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Pertussis toxin inhibits chemotactic peptide-stimulated generation of inositol phosphates and lysosomal enzyme secretion in human leukemic (HL-60) cells

(signal transduction/GTP-binding proteins/phospholipase C/inositol phospholipids)

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ABSTRACT **The binding of the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine to its cell surface receptor rapidly elicits the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C to form the putative second messengers inositol 1,4,5-trisphosphate and *sn*-1,2-diacylglycerol. To investigate the possible role of a guanine nucleotide binding protein in transduction of this membrane signal, we examined the effects of pertussis toxin on chemotactic peptide-stimulated inositol phospholipid metabolism in differentiated HL-60 cells labeled with [^{3}H]inositol. Pertussis toxin inhibited the chemotactic tripeptide-stimulated production of inositol mono-, bis-, and trisphosphates and secretion of *N*-acetyl-β-D-glucosaminidase in a time- and concentration-dependent manner. Treatment with pertussis toxin did not alter the total incorporation or the distribution of [^{3}H]inositol in inositol phospholipid. Chemotactic peptide receptor number was unchanged, although a slight decrease in binding affinity was observed. These findings suggest a role for a guanine nucleotide binding protein in coupling the chemotactic peptide receptor to phospholipase C.**

Binding of the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) to its receptor on the leukocyte membrane triggers multiple cellular responses, including chemotaxis, secretion of lysosomal enzymes, and generation of bactericidal oxygen species (1). One of the earliest biochemical consequences of receptor occupancy is the phosphodiesteratic hydrolysis of inositol phospholipids, also termed phosphoinositides (2–5). Evidence is rapidly accumulating that phospholipase C-mediated hydrolysis of one of these lipids, phosphatidylinositol 4,5-bisphosphate (PtdInsP_2), creates two second messengers: *sn*-1,2-diacylglycerol, which activates protein kinase C (reviewed in ref. 6), and inositol 1,4,5-trisphosphate (InsP_3), which releases calcium from intracellular stores (reviewed in ref. 7). However, little is known about the mechanism by which receptor occupancy leads to phospholipase C activation.

We have approached this problem by examining the effect of pertussis toxin (PT) on chemotactic peptide-stimulated inositol phospholipid metabolism in differentiated HL-60 cells. PT catalyzes the ADP ribosylation of specific guanine nucleotide binding proteins, and it has been widely used to study the coupling of receptors to adenylate cyclase (8–10). Recent work has suggested that guanine nucleotide binding proteins may be involved in cellular responses that utilize calcium as a second messenger (11, 12). We chose to study the HL-60 leukemic cell line because these cells avidly incorporate [^{3}H]inositol into inositol phospholipids (5) and, after treatment in culture with dibutyryl cAMP (Bt_2cAMP), express large numbers of biologically active formyl peptide receptors (13). We report that PT inhibits fMet-Leu-Phe-stimulated generation of inositol phosphates and lysosomal enzyme secretion in HL-60 cells induced to differentiate with Bt_2cAMP. Our results are consistent with the hypothesis that the chemotactic peptide receptor is coupled to phospholipase C by a guanine nucleotide binding protein.

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MATERIALS AND METHODS

Materials. fMet-Leu-Phe, Hepes, bovine serum albumin (Cohn fraction V), cytochalasin B, *p*-nitrophenyl-β-D-glucosaminide, Bt_2cAMP, phosphatidylinositol 4-monophosphate (PtdIns4*P*), and PtdInsP_2 were purchased from Sigma. Phosphatidylinositol (PtdIns) was from Avanti. Myo-[2-^{3}H]inositol (specific activity, 14 and 15.6 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham. It was passed over Dowex (Bio-Rad) anion exchange resin (AG 1-X8, 100-200 mesh) in the formate form and was eluted with water prior to use. All media were obtained from GIBCO. Fetal bovine serum was from HyClone (Logan, UT). Precoated silica gel (G-25) thin-layer chromatography plates were from Brinkman. *N*-formylnorleucylleucylphenylalanyl[^{3}H]$_2$tyrosine-(fNle-Leu-Phe-[^{3}H]$_2$Tyr) (specific activity, 71 Ci/mmol) was a gift of Richard Freer (Medical College of Virginia). Initial experiments were done using purified PT provided by Erik Hewlett (University of Virginia). Toxin was also purchased from List Biological Laboratories (Campbell, CA). Similar results were obtained with both preparations, although a difference in potency was noted. All other chemicals were of reagent grade.

