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Stimulation of a Histone H4 Protein Kinase in Triton X-100 Lysates of Rabbit Peritoneal Neutrophils Pretreated With Chemotactic Factors: Lack of Requirements of Calcium Mobilization and Protein Kinase C Activation

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The characteristics of the activation of a histone H4 kinase activity in Triton X-100 lysates of rabbit peritoneal neutrophils pretreated with fMet-Leu-Phe were studied: The activation of the kinase was a) inhibited by the antagonist of formylpeptide, *t*-Boc-(Phe-Leu)$_2$-Phe, b) completely inhibited by pertussis toxin pretreatment, c) not affected by the pretreatment of neutrophils with an activator of protein kinase C, phorbol-12-myristate-13-acetate, or an inhibitor of protein kinase C, 1-(5-isoquinoline-sulfonyl)-2-methyl-piperazine, and d) not inhibited in the cells preloaded with the intracellular calcium chelators, bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetra acetic acid acetoxymethyl-ester (BAPTA/AM). These results suggest that the stimulus-induced activation of H4 kinase requires functional receptor and GTP-binding protein but neither calcium mobilization nor protein kinase C activation.

Key words: H4 kinase, signal transduction, neutrophil activation

Neutrophils undergo various responses such as chemotaxis, degranulation, and superoxide generation upon stimulation with chemotactic factors (for review, [1,2]). The increases of the levels of free calcium and diacylglycerol in stimulated neutrophils have been linked to the activation of a calcium-phospholipid-diacylglycerol-dependent

The abbreviations used are: fMet-Leu-Phe, N-Formyl-methionyl-leucylphenylalanine; PMA, probol-12-myristate-13-acetate; H7, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine; SDS, sodium dodecyl sulfate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; BAPTA/AM, bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid/(acetoxymethyl)-ester.

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protein kinase C. Protein kinase C was postulated as the key enzyme involved in neutrophil activation. However, recent studies raise questions for this hypothesis: a) Inhibitors of protein kinase C such as l-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) inhibit poorly many of the responses [3]. b) Locomotion [4], superoxide generation [5], and actin polymerization [6] stimulated by fMet-Leu-Phe can occur without calcium elevation in neutrophils. c) Cytochalasin B (CB), which potentiates the degranulation and superoxide generation of fMet-Leu-Phe-treated neutrophils, does not enhance protein kinase C–mediated phosphorylation [7,8]. d) Pretreatment of the neutrophils with a protein kinase C activator, phorbol-12-myristate-13-acetate (PMA), inhibits the calcium elevation and degranulation stimulated by fMet-Leu-Phe. This inhibition can be blocked by the pretreatment of the cells with H-7 [9]. And e) the gradual, prolonged elevation of diacylglycerol in stimulated neutrophils does not match with the rapid kinetics of most of the biological responses [10].

In searching for an fMet-Leu-Phe-stimulated protein kinase unrelated to protein kinase C, we have developed a lysed cell assay system. In this assay, the neutrophils are stimulated for a fixed period of time and then Triton X-100 and EGTA are added to quench the stimulated responses. EGTA and H-7 are included in the assay mixture to inhibit the calcium-dependent and cyclic-nucleotide-dependent protein kinase activity. Using this method, we have observed a transient increase of histone H4 kinase activity in the Triton X-100 lysate of fMet-Leu-Phe but not PMA-stimulated neutrophils [11]. The effect of fMet-Leu-Phe on the kinase activity is very rapid, reaching a maximum level within 10 s of stimulation [11].

In this paper, we have studied the possible role of calcium mobilization and protein kinase C in the activation of H4 protein kinase by fMet-Leu-Phe.

EXPERIMENTAL PROCEDURES

Material

t-Boc-(Phe-Leu)$_2$-Phe was obtained from Sigma. Pertussis toxin was a gift from Dr. John Munoz. Other chemicals were obtained as described earlier [11].

Methods—Stimulation and Various Pretreatments of Neutrophils

Rabbit peritoneal neutrophils were prepared as described in the previous paper [11], and the cells (2.5×10^7 cells/ml) were suspended in Hanks' buffer containing 10 μM $CaCl_2$ before use. In some experiments, the cells were suspended in Hanks' buffer without adding $CaCl_2$. Neutrophils were incubated with 0.5 μg/ml pertussis toxin for 1 h as described [12]. The treatment completely inhibits granule enzyme secretion and actin polymerization induced by fMet-Leu-Phe. The inhibition parallels ADP-ribosylation of a membrane protein of molecular weight of 41,000 [12].

