Chemotactic Peptide Activation of Human Neutrophils and HL-60 Cells

Pertussis Toxin Reveals Correlation between Inositol Trisphosphate Generation, Calcium Ion Transients, and Cellular Activation

Karl-Heinz Krause, Werner Schlegel, Claes B. Wollheim, Tommy Andersson, Francis A. Waldvogel, and P. Daniel Lew
Division des maladies infectieuses, Hopital Cantonal Universitaire, Fondation pour recherches medicales; Institut de biochemie clinique, Centre Medical Universitaire, Geneva, Switzerland; Department of Medical Microbiology, University of Linköping, Sweden

Abstract

The mechanism of neutrophil activation by the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (FMLP) has been studied by pretreatment of human neutrophils with pertussis toxin. Upon stimulation with FMLP, the cytosolic-free calcium concentration, $[Ca^{2+}]_i$, is increased both by stimulation of calcium influx and mobilization of cellular calcium. We have measured $[Ca^{2+}]_i$ as well as the generation of the phospholipid breakdown product inositol trisphosphate (IP_3), which is thought to mediate Ca^{2+} mobilization. As the phosphoinositide pool in human neutrophils is difficult to prelabel with [^{3}H]myoinositol, experiments were also carried out in the cultured human promyelocytic leukemia cell line HL-60 after differentiation with dimethylsulfoxide.

Pertussis toxin pretreatment of both cell types inhibited FMLP stimulated membrane depolarization, exocytosis, and superoxide production in a dose-dependent manner. This toxin effect was selective for the receptor agonist, since stimulation of these parameters by two substances bypassing the transduction mechanism, the calcium ionophore ionomycin and the phorbolester phorbol myristate acetate, were unaffected.

Rises in $[Ca^{2+}]_i$, as well as generation of IP_3 in response to FMLP, were inhibited in parallel; for the inhibition of functional responses, slightly lower toxin concentrations were required. The attentuation of the $[Ca^{2+}]_i$ rise was more marked in the absence of extracellular calcium, i.e., when the rise is due only to calcium mobilization.

The results provide evidence that phospholipase C stimulation by FMLP resulting in IP_3 generation is involved in the signal transduction mechanism. Coupling of FMLP receptor occupancy to phospholipase C activation is sensitive to pertussis toxin, suggesting the involvement of a GTP binding protein (N protein), which has been shown to be a pertussis toxin substrate. The parallel changes in $[Ca^{2+}]_i$ and IP_3 further support the hypothesis that IP_3 is the calcium-mobilizing mediator in FMLP-activated cells.

Dr. Krause is a recipient of a fellowship of the Paul-Martini-Stiftung. Dr. Lew is a recipient of a Max Cloetta Career Development award. Dr. Andersson is a recipient of a postdoctoral fellowship from the Swedish Society of Medicine. Address reprint requests to Dr. Krause, Hopital Cantonal Universitaire.

Received for publication 3 May 1985.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/85/10/1348/07 $1.00
Volume 76, October 1985, 1348–1354

Introduction

The interaction of the chemotactic peptide formyl-methionyl-leucyl-phenylalanin (FMLP)[1] with specific cell surface receptors in neutrophils leads to a rapid rise in cytosolic-free calcium ($[Ca^{2+}]_i$), which appears to be necessary for cellular activation (1, 2). Two recent observations suggest that inositol trisphosphate (IP_3), a product of the hydrolysis of the plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (also referred to as polyphosphoinositide phosphodiesterase), acts as a second messenger: IP_3 is generated within seconds after chemotactic peptide stimulation of neutrophils (3, 4) and HL-60 cells (5) without the need for a previous rise in $[Ca^{2+}]_i$ (3); IP_3 releases Ca^{2+} from intracellular stores in neutrophils permeabilized by digitonin treatment (6).

It remains, however, unclear how the stimulus receptor complex activates phospholipase C. Recent data in several systems suggest the involvement of nucleotide regulatory proteins, called N proteins (7) or G proteins (8), in receptor stimulation of PIP_2 breakdown (9–11). Bacterial toxins, namely cholera toxin and pertussis toxin, have been used in the past to investigate the role of N proteins in receptor coupling, since they modify N protein function by ADP ribosylation (8). Pertussis toxin has been shown to impair the function of the inhibitory N protein linked to adenylate cyclase, N_i (12, 13), and more recently it has been suggested that pertussis toxin may interfere with receptor-mediated alterations of phospholipid metabolism. In neutrophils, it has been shown that pertussis toxin interacts with the FMLP-induced rise in $[Ca^{2+}]_i$ (14) and alters the transient decrease in PIP_2 assessed by short-term labeling with ^{32}P (15, 16).

