Pertussis toxin inhibits chemotactic factor-induced phospholipase C stimulation and lysosomal enzyme secretion in rabbit neutrophils

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Pertussis toxin suppressed [^{32}P]polyphosphoinositide breakdown and lysosomal enzyme secretion induced by fMet-Leu-Phe in rabbit neutrophils. Likewise, fMet-Leu-Phe- or leukotriene B_4-evoked [^{3}H]inositol trisphosphate accumulation was inhibited by the toxin. These findings, taken together with evidence that pertussis toxin specifically causes inactivation of the guanine nucleotide binding protein (N_i), suggests that guanine nucleotide binding proteins may mediate coupling between calcium-mobilising receptors and phospholipase C-mediated reactions in rabbit neutrophils.

Pertussis toxin *Rabbit neutrophil* *Phospholipase C* *Inositol trisphosphate* *Lysosomal enzyme secretion*

1. INTRODUCTION

In the rabbit neutrophil, an initial response to an fMet-Leu-Phe challenge is the phospholipase C-mediated breakdown of PtdIns(4,5)P_2 to form diacylglycerol and InsP_3 [1–3]. Stimulation of phospholipase C may be a general mechanism of neutrophil response to chemotactic factors as suggested by the ability of LTB_4 also to elicit InsP_3 accumulation to a degree commensurate with its degranulating capacity [3]. (1,4,5)InsP_3, the product of the phosphodiesteratic hydrolysis of PtdIns(4,5)P_2, induces Ca^{2+} release from non-mitochondrial vesicular pools in permeable DMSO-differentiated HL-60 cells, which is a model for the human neutrophil [4]. Elevated cytosolic Ca^{2+} activates Ca^{2+}-dependent processes which act synergistically with protein kinase C to elicit a maximal secretory response by the neutrophil [5].

Further insight into the mechanism of signal transduction was obtained by recent studies employing PT. The toxin which specifically ADP-ribosylates the α subunit of the guanine nucleotide binding (N_i) protein [6], inhibits the quin2-sensitive rise in intracellular free Ca^{2+} in stimulated rabbit neutrophils [7] and the activation of phospholipase A_2 and lysosomal enzyme secretion in guinea-pig neutrophils [8,9].

In this report, we provide further insight into the mechanism by which PT alters rabbit neutrophil function by demonstrating that the toxin inhibits phospholipase C activation induced by fMet-Leu-Phe and LTB_4. These findings support the hypothesis that the N_i protein may function to couple chemotactic factor receptors and phospholipase C activity in polymorphonuclear cells.

2. METHODS

2.1. *Materials*

[^{3}H]Inositol (16 Ci/mmol) was purchased from

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Abbreviations: PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; InsP_3, inositol trisphosphate; fMet-Leu-Phe, formylmethionylleucyl-phenylalanine; LTB_4, leukotriene B_4; PT, pertussis toxin; NAGA, *N*-acetylglucosaminidase; DMSO, dimethyl sulphoxide

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American Radiolabeled Chemicals (St Louis, MO) and $^{32}PO_4$ (35 μCi/ml) was obtained from New England Nuclear (Boston, MA). Pertussis toxin was generously provided by Dr Frederick Darfler (National Institutes of Health). fMet-Leu-Phe was obtained from Sigma (St Louis, MO), and LTB_4 was a generous gift from Dr J. Rokach of Merck Frosst Canada, Inc.

2.2. *Preparation and incubation of rabbit neutrophils*

Neutrophils were harvested from New Zealand white rabbits by peritoneal saline lavage 4–10 h after an intraperitoneal injection of 150 ml 0.1% molluscan glycogen. The cells (>95% neutrophils) were washed, resuspended, and maintained at 37°C under O_2/CO_2 (95/5) in a modified Hank's balanced salt solution which had the following composition (mM): NaCl, 128; KCl, 4.6; Na_2HPO_4, 0.64; KH_2PO_4, 0.66; $NaHCO_3$, 15.2; $CaCl_2$, 0.5; $MgSO_4$, 0.2; glucose, 5.6; Hepes, 10 (pH 7.4) and 0.025% bovine serum albumin. Neutrophils were treated for 1 h at 37°C with either PT (200 ng/ml) or vehicle as indicated. For inositol phosphate studies, cells (200–300 × 10^6/2–3 ml) were labeled for 2 h with 100 μCi [^{3}H]inositol. During the second hour, PT or vehicle was added to the incubation medium. After labeling, the cells were washed, aliquoted, and exposed to agonist for 20 s. For [^{32}P]phosphoinositide studies, neutrophils were treated for 1 h with toxin or vehicle, washed, and then labeled for 45 min in low phosphate buffer with $^{32}PO_4$. The cells were then stimulated with agonist for 20 s.