The cells were treated with 0.1 μg/ml PMA for 3 min as described [13]. The treatment strongly inhibits the rise of intracellular calcium and partially inhibits the degranulation of primary granules induced by fMet-Leu-Phe [13]. The procedure also causes translocation of protein kinase C from cytosol to membrane [2] and stimulates protein kinase C–mediated protein phosphorylation [8] A protein of molecular weight of 50,000 (pp50) has previously been identified as a substrate for protein kinase C in neutrophils [8]. H-7 was used at 25 μM for 5 min as described [9]. The treatment inhibits the phosphorylation of pp50 and blocks the inhibition of fMet-Leu-Phe

responsiveness caused by PMA [9]. It does not inhibit the degranulation or superoxide generation induced by fMet-Leu-Phe [3,9].

The cells were preloaded with BAPTA/AM (50 μM) at 37°C for 30 min. The viability of the cells was greater than 90% as determined by Trypan Blue dye exclusion. The intracellular concentration of free calcium was measured by using the fluorescence dye fura-2 as described [14].

The assay for the H4 kinase activity was done as described [11] with slight modification. Cells (2.5×10^7 cells/ml) with or without various pretreatments were stimulated with fMet-Leu-Phe at 37°C for various periods of time as indicated in the figure legends. Aliquots (30 μl) were withdrawn and mixed with the assay mixture (30 μl) containing 10 mM HEPES, pH 7.2, 25 mM $MgCl_2$, 20 μM sodium orthovanadate, 5 mM p-nitrophenylphosphate, 70 μM [γ^{32}P]ATP($0.7–1.4 \times 10^3$ dpm/pmol), 0.1% Triton X-100, 2 mM EGTA, 100 μM H-7, and 10 μg histone H4. The phosphorylation reaction was carried out at room temperature for 10 min. Aliquots of the reaction mixture (30 μl) were then spotted on phosphocellulose paper. The papers were washed, dried, and counted. The radioactivity of samples with and without histones was measured by scintillation spectrometry. The amount of [^{32}P] incorporated into H4 was then determined as described [11].

Others

Preparation of [^{32}P]-labeled intact cells, stimulation of the cells with fMet-Leu-Phe or PMA, and analysis of [^{32}P]-labeled phosphoproteins by SDS-gel electrophoresis were done as described [8]. Measurement of generation of phosphatidic acid was carried out as described previously [15]. The data shown are either representative results or average values ± S.E. of three or more experiments.

RESULTS AND DISCUSSION

Effect of BAPTA/AM

The H4 kinase activity in the Triton X-100 lysate of fMet-Leu-Phe-stimulated neutrophils has been reported [11]. The effect of fMet-Leu-Phe on the kinase activity is very rapid, reaching a maxium level within 10 s of stimulation. To test whether the rise of intracellular calcium is required for stimulation of the kinase by fMet-Leu-Phe, cells were suspended in Hanks' buffer without $CaCl_2$ and preloaded with BAPTA/AM before stimulation. As shown in Figure 1, loading of the cells with BAPTA/AM (50 μM) completely inhibits the rise of intracellular calcium stimulated by fMet-Leu-Phe. However, the effect of fMet-Leu-Phe (10^{-8}M, 10 s) on the kinase activity in the BAPTA/AM-treated cells was not inhibited (Table I). BAPTA/AM also inhibited the generation of phosphatidic acid stimulated by fMet-Leu-Phe (Table II). The rapid generation of phosphatidic acid suggests that diacylglycerol was produced by short exposure to fMet-Leu-Phe as has been reported previously [12,15]. BAPTA/AM may inhibit the production of diacylglycerol without inhibiting the stimulation of H4 kinase (Tables I and II).

The effect of fMet-Leu-Phe-stimulated H4 kinase activity was completely inhibited by the pretreatment of the cells with pertussis toxin. The synthetic peptide *t*-BOC(Phe-Leu)$_2$-Phe (10^{-6}M,) a specific antagonist of the binding of fMet-Leu-Phe [4], inhibited the stimulation of fMet-Leu-Phe by 47 ± 12% (N = 3). The antagonist itself had no effect on the kinase (Table I).