In the present study we directly measured the generation of the putative second messenger IP_3 after long-term labeling of the phosphoinositides in human neutrophils and in the differentiated human promyelocytic leukemic cell line HL-60. Our results demonstrate that pertussis toxin blocks the FMLP-induced rise in IP_3 in both cell types, and provide substantial evidence for the involvement of a pertussis toxin substrate, presumably a N protein, in the coupling of FMLP receptors to the stimulation of phospholipase C in two cell types. The concom-

1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, cytosolic-free calcium; Di-*O*-C_5(3), 3-3′dipentyloxacarbocyanine; DMSO, dimethylsulfoxide; FMLP, formyl-methionyl-leucyl-phenylalanin; GTP, guanosin-triphosphate; IP_1, inositol phosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; PI, phosphatidylinositol; PIP_1, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PMA, phorbol myristate acetate; quin2/AM, quin 2 acetoxy-methylester.

itant inhibition by pertussis toxin of a rise in $[Ca^{2+}]_i$ and several functional responses to FMLP but not to receptor independent stimulation allowed the assessment of receptor-stimulated IP_3 production in neutrophil activation.

Methods

The materials and their sources were as follows: pertussis toxin (List Biological Laboratories Inc., Campbell, CA), FMLP, phorbol myristate acetate (PMA), cytochalasin B, cytochrome *c* (Sigma Chemical Co., St. Louis, MO), myo-(2-^{3}H)inositol and quin2 acetoxy-methylesther (quin2/AM) (Amersham Int'l. plc, Amersham, England), and Dextran T500 and Ficoll Hypaque (Pharmacia Fine Chemicals, Upsala, Sweden). 3-3'dipentyloxacarbo-cyanine iodide, Di-*O*-C_5(3) (Molecular Probes Inc., Junction City, OR), and 4-methylumbelliferyl substrates were from Koch Laboratories, Haverhill, England. Ionomycin was a kind gift of Dr. C. M. Liu of Hoffmann-La Roche, Inc., Nutley, NJ.

Preparation of human neutrophils. Neutrophils were prepared from fresh blood samples (usually 90 ml) obtained from healthy volunteers. Fresh neutrophils were purified by dextran sedimentation followed by centrifugation through a layer of Ficoll as described previously (17, 18). The cells were suspended in a medium containing 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 100 μM EGTA, 1 mM $NaHPO_4$, 5 mM $NaHCO_3$, 5.5 mM glucose, and 20 mM Hepes, pH 7.4 (This medium will be referred to as calcium medium; calcium-free medium will refer to the above medium without $CaCl_2$, supplemented with 1 mM EGTA).

Culture of HL-60 cells. The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were passaged once every week, and only cells with passage number below 35 were used for the experiments. Differentiation of the cells was induced by adding dimethylsulfoxide (DMSO) (final concentration 1.3%, vol/vol) to the cell suspension. Cells were used for all experiments after 4 d of differentiation.

Preparation of cells for labeling experiments. Phosphoinositides and inositol phosphates were labeled by incubating cells in RPMI medium containing myo-(2-^{3}H)inositol (1 μCi/ml) and 10 and 3% heat-inactivated serum, respectively, for HL-60 cells and human neutrophils. HL-60 cells were incubated under these conditions for 48 h and human neutrophils for 18 h, as described (3, 5).

Incubation with pertussis toxin. 50 μg of pertussis toxin were diluted in 500 μl of a buffer containing 0.1 M sodium phosphate, pH 7.0, and 0.5 M NaCl, yielding a stock concentration of 100 μg/ml. Cells were suspended in calcium medium at a concentration of 10^7 cells/ml and incubated for 2 h at 37°C with the respective concentration of pertussis toxin. Control cells were incubated with the equal amount of buffer.

Measurement of cytosolic-free calcium. Quin2 loading, fluorescent measurement, and calibration were performed as described previously (1).

Degranulation. 1.25×10^6 cells were suspended in 500 μl of calcium medium containing 2.5 μg cytochalasin B and warmed at 37°C for 5 min before addition of stimuli at the indicated concentration and time. Incubation was terminated by rapid cooling in ice and centrifugation (800 *g* for 10 min). Enzymes and vitamin B_{12}-binding protein were assayed in the supernatants and calculated as a percentage of control cells (cells incubated without pertussis toxin). For control cells, percentage of total cell protein released from an aliquot of the same cell suspension treated with 0.025% Triton X-100 for 5 min at 37°C is shown in the figure legend.