Cell Cultures. HL-60 promyelocytic leukemia cells were obtained from Robert Gallo (National Cancer Institute) and were routinely passaged as described (13). Viability, as assessed by trypan blue exclusion, was >90% under all conditions tested. Efficient labeling with [^{3}H]inositol was achieved using 4 μCi/ml in Ham's F-10 medium supplemented with 1% fetal bovine serum/10 mM Hepes. Cells were seeded in this medium at a concentration of 7.5×10^5 cells per ml and were induced to differentiate with 500 μM Bt_2cAMP (13). After a 36-hr incubation at 37°C in an atmosphere of 95% air/5% CO_2, the cells were collected by low speed centrifugation, resuspended in fresh medium without [^{3}H]inositol or Bt_2cAMP, to which was added PT or buffer, and returned to the incubator. Cells used in secretion experiments were handled in the same way, except that RPMI 1640 medium with 10% fetal bovine serum/10 mM Hepes/500 μM Bt_2cAMP was used.

Abbreviations: PT, pertussis toxin; PtdInsP_2, phosphatidylinositol 4,5-bisphosphate; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol; Ins*P*, inositol 1-phosphate; InsP_2, inositol 1,4-bisphosphate; InsP_3, inositol 1,4,5-trisphosphate; Bt_2cAMP, N^6,$O^{2'}$-dibutyryl cAMP.

Measurement of [^{3}H]Inositol Phosphates and [^{3}H]Inositol Phospholipids. After treatment with PT or buffer, cells were washed with a balanced salt solution consisting of 135 mM NaCl/4.5 mM KCl/1.5 mM $CaCl_2$/0.5 mM $MgCl_2$/5.6 mM glucose/10 mM Hepes/0.025% bovine serum albumin, pH 7.4. Typically, for each data point, 2–3 million cells were mixed with the indicated concentration of fMet-Leu-Phe in a vol of 500 μl. The cell suspension was incubated in a 37°C water bath and the reaction was stopped by the addition of 1.88 ml of chloroform/methanol/HCl (100:200:2). In some experiments, the cells were pretreated with 10 mM lithium chloride for 5 min before adding fMet-Leu-Phe. Lithium inhibits the enzyme inositol 1-phosphate phosphatase (14) and has been used to amplify receptor-stimulated generation of inositol phosphates. Similar results were obtained with or without its use. Chloroform (0.62 ml) and water (0.62 ml) were added to separate the phases, and the contents of the tubes were mixed and then centrifuged at 800 × *g* for 10 min.

The inositol phosphates were separated as described by Berridge (15) and as modified by Watson *et al.* (16). A 1.5-ml aliquot of the upper aqueous phase was mixed with 2.5 ml of water and applied to columns containing 1 ml of Dowex AG 1-X8 in the formate form. [^{3}H]Inositol and [^{3}H]glycerophosphorylinositol were eluted with 16 ml of 60 mM ammonium formate/5 mM disodium tetraborate and discarded. [^{3}H]Inositol phosphate (Ins*P*) was eluted with 16 ml of 200 mM ammonium formate/100 mM formic acid/[^{3}H]inositol 1,4-bisphosphate (InsP_2) with 16 ml of 400 mM ammonium formate/100 mM formic acid; and [^{3}H]InsP_3 with 12 ml of 1 M ammonium formate/100 mM formic acid. The InsP_3 fraction may have included both the recently described 1,3,4-isomer (17) and the 1,4,5-isomer on prolonged stimulation with fMet-Leu-Phe. Radioactivity was determined by counting 1-ml aliquots of pooled column fractions with 15 ml of Ultrafluor in a liquid scintillation counter. Because the cells displayed considerable basal inositol phosphate production, in most cases companion tubes without fMet-Leu-Phe were run to determine fMet-Leu-Phe-stimulated activity.