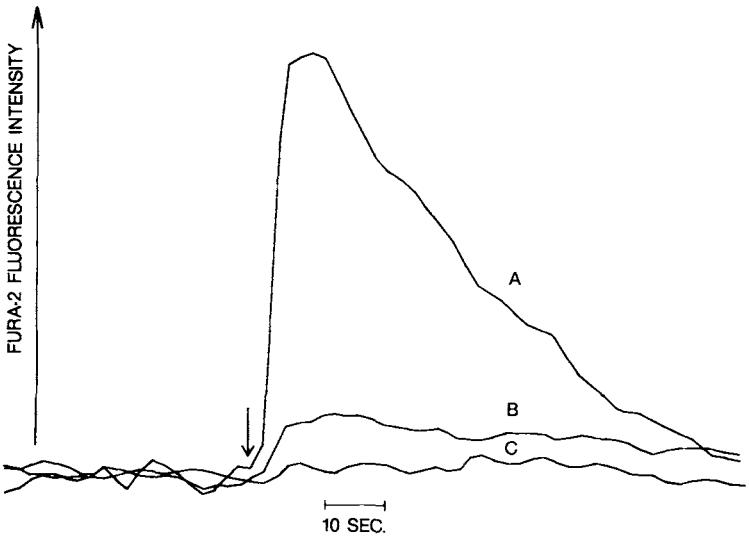

Fig. 1. Effect of intracellular loading of BAPTA/AM on fMet-Leu-Phe-induced calcium mobilization in rabbit peritoneal neutrophils. Neutrophils were pretreated with different concentrations of BAPTA/AM (**A** (control), **B** (25 μM), and **C** (50 μM)), and then washed and loaded with the fluorescent dye fura-2 (1 μM). Cells were then stimulated with fMet-Leu-Phe (10^{-8}M) and the increases of intracellular calcium concentrations were recorded as described [14].

TABLE I. Effects of Various Treatments on the Histone H4 Kinase Activity Stimulated by fMet-Leu-Phe in Rabbit Peritoneal Neutrophils*

	Histone H4 Kinase Activity	
Pretreatment	Basal	fMet-Leu-Phe-treated
A. None	100%	318 ± 15%
Pertussis toxin (0.5 μg/ml, 1 h)	72 ± 4%	76 ± 5%
B. None	100%	344 ± 40%
t-BOC-(Phe-Leu)$_2$-Phe (10^{-6}M, 0 min)	96 ± 3%	180 ± 3%
C. None	100%	215 ± 22%
BAPTA/AM Loading (50 μM, 30 min)	106 ± 12%	227 ± 32%
D. None	100%	279 ± 27%
PMA (0.1 μg/ml, 3 min)	86 ± 7%	299 ± 30%
E. None	100%	288 ± 35%
H-7 (25 μM, 3 min)	95 ± 8%	273 ± 60%

*Cells were treated with various reagents and then stimulated with fMet-Leu-Phe (10^{-8}M, 10 s). The H4 kinase activity was assayed from control and treated cells as described in the text. The basal kinase activity measured in control cells was used as 100%. The absolute values of basal activity are A) 37.5 ± 7.8, B) 48.5 ± 3.2, C) 31.8 ± 0.6, D and E) 26.3 ± 2.8 (pmol/min/2.5 × 10^7 cells) respectively. Each value represents the mean ± SEM of three or four experiments. In the experiments using the antagonist *t*-BOC-(Phe-Leu)$_2$-Phe, the antagonist was added together with fMet-Leu-Phe to the cells.

Effect of PMA and H-7 Pretreatments

We (unpublished result) and others [2] have observed that treatment of neutrophils with PMA causes the translocation of protein kinase C from cytosol to membrane, and the increase of protein phosphorylation. Pretreatment of the cells with PMA results in the inhibition of intracellular calcium rise as well as the degranulation

TABLE II. The Effect of BAPTA/AM on the Geration of Phosphatidic Acid in Neutrophils Stimulated With fMet-Leu-Phe*

Pretreatment	Basal	Phosphatidic acid production	
		fMet-Leu-Phe (10s)	fMet-Leu-Phe (2 min)
None	100%	130 ± 4%	180 ± 7%
BAPTA/AM (50 μM, 30 min)	109 ± 10%	110 ± 6%	136 ± 10%

