the extracellular medium (28).

The results obtained with 5 nM fMet-Leu-Phe represent the functioning of a system well below the point of maximum stimulation or saturation as indicated by the dose response curve in Figure 3. The effect is detectable at 0.05 nM fMet-Leu-Phe and increases at higher concentrations up to a 110% elevation of the cyclic AMP levels at 0.1 μM. The synthetic peptide fMet-Leu-Phe is highly chemotactic (3); significant chemotactic activity in rabbit neutrophils can be observed at a concentration as low as 0.01 nM. This activity increases with increasing fMet-Leu-Phe concentrations reaching a maximum at 0.5 nM and then drops off at high concentrations (≥10 nM). The observed increase in the cyclic AMP levels and the corresponding decrease in

TABLE 3

Influence of extracellular Ca^{2+} on cyclic AMP levels (pmoles/10^7 cells)

Experimental Condition	with Ca^{2+}	% increase	no added Ca^{2+}	% increase
Control	8.7 ± 0.4 (5)[c]	—	7.1 ± 1.4 (5)	—
+MIX[a] 1×10^{-4} M	11.8 ± 2.1 (5)	36	9.5 ± 0.6 (5)	34
+fMet-Leu-Phe[b] 5×10^{-9} M	12.9 ± 0.4 (5)	48	7.6 ± 1.8 (5)	7

[a] Cells incubated with MIX for 5 min at 37° before initiation of the experiments.

[b] Determined 1 min after fMet-Leu-Phe addition.

[c] Means are given with the standard error of the mean. The number in parentheses refers to the total number of experiments.

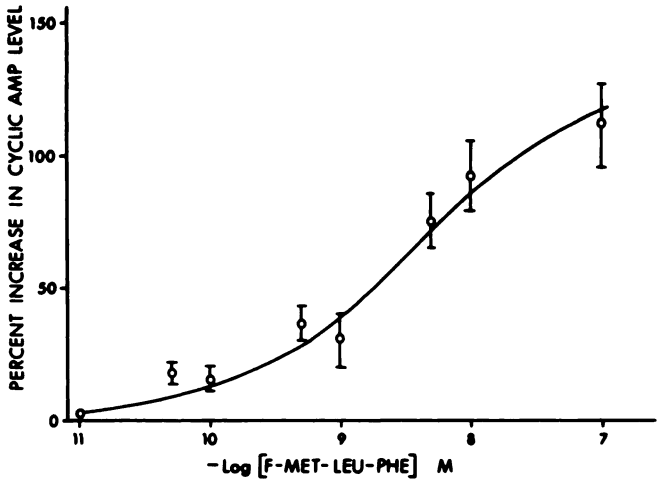

FIG. 3. *A dose-response curve for the percent increase of the cyclic AMP levels by fMet-Leu-Phe* Data represent the mean ± S.E.M. of at least 6 experiments.

the responsiveness of the neutrophils at very high concentrations of the chemotactic factor may be related. The cyclic AMP elevation, though <2-fold at 0.1 μM fMet-Leu-Phe, is equivalent to that previously reported for rabbit peritoneal neutrophils in response to isoproterenol, PGE_1 and norepinephrine (33). In addition, comparable changes in the cyclic AMP levels of other cell types, including lymphocytes (35), myeloid leukemia cells and mature macrophages (27), have not only been described as coincident with mitogenic or hormonal stimuli but also have been intrinsically implicated in the biological responses of these cells.

The role of cyclic AMP in the response of neutrophils to the chemotactic peptide cannot be deduced from the present studies. Nor does the data strongly support any one mechanistic explanation of the stimulation of the cyclic AMP level by fMet-Leu-Phe. In view of the temporal coincidence of the cyclic AMP response with those previously reported for fMet-Leu-Phe (1, 20), the authors feel that the effect of fMet-Leu-Phe on cyclic AMP is important and warrants further investigation.

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