In some experiments, the organic phase was taken, the remaining material was washed with 2 ml of chloroform, and the phases were separated by centrifuging at 800 × *g* for 10 min. The combined organic phases were dried under nitrogen and resuspended in 0.05 ml of chloroform. The inositol phospholipids PtdIns, PtdIns4*P*, and PtdInsP_2 were separated on oxalate-impregnated silica gel thin-layer chromatography plates (18) developed in chloroform/methanol/ammonium hydroxide (4 M) (45:35:12). Lipid standards were run in parallel and were visualized with iodine vapor. The areas corresponding to the three inositol phospholipids were scraped into vials and counted in a liquid scintillation counter.

Other Assays. fMet-Leu-Phe-stimulated release of the lysosomal enzyme *N*-acetyl-β-D-glucosaminidase from cytochalasin B-treated HL-60 cells was measured as described (5). Binding of the radiolabeled chemotactic peptide fNle-Leu-Phe[^{3}H]$_2$Tyr was carried out as described (19). Measurements of inositol phosphates and lysosomal enzyme secretion were done in duplicate. These consistently differed by <10%. Binding studies were done in triplicate.

RESULTS

Dougherty *et al.* used dimethyl sulfoxide-differentiated HL-60 cells to study fMet-Leu-Phe-stimulated inositol phospholipid metabolism (5). Preliminary experiments demonstrated a similar pattern of fMet-Leu-Phe-stimulated production of inositol phosphates in HL-60 cells that had been induced to differentiate by treatment with Bt_2cAMP. As can be seen in Fig. 1, the concentration dependence for fMet-Leu-Phe-stimulated secretion and for generation of inositol phosphates was similar. Treatment of the cells with 400 ng of PT per ml for 2 hr resulted in almost total inhibition of both stimulated lysosomal enzyme secretion and generation of inositol phosphates. This inhibition could not be overcome with higher concentrations of fMet-Leu-Phe. In some experiments, PT lowered the basal level of tritiated inositol phosphates. This was particularly evident when high concentrations of PT were used.

The inhibitory effects of PT were time and concentration dependent. Increasing inhibition with time was noted, such that secretion was 20% of control and inositol phosphate generation was 45% of control after a 6-hr incubation with PT at 50 ng/ml (data not shown). The concentration dependence is shown in Fig. 2. A close, although not exact, correlation was evident between inhibition of fMet-Leu-Phe-stimulated secretion and generation of inositol phosphates.

Inhibition of inositol phosphate formation could result from the inhibition of PtdIns and/or PtdIns4*P* kinase or from the stimulation of PtdInsP_2, and/or PtdIns4*P* phosphomonoesterase, thus decreasing the amount of substrate available to phospholipase C. To investigate these possibilities, cells were treated with 400 ng of PT per ml for 2 hr and PtdIns,