*Measurement for the generation of phosphatidic acid was carried out as described [15]. Cells were incubated at 37°C for 1 h in Hanks' solution containing [^{32}P]-H_2PO_3 (33 μCi/ml). Cells were then treated with or without BAPTA/AM (50 μM, 30 min), washed, resuspended in Hanks' buffer, and stimulated with fMet-Leu-Phe (10^{-8}M) (10 s or 2 min). The reaction was stopped by the addition of hexane/isopropanol/HCl and the phospholipids were analyzed by TLC plate as described [15]. The basal value of [^{32}P] phosphatic acid measured in control cells was used as 100%. Each value represent the mean ± Sem of three experiments.

of primary granules stimulated by fMet-Leu-Phe [13]. The antagonistic effect of PMA is most likely due to the disruption of the coupling between GTP binding protein and phospholipase C [16]. We have previously presented evidence that a pp50 phosphoprotein is a substrate for protein-kinase C in fMet-Leu-Phe and PMA-treated rabbit neutrophils [8]. fMet-Leu-Phe (10^{-8}M) stimulates the pp50 phosphorylation within 20 s of treatment while PMA (0.1 μg/ml) takes several minutes for a detectable effect [8]. A similar phosphopeptide derived from pp50 by limited proteolysis was phosphorylated by both stimuli [8]. We tested whether PMA pretreatment affected fMet-Leu-Phe-stimulated pp50 phosphorylation. Pretreatment of [^{32}P]-labeled cell with PMA (0.1 μg/ml) gradually inhibits the stimulation of pp50 phosphorylation by fMet-Leu-Phe (Fig. 2). The inhibition is maximum after 3 min of PMA pretreatment. We then tested the effect of pretreatment of neutrophils of PMA on the stimulation of histone H4 kinase by fMet-Leu-Phe. As shown in Figure 3 and Table I, pretreatment of the cells with PMA had no significant effect on the stimulation of the kinase by fMet-Leu-Phe (10^{-8}M, 10 s). It should be mentioned that PMA pretreatment also did not change the dose-response curve of fMet-Leu-Phe stimulation of H4 kinase (data not shown).

Treatment of neutrophils with H-7 or its analogues inhibits protein kinase C–mediated phosphorylation of pp50 [17] but has little effect on the degranulation and superoxide generation responses stimulated by fMet-Leu-Phe [17]. Figure 3 and Table I show that pretreatment of neutrophils with H-7 had no significant effect on the stimulation of H4 kinase by fMet-Leu-Phe.

Possible Mode of Activation of H4 Kinase by fMet-Leu-Phe

The possibility that a small change of localized levels of calcium and diacylglycerol is related to H4 kinase activation cannot be ruled out. However, the data presented in this paper suggest that increases of the mean intracellular levels of free calcium and possibly the full activation of phospholipase C and protein kinase C are not required for H4 kinase activation. However, functional receptor and a pertussis-toxin-sensitive GTP binding protein are both necessary. These results suggest several possible modes of activation of H4 kinase by fMet-Leu-Phe:

a. Activation by a pathway similar to actin polymerization stimulated by fMet-Leu-Phe [18]. The actin polymerization induced by fMet-Leu-Phe does not require intracellular calcium mobilization and is most likely regulated by phosphatidylinositol bisphosphate [1]. Profilactin [19] and actin binding protein such as gelsolin (20) have