β-glucuronidase and *N*-acetyl-β-glucosaminidase were measured fluorimetrically with 4-methylumbelliferyl substrates (19). The unsaturated vitamin B_{12}-binding protein was assayed by a slight modification of the method of Kane et al. (19).

Superoxide production. Superoxide production was monitored continuously in a double beam spectrophotometer, thermostated at 37°C as previously described (20). Data are shown as percentage of control cells (cells not treated with pertussis toxin). For control cells, absolute values are given in the figure legend.

Depolarization. Changes in membrane potential were measured by a flourimetric assay, using the membrane potential-sensitive Cyanine Dye Di-*O*-C_5(3) as described by Seligman (21). A Perkin-Elmer fluorimeter was used. Excitation and emission wavelength were 460 nm and 520 nm, respectively. Di-*O*-C_5(3) was added to a final concentration of 10^{-7} M and cells were added to a final concentration of 5×10^5 cells/ml. The temperature of the cuvette was maintained at 37°C and the suspension was magnetically stirred. Fluorescence changes are reported as a percentage of the maximal fluorescence, taking the fluorescence of Di-*O*-C_5(3) without cells as 0% and the fluorescence after the addition of nonstimulated cells (equal maximal fluorescence) as 100%.

Measurement of phosphoinositides and inositolphosphates. For the determination of stimulus-induced changes in phosphoinositides and inositol phosphates the prelabeled cells were washed by suspending twice at 37°C for 10 min in RPMI medium without inositol. The cells were resuspended in calcium medium and incubated for 2 h with or without pertussis toxin. The cells were finally resuspended in calcium medium, warmed for 5 min at 37°C, followed by the addition of various stimuli for the indicated time; control cells were treated in parallel with appropriate solutions. For experiments with human neutrophils and HL-60 cells, the number of cells used for each incubation condition was, respectively, 12×10^6 cells and 3×10^6 cells. Preincubation of HL-60 cells with lithium (10 mM $LiCl_2$ for 10 min at 37°C) in some experiments did not alter basal or stimulated IP_3 levels. Therefore, all our experiments reported here were performed in its absence.

Incubations were terminated by addition of 10% (vol/vol) trichloroacetic acid. The samples were kept on ice 5–10 min and centrifuged. The phospholipids in the trichloroacetic acid precipitate were extracted with 5.5 ml of chloroform/methanol/HCl, 12 N (2:1:0.0075), the extracts washed three times with 1 ml of chloroform/methanol/HCl, 0.6 N (3:48:47), and dried under a stream of air. Phosphoinositides were analyzed by thin-layer chromatography according to Jolles et al. (22).

The supernatant was washed three times with a fivefold excess of diethylether. The washed extract was adjusted to pH 7.5 with Tris 0.2 M and inositol phosphates separated by stepwise elution from Dowex (formate) columns as described (23). Radioactivity in the fractions was determined by liquid scintillation counting with 67% (vol/vol) aquasol. The inositol trisphosphate fraction may have included both the active calcium-mobilizing isomer 1,4,5 and the isomer 1,3,4. No attempt has been performed to distinguish between these two possibilities.

Results

Human neutrophils

Functional studies. Because of quantitative differences of the effects of pertussis toxin on neutrophils from different donors, the results presented here were all obtained from cells of the same donor in order to allow quantitative correlation among various functions. All parameters were qualitatively reproduced in cells from two additional donors.

In Fig. 1 is shown the effect of preincubation of human neutrophils for 2 h at 37°C with various concentrations of pertussis toxin on degranulation of secondary granules upon stimulation with the chemotactic peptide FMLP (1 μM), the calcium ionophore ionomycin (250 nM), and the phorbol ester PMA (100 nM), as assessed by release of vitamin B_{12} binding protein. The response to ionomycin and PMA, which bypass cell surface receptors, is unaltered, whereas the response to FMLP is inhibited in a dose-dependent manner.