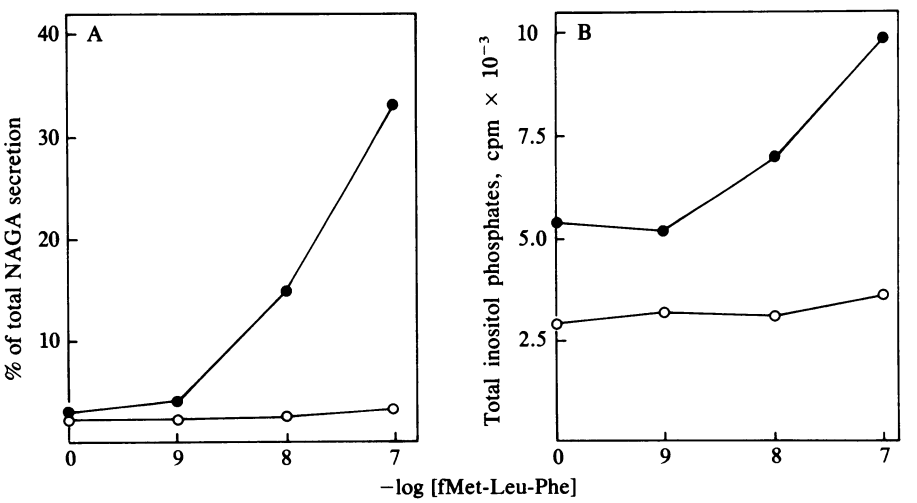

FIG. 1. *N*-Acetyl-β-D-glucosaminidase (NAGA) secretion (*A*) and generation of inositol phosphates (*B*) as a function of fMet-Leu-Phe concentration in the presence or absence of PT. Cells were treated with 400 ng of PT per ml (○) or buffer (●) for 2 hr and then assayed as described. Total radioactivity in inositol phosphates was derived from the sum of that in Ins*P*, InsP_2, and InsP_3 after sequential treatment with 10 mM LiCl for 5 min and fMet-Leu-Phe for 15 min.

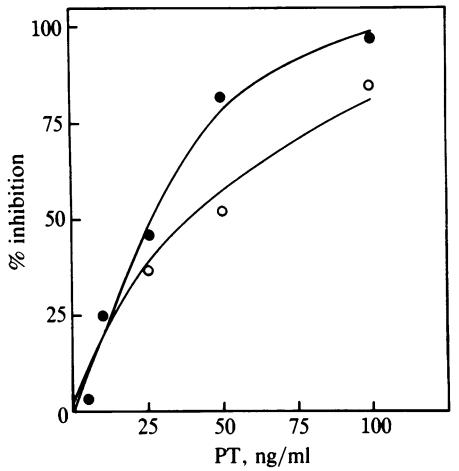

FIG. 2. Inhibition of *N*-acetyl-β-D-glucosaminidase secretion (●) and total inositol phosphate generation (○) as a function of PT concentration. Cells were treated with buffer or PT at the indicated concentrations for 6 hr and then assayed as described, using 100 nM fMet-Leu-Phe. Total radioactivity in inositol phosphates was derived from the sum of that in Ins*P*, InsP_2, and InsP_3 after a 1-min incubation with fMet-Leu-Phe.

PtdIns4*P*, and PtdInsP_2 were separated by thin-layer chromatography. No differences were noted in either the total amount of [^{3}H]inositol incorporated (data not shown) or in the distribution of label among these three inositol phospholipids (Table 1). These data largely exclude an effect of PT on one of the phosphoinositide kinases or monoesterases and indicate that the toxin was acting at the level of phospholipase-mediated hydrolysis.

InsP_3 increased within the first 5 sec of stimulation, in agreement with the results of Dougherty *et al.* (5). PT inhibited the fMet-Leu-Phe-stimulated increase in all three inositol phosphates (Fig. 3). This inhibition was evident at the earliest time point studied (5 sec). These data indicate that PT inhibited the phosphodiesteratic cleavage of PtdInsP_2 by phospholipase C. The decrease in InsP_2 and Ins*P* could have resulted from inhibition of phospholipase C-mediated hydrolysis of PtdIns4*P* and PtdIns, respectively, or could simply reflect the decrease in InsP_3, the initial substrate for phosphomonoesterases that catalyze the sequential hydrolysis of InsP_3 to InsP_2 and Ins*P*.