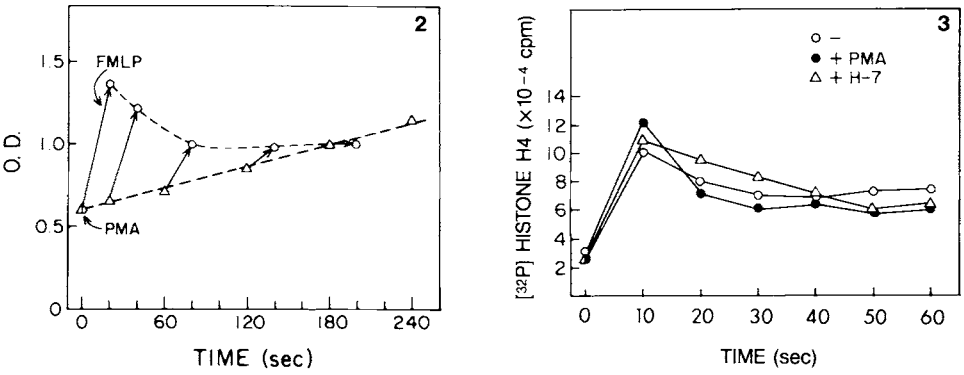

Fig. 2. Effect of PMA pretreatment on the stimulation of the phosphorylation of pp50 by fMet-Leu-Phe. [^{32}P]-labeled intact cells (2.5 × 10^7 cells/ml) in Hanks' buffer were treated with PMA (0.1 μg/ml (Δ)). At various time periods, aliquots of cells were withdrawn and stopped by SDS (Δ) or stimulated with fMet-Leu-Phe (10^{-8}M, 20 s) (○). The stimulation was stopped by SDS and [^{32}P]-labeled phosphoproteins analyzed by gel electrophoresis and autoradiography. Optical density of pp50 in the autoradiograph was determined by densitometry as described [8].

Fig. 3. Effect of PMA and H-7 pretreatment on the H4 kinase activation. Cells (2.5 × 10^7 cells/ml, 0.5 ml) were pretreated with buffer (○) or PMA (0.1 μg/ml; 3 min) (●) or H-7 (25 μM, 5 min) (Δ) before stimulating with fMet-Leu-Phe (10^{-8}M). Aliquots (30 μl) of cells were withdrawn at various time periods and assayed for H4 kinase activity as described in the text. Direct addition of PMA or H-7 to the assay mixture did not affect the H4 kinase activity [11].

been shown to bind to phosphatidylinositol bisphosphate specifically and they may be involved in actin polymerization. We have recently observed that H4 kinase was inhibited by phosphatidylinositol, phosphatidylinositol-4-phosphate, and phosphatidylinositol 4,5 bisphosphate (see below). The rapid changes in phosphatidylinositol metabolism may induce both actin polymerization and H4 kinase activation.

b. Activation by interaction with a GTP binding protein. Pretreatment of neutrophils with PMA disrupts the coupling between GTP binding proteins and phospholipase C [16]. The lack of effect of PMA pretreatment on fMet-Leu-Phe-stimulated H4 kinase activity suggests that fully activation of phospholipase C is not required for the stimulation of H4 kinase. Whether there is a GTP binding protein which can activate directly the H4 kinase is unknown. The possibility that one GTP binding protein can interact with more than one effector protein has recently been demonstrated [21,22].

c. Reversible covalent modification of the H4 kinase. Masarachia and her colleague have reported the activation of H4 kinase by preincubation of the kinase with MgATP [23]. We recently observed a similar effect of MgATP preincubation on partially purified H4 kinase from neutrophils (data not shown). Whether this is due to autophosphorylation or phosphorylation mediated by other kinases remains to be studied.

We have previously determined the activity of H4 kinase in the soluble and particulate fractions of neutrophils [11]. Of the kinase activity, 33% were observed in the cytosol and 67% in the particulate fraction. Both were stimulated by fMet-Leu-Phe treatment of intact cells. Recently, we have purified the cytosolic kinase several-

hundred-fold by DEAE-cellulose, H4-Sepharose, and histone-agarose chromatography. The partially purified enzyme was strongly inhibited by phosphatidylserine, phosphatidylinositol, phosphatidylinositol-4-phosphate, and phosphatidylinositol 4,5-bisphosphate. The physiological significance of this finding remains to be defined.

The rapid and transient activation of H4 kinase suggests that it may play a role in triggering the biological responses induced by chemotactic factor. It may represent a novel pathway which is calcium and protein kinase C independent. Recent studies on neutrophil activation support the existence of such a pathway [5,24].

Using methods similar to ours, Slonczewski et al. [25] reported the activation of phosphorylase a by fMet-Leu-Phe in human neutrophils. In contrast to the H4 kinase activation, phosphorylase a activation is closely related to the cytoplasmic calcium levels in neutrophils. We recently observed the stimulation of protein tyrosine phosphorylation by fMet-Leu-Phe [26]. This latter response is also related to the cytoplasmic calcium levels in neutrophils (manuscript in preparation). Recently, a histidine protein kinase which can also phosphorylate histone H4 has been found to play a role in bacterial chemotaxis [27]. It remains to be shown whether histidine phosphorylation in addition to serine phosphorylation [11] also occurs in the histone H4 kinase assay described in this paper.

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