2.3. *Analytical methods*

The inositol phosphates were extracted with 2 vols ice-cold 4.5% perchloric acid, separated by anion exchange chromatography, and [^{3}H]$InsP_3$ analysed as previously described [3]. For analysis of [^{32}P]polyphosphoinositides, the lipids were extracted with cold chloroform/methanol/6 N HCl (1:2:0.025) as in [10]. Polyphosphoinositides were separated by thin layer chromatography on silica gel 60 (EM Reagents) using a solvent system of chloroform/methanol/NH_4OH/H_2O (90:90:7:20). Lipid spots were visualized by autoradiography, scraped, and quantitated by liquid scintillation spectrometry. Lysosomal enzyme activity (NAGA and lysozyme) was assayed in the supernatant of neutrophils suspended in medium containing cytochalasin B (1 μg/ml); the latter drug is required for expressing the stimulatory effect of chemotactic factors on secretion [3].

3. RESULTS AND DISCUSSION

To test the hypothesis that guanine nucleotide binding proteins mediate phospholipase C stimulation, rabbit neutrophils were treated with PT and parameters reflecting phospholipase C activity examined. The effect of PT on chemotactic factor-induced [^{32}P]polyphosphoinositide breakdown was studied first (table 1). fMet-Leu-Phe caused a small, but significant, concentration-dependent decline in ^{32}P-PtdIns(4,5)P_2 levels; minimum levels were observed 10–20 s following stimulation [1]. A 60-min preincubation with PT almost completely blocked this stimulated breakdown (table 1).

In cells prelabeled with [^{3}H]inositol, [^{3}H]$InsP_3$ can arise only from the phosphodiesteratic cleavage of [^{3}H]PtdIns(4,5)P_2 and is therefore an accurate measure of phospholipase C activity. fMet-Leu-Phe caused a rapid and dose-dependent accumulation of [^{3}H]$InsP_3$ (table 2), confirming our previous finding [3] that one of the initial cellular responses to this peptide is the activation of phospholipase C. A maximum secretory dose of fMet-Leu-Phe (10^{-8} M) raised [^{3}H]$InsP_3$ levels

Table 1

Effect of pertussis toxin on stimulated [^{32}P]polyphosphoinositide metabolism in rabbit neutrophils

fMet-Leu-Phe concentration (M)	[^{32}P]PtdIns(4,5)P_2 levels (% basal)	
	Control	+ Toxin
10^{-10}	98.2 ± 0.3	99.7 ± 0.2
10^{-9}	95.9 ± 1.5	98.5 ± 1.0
10^{-8}	89.1 ± 2.4	99.3 ± 2.2

Values are % of DMSO-treated samples and represent means ± SE of 4 determinations each done in duplicate. All concentrations of fMet-Leu-Phe caused significant reductions in [^{32}P]PtdIns(4,5)P_2 levels ($P < 0.025$), and these effects were obtunded by pertussis toxin ($P < 0.025$)

Table 2

Effect of pertussis toxin on stimulated [^{3}H]InsP$_3$ levels in rabbit neutrophils

Additions	[^{3}H]InsP$_3$ levels (% [^{3}H]phosphoinositide)	
	Control	+ Toxin
DMSO (0.1%)	0.39 ± 0.06	0.34 ± 0.05
fMet-Leu-Phe (10^{-10} M)	0.47 ± 0.07	0.33 ± 0.06
fMet-Leu-Phe (10^{-9} M)	0.75 ± 0.07	0.43 ± 0.06
fMet-Leu-Phe (10^{-8} M)	1.19 ± 0.10	0.56 ± 0.07
LTB$_4$ (10^{-7} M)	0.52 ± 0.04	0.39 ± 0.06