To explore the possibility that the effect of PT on inositol phosphate generation may be mediated by an increase in cAMP, we compared the effects of PT and cholera toxin, an agent known to increase intracellular cAMP levels. Cholera toxin stimulates adenylate cyclase by activating a specific GTP-binding protein (20), and previous work had demonstrated that neutrophil membranes possess this protein (21). Cells were treated with PT or cholera toxin at 100 ng/ml for 6 hr, and total inositol phosphate release was measured after a 1-min incubation with 100 nM fMet-Leu-Phe. Release of inositol phosphates was inhibited 76% by PT and 24% by cholera toxin (data not shown). Okajima and Ui (22) and Bokoch and Gilman (23) reported virtually identical inhibition by PT and cholera toxin of fMet-Leu-Phe-stimulated arachidonic acid liberation in guinea pig neutrophils. They also showed directly that PT treatment did not cause an increase in either basal or fMet-Leu-Phe-stimulated cAMP levels. Cholera toxin, which caused a large increase in intracellular cAMP concentration, was not as effective as PT in blocking arachidonic acid release.

Table 1. Effect of PT on distribution of [^{3}H]inositol in inositol phospholipids

	Control (n = 3)	PT (n = 4)
PtdIns	94.1 ± 1.4	93.8 ± 1.4
PtdIns*P*	3.8 ± 0.1	3.9 ± 1.0
PtdInsP_2	2.1 ± 1.4	2.4 ± 0.5

Data represent percent ± SD of radioactivity in each of the indicated inositol phospholipids after treatment of cells with PT (400 ng/ml) or buffer for 2 hr. Results from two similar experiments were pooled (n = number of determinations).

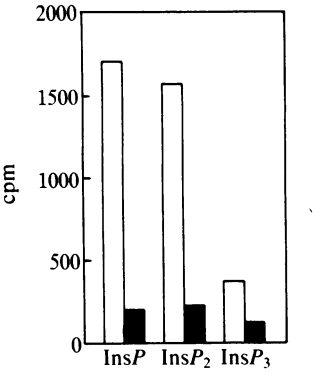

FIG. 3. Effect of PT on formation of individual inositol phosphates after a 1-min incubation with 100 nM fMet-Leu-Phe. Cells were treated with 100 ng of PT per ml (shaded bars) or buffer (open bars) for 6 hr and were assayed as described.

Finally, the effect of PT on the binding of chemotactic peptide to its receptor on HL-60 cells was assessed with the agonist fNle-Leu-Phe-[^{3}H]$_2$Tyr. Previous work with differentiated HL-60 cells had established that this ligand binds to a single class of formyl peptide receptors (19). Receptor numbers per cell and dissociation constants (K_d) were calculated by Scatchard analysis of binding data from control cells and from cells treated with PT at 400 ng/ml for 2 hr. PT, under these conditions, increased the K_d from 0.99 ± 0.09 × 10^{-9} M (mean ± SD; n = 5) to 1.63 ± 0.11 × 10^{-9} M (n = 4). The number of binding sites was unchanged (13,910 ± 693 sites per cell for control and 13,124 ± 1040 sites per cell for PT-treated cells). Decreased binding affinity of α-adrenergic, muscarinic cholinergic, and opiate receptors has been reported after treatment with PT (9, 10).

DISCUSSION

An understanding of the mechanism by which *N*-formylated chemotactic peptides activate phagocytic leukocytes has been slowly developing over the past few years. Workers in several laboratories have demonstrated rapid fMet-Leu-Phe-stimulated hydrolysis of inositol phospholipids by phospholipase C; the most extensive studies have recently been reported using differentiated HL-60 cells (5). Both products of the phospholipase C-mediated hydrolysis of PtdInsP_2, *sn*-1,2-diacylglycerol and InsP_3, are likely to function as intracellular second messengers; the former by activating protein kinase C and the latter by causing release of intracellular calcium (for reviews, see refs. 6 and 7). The actions of InsP_3 may be mediated by protein phosphorylation (24, 25).

That a guanine nucleotide binding protein might have a role in transmembrane signaling via the formyl peptide receptor was suggested by the observations that nonhydrolyzable GTP analogs converted high-affinity fMet-Leu-Phe binding sites to low-affinity sites (26, 27) and that fMet-Leu-Phe stimulated a GTPase activity in neutrophil homogenates (28). However, no data linking the GTP-binding proteins with receptor-mediated phospholipase C activation had been presented.