FMLP-induced degranulation of primary granules, as assessed in the same supernatants by the release of β-glucuronidase, was 44%±2, 5±5%, and 2±5% of control (without pertussis toxin)

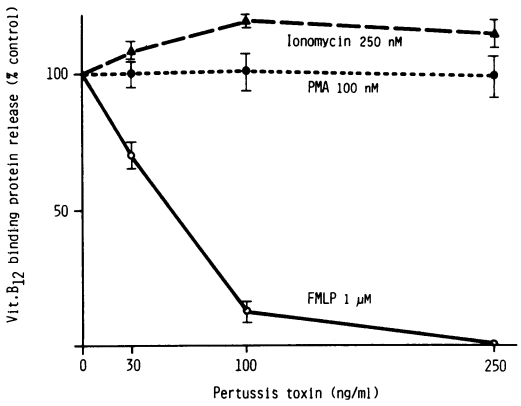

Figure 1. Effect of increasing concentrations of pertussis toxin on vitamin B_{12} binding protein release after 5 min incubation with 1 μM FMLP, 250 nM ionomycin, and 100 nM PMA in human neutrophils. Results are expressed as percentage of control cells (cells incubated without pertussis toxin). Control cells released 39±1.8% (FMLP), 63±1.9% (ionomycin), and 49±1.5% (PMA) of their total vitamin B_{12} binding protein content (after subtraction of basal values). Results are mean±SD of 3–6 determinations.

values after preincubation with 30, 100, and 250 ng/ml of pertussis toxin, respectively.

FMLP-induced superoxide production was 9±9% of control (without pertussis toxin) values after preincubation with 30 ng/ml, and was completely abolished after preincubation with 100 and 250 ng/ml of pertussis toxin.

Changes in the average membrane potential of human neutrophils were assessed with the fluorescent probe Di-*O*-C_5(3) (21). As is shown in Fig. 2, neutrophils are depolarized by FMLP (panel *A*), ionomycin (panel *B*), and PMA (panel *C*). Pertussis toxin treatment suppresses in a dose-dependent manner the response to FMLP, while the depolarization induced by PMA or ionomycin are unaffected.

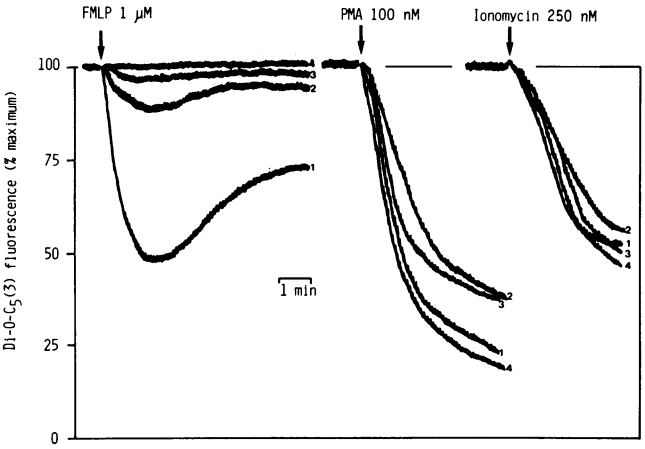

Figure 2. Effect of increasing concentrations of pertussis toxin on depolarization of human neutrophils after the addition of 1 μM FMLP (*A*), 100 nM PMA (*B*), and 250 nM ionomycin (*C*). The scale to the left shows percentage of maximal fluorescence (medium plus Di-*O*-C_5(3) = 0%; medium plus Di-*O*-C_5(3) plus unstimulated cells = 100%). Traces (1), (2), (3), and (4) are done with neutrophils preincubated with 0, 30, 100, and 250 ng/ml pertussis toxin, respectively. Traces are representative of 3–6 experiments.

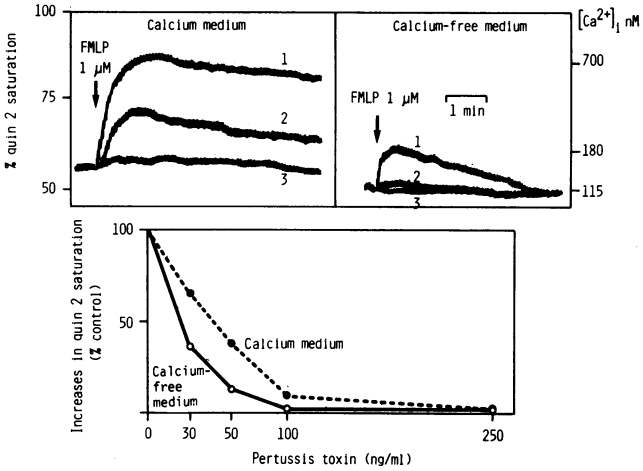

Figure 3. (*A*) Quin2 traces recorded from human neutrophils in calcium medium (*left*) and calcium-free medium (*right*), in cells preincubated without (1), with 30 (2), and 100 (3) ng/ml pertussis toxin. Traces typical for at least three other experiments. (*B*) Effect of increasing concentrations of pertussis toxin on $[Ca^{2+}]_i$ in human neutrophils after stimulation with 1 μM FMLP in calcium and calcium-free medium. Values are given as percentage of quin2 changes of control cells (same experiment as Fig. 4 *A*).