All concentrations of fMet-Leu-Phe and LTB$_4$ significantly elevated [^{3}H]InsP$_3$ levels ($P < 0.01$), and this effect was significantly decreased by pertussis toxin ($P < 0.025$). The toxin had no effect on inositol incorporation into lipid. Total ^{3}H incorporation into phosphoinositides averaged 50000 cpm/sample. Values are means ± SE of 4 separate experiments, each done in duplicate

from 0.39 to 1.19% of the total [^{3}H]phosphoinositide radioactivity. When neutrophils were pretreated for 1 h with PT, the stimulatory effect of fMet-Leu-Phe on [^{3}H]InsP$_3$ levels was greatly attenuated, indicative of obtunded phospholipase C activity (table 2). At the lowest concentration of fMet-Leu-Phe employed (10^{-10} M), PT completely blocked the elevation in [^{3}H]InsP$_3$ accumulation; while at the highest peptide concentration (10^{-8} M), the toxin caused more than a 70% depression of stimulated [^{3}H]InsP$_3$ levels.

LTB$_4$ also promoted [^{3}H]InsP$_3$ accumulation, although the observed effect was smaller in magnitude than those observed with fMet-Leu-Phe (table 2). A near maximal dose of LTB$_4$ (10^{-7} M) raised [^{3}H]InsP$_3$ levels to 0.52% of the total ^{3}H-lipid. PT pretreatment also markedly depressed the elevation of [^{3}H]InsP$_3$ levels elicited by LTB$_4$ (table 2), suggesting that fMet-Leu-Phe- and LTB$_4$-induced neutrophil activation are mediated by a similar mechanism. Despite the fact that LTB$_4$ generated a significant rise in [^{3}H]InsP$_3$ levels, a significant breakdown of [^{32}P]PtdIns(4,5)P$_2$ was not observed (not shown) (see also [11]). Measurement of InsP$_3$ levels thus appears to be a more sensitive indicator of phospholipase C activity than [^{32}P]inositide breakdown.

PT also depressed fMet-Leu-Phe- and LTB$_4$-stimulated NAGA and lysozyme secretion from primary and secondary granules, respectively (table 3). The dose-dependent secretion of NAGA and lysozyme was markedly suppressed to a similar extent following a 60-min preincubation with PT. A toxic effect of the toxin can be discounted because the neutrophils retained their lactate dehydrogenase complement and also maintained a modest ability to secrete in a dose-dependent man-

Table 3

Effect of pertussis toxin on stimulated enzyme secretion by rabbit neutrophils

Additions	Lysosomal enzyme release (% total cell content)			
	NAGA		Lysozyme	
	Control	+ Toxin	Control	+ Toxin
fMet-Leu-Phe (10^{-10} M)	12.1 ± 1.1	2.0 ± 0.3	23.3 ± 1.8	3.2 ± 0.9
fMet-Leu-Phe (10^{-9} M)	24.0 ± 1.3	4.5 ± 0.2	36.5 ± 2.3	7.0 ± 0.8
fMet-Leu-Phe (10^{-8} M)	30.6 ± 1.5	7.3 ± 0.9	55.2 ± 1.4	16.3 ± 2.9
LTB$_4$ (10^{-7} M)	11.1 ± 2.3	1.7 ± 0.3	17.3 ± 2.0	4.8 ± 1.3

Neutrophils were treated for 60 min with 200 ng/ml pertussis toxin or vehicle, washed and then exposed to fMet-Leu-Phe or LTB$_4$ for 5 min. Values are means ± SE of 4 determinations, each done in duplicate. Basal secretion (10–15% of total) was subtracted in all cases. Under all conditions, chemotactic agents stimulated significant enzyme secretion ($P < 0.01$), and the toxin caused inhibition of secretion ($P < 0.01$). Lactate dehydrogenase release was less than 10% in all experiments

ner after a 60-min exposure to the toxin.

The present findings indicate that PT inhibits secretion from rabbit neutrophils by suppressing chemotactic factor-induced phospholipase C activation. Subsequent metabolic steps that may be dependent upon this reaction, including Ca^{2+} mobilization, phospholipase A_2 activation, and enzyme secretion are all inhibited by this toxin [1,8,9]. The hypothesis that guanine nucleotide regulatory proteins couple chemotactic factor receptors in a stimulatory way to phospholipase C [12], is supported by our findings that guanine nucleotides are capable of potentiating fMet-Leu-Phe-stimulated hydrolysis of PtdIns(4,5)P_2 in permeabilized neutrophils (in preparation).

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