While this manuscript was in preparation, several related reports have appeared. Goldman *et al.* (29) and Molski *et al.* (30) showed that PT blocked the increase in intracellular free calcium induced by fMet-Leu-Phe and leukotriene B_4, as

measured by Quin-2 fluorescence changes. Okajima and Ui (22) and Bokoch and Gilman (23) independently demonstrated that PT inhibited fMet-Leu-Phe-stimulated superoxide production, phospholipase A_2 activation, and lysosomal enzyme release in guinea pig neutrophils. Both groups conclusively demonstrated that these actions were not due to changes in cAMP, because PT did not increase basal or stimulated intracellular cAMP concentrations and agents that caused large increases in cAMP were relatively poor inhibitors of fMet-Leu-Phe-stimulated responses. They demonstrated that PT caused the ADP ribosylation of a M_r 41,000 membrane protein. We have also found a M_r 41,000 protein to be the only substrate for PT-mediated ADP ribosylation in particulates prepared from differentiated HL-60 cells.

Independently, Verghese *et al.* have obtained data that also lead to the conclusion that PT blocks fMet-Leu-Phe-stimulated phospholipase C in human neutrophils. They showed that PT inhibited fMet-Leu-Phe-stimulated Ca^{2+} mobilization, cAMP increase, chemotaxis, superoxide production, and lysosomal enzyme release. More importantly, they showed that PT completely abolished fMet-Leu-Phe-stimulated breakdown of PtdInsP_2, demonstrating that the inhibition of inositol phosphate production that we observed was due to specific inhibition of the phosphodiesteratic cleavage of PtdInsP_2 (M. W. Verghese, C. D. Smith, and R. Snyderman, personal communication).

The data presented here and that of Verghese *et al.* provide an explanation for the inhibition by PT of multiple biological and biochemical processes elicited by fMet-Leu-Phe in leukocytes. All of the processes studied—lysosomal enzyme release, superoxide generation, phospholipase A_2 activation, and increased Quin-2 fluorescence—are associated with an increase in intracellular calcium and/or diacylglycerol. By blocking the phospholipase C-mediated hydrolysis of PtdInsP_2, PT would inhibit the generation of both the intracellular calcium mobilizing and diacylglycerol signals. Hence, any cellular response dependent on the release of either of these second messengers would be inhibited.

The biochemical mechanism by which PT inhibits phosphoinositide hydrolysis has not been determined. However, Okajima and Ui (22) and Bokoch and Gilman (23) correlated inhibition of fMet-Leu-Phe-elicited biological responses with ADP ribosylation of a M_r 41,000 protein. This information, considered in the context of the guanine nucleotide-induced changes in fMet-Leu-Phe receptor affinity (26, 27) and the fMet-Leu-Phe-activated GTPase activity (28), suggests that formyl peptide receptor activation of phospholipase C may be analogous to other receptor-coupled enzyme responses. For instance, PT-catalyzed ADP ribosylation of transducin inhibits a light-stimulated cGMP phosphodiesterase (31). PT uncouples inhibitory receptors from adenylate cyclase by modification of the regulatory protein designated N_i (or G_i) (9, 10). PT also inhibits insulin activation of a cAMP phosphodiesterase (32). In these examples, PT catalyzes the ADP ribosylation of a GTP-binding protein essential for receptor–enzyme coupling. Our data and that of Verghese *et al.* support the hypothesis that the formyl peptide receptor is coupled to phospholipase C by a GTP-binding protein (33); most probably, the M_r 41,000 membrane protein identified in neutrophils and differentiated HL-60 cells. This protein could be the α subunit of N_i or a subunit of one of the recently described GTP-binding proteins for which no function has yet been determined (34, 35).

Note Added in Proof. Shefcyk *et al.* (36) and Verghese *et al.* (37) have reported PT inhibition of PtdInsP_2 hydrolysis in rabbit and human neutrophils, respectively.

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