Intracellular signals. Human neutrophils loaded with the fluorescent calcium indicator quin2 were used to assess the FMLP-induced calcium transients in neutrophils preincubated with increasing concentrations of pertussis toxin. In calcium containing medium the calcium transients are inhibited by increasing concentrations of pertussis toxin. To elucidate, whether this inhibition is due to an effect on calcium influx or mobilization from cellular stores, the experiments were repeated in calcium-free medium. In the latter condition, $[Ca^{2+}]_i$ rises reflect release from internal stores, since calcium influx is negligible. In calcium-free medium, the FMLP effect was attenuated at lower pertussis toxin concentrations than in the presence of calcium (Fig. 3). In contrast, the rises in $[Ca^{2+}]_i$ induced by the calcium ionophore ionomycin, which equilibrates Ca^{2+} along the electrochemical gradients, were unaffected by pretreatment of cells with pertussis toxin (data not shown).

Extensive experimental evidence indicates that IP_3 is a second messenger, which exerts its action by releasing calcium from internal stores in a wide variety of cells, including phagocytes (6, 24, 25). We therefore measured the production of IP_3 and its products of degradation inositol bisphophate (IP_2) and inositol monophosphate (IP_1), upon stimulation with 1 μM FMLP in cells incubated without and with a maximally inhibitory dose of pertussis toxin (250 ng/ml). As IP_3 is generated from the phospholipid PIP_2, and changes in the level of this phospholipid might be an important reason for changes in the receptor-mediated production of IP_3, we also measured the levels of PIP_2 and its precursors phosphatidylinositol 4-phosphate (PIP_1) and phosphatidylinositol (PI). In Table I is shown the effect of pertussis-toxin on inositol phosphates and phosphoinositides in neutrophils stimulated with 1 μM FMLP for 20 s. A doubling of the production of inositol-phosphates (IP_3, IP_2, IP_1) is observed after stimulation. This rise is completely inhibited by the preincubation of the cells with pertussis toxin. No major changes due to pertussis toxin or FMLP are seen in the levels of the phosphoinositides.

Table I. Effect of Pertussis Toxin (250 ng/ml) on Levels of Inositolphosphates and Phosphoinositides in Human Neutrophils after Stimulation with FMLP

	Pertussis toxin	
	ng/ml	*ng/ml*
	0	250
IP_3	269±16	110±3
IP_2	183±10	99±5
IP_1	215±15	103±3
PIP_2	113±18	105±14
PIP_1	128±18	142±11
PI	102±9	113±3

Neutrophils were cultured in the presence of myo-[2-^{3}H]inositol (1 μCi/ml) for 18 h and with pertussis toxin 250 ng/ml for 2 h. 1 μM FMLP was added. The reaction was stopped after 20 s by adding trichloracetic acid. Inositolphosphates were determined in the supernatant, and phosphoinositides in the precipitate. The values are given as percentage of basal values of control cells (cells incubated without pertussis toxin). Basal values of control cells were 66±4, 70±5, and 104±4 dpm for IP_3, IP_2, and IP_1, respectively, and 229±9, 449±26, and 4131±30 dpm for PIP_2, PIP_1, and PI, respectively. Values are mean±SD of three determinations.

HL-60 cells

In order to be able to correlate the effect of pertussis toxin on functional results with the effect on intracellular signals, the functional studies with HL-60 cells were always carried out on the same day with the same batch of cells as studies of inositolphosphates and phosphoinositides. The extent of inhibition of the different parameters by any dose of pertussis toxin did—in contrast to human neutrophils—not vary between different batches of HL-60 cells, reflecting the properties of a homogenous cell line.

Functional studies. As differentiated HL-60 cells do not possess secondary granules, only the degranulation of primary granules was determined, as assessed by the release of glucosaminidase (26). Although the amount of granule content released is substantially lower than in human neutrophils, similar results are observed, i.e., degranulation induced by 250 nM ionomycin is not impaired by increasing concentrations of pertussis toxin, whereas the degranulation induced by 1 μM FMLP is almost completely abolished in a dose dependent manner (Fig. 4 *A*). Superoxide production in HL-60 cells in response to 100 nM PMA is unchanged by increasing concentrations of pertussis toxin, whereas superoxide production in response to 1 μM FMLP is markedly reduced at 30 ng/ml and is completely abolished at 100 and 250 ng/ml (Fig. 4 *B*).

Intracellular signals. In Fig. 5, left panel, the effects of pertussis toxin on IP_3 levels in differentiated HL-60 cells before and 30 s after stimulation with 1 μM FMLP are shown. IP_3 levels reach 318±16% upon stimulation in control cells. This response is inhibited by pertussis toxin in a dose-dependent manner, such that in cells preincubated with 250 ng/ml of pertussis toxin, stimulated IP_3 levels are only 112±5% of unstimulated control cells. In parallel, there is a gradual decline of basal IP_3 values from 100±5% to 79±6% after preincubation of cells with the maximal dose of pertussis toxin.

Fig. 5, left panel, shows changes in FMLP-induced calcium

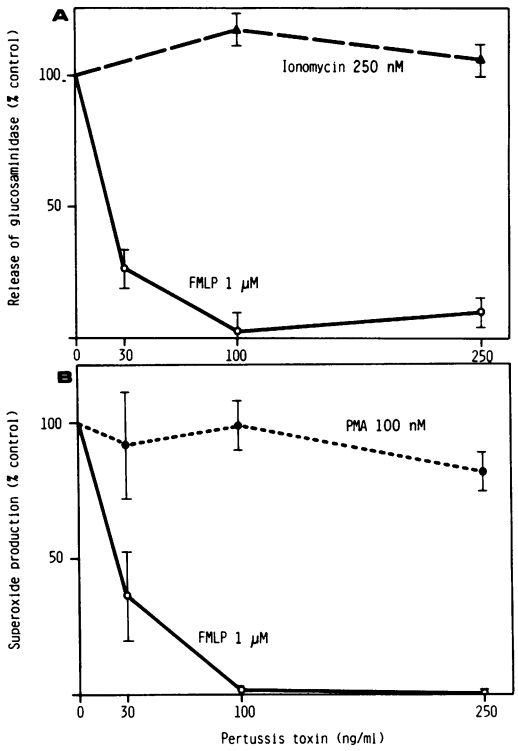

Figure 4. (*A*) Effect of increasing concentrations of pertussis toxin on glucosaminidase release after 5 min incubation with 1 μM FMLP and 250 nM ionomycin in HL-60 cells. Results are expressed as percentage of control cells (cells incubated without pertussis toxin). Control cells released 16±5 and 9±3% of their total glucosaminidase, respectively, upon stimulation with FMLP or ionomycin (after subtraction of basal values). The results are mean±SD of 3–6 determinations. (*B*) Effect of increasing concentrations of pertussis toxin on initial rates of superoxide production in HL-60 cells in response to 1 μM FMLP or 100 nM PMA. The results are expressed as percentage of control cells (cells preincubated without pertussis toxin). Control cells released 6.1±1.4 and 2.5±0.5 nmol $O^{2-}/10^6$ cells/min upon stimulation with PMA and FMLP, respectively. The results are mean±standard deviation of 3–6 determinations.

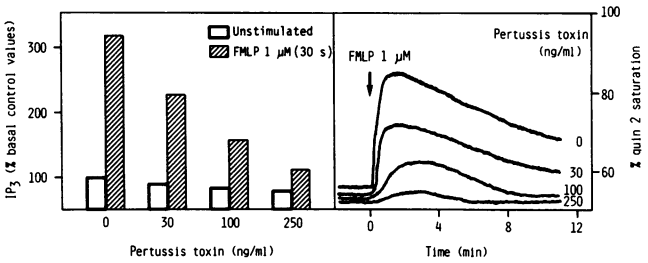

Figure 5. (*Left*) Effect of increasing concentrations of pertussis toxin on IP_3 generation in unstimulated and stimulated (FMLP, 1 μM, 30 s) HL-60 cells. Results are expressed as percentage of IP_3 values of unstimulated control (preincubation without pertussis toxin) cells and are mean of the triplicates of one experiment, typical for three experiments (for SD see Table II). (*Right*) Effect of preincubation with increasing concentrations of pertussis toxin on calcium transients in differentiated HL-60 cells, loaded with the fluorescent calcium indicator quin2. All traces were done on the same day and with the same batch of cells as in the *left* panel; to facilitate comparison they are superimposed. The scale on the *right* side shows the percentage of maximal changes in Quin 2 flourescence. The traces are typical for three experiments.

transients monitored on the same day with the same batch of cells as that for the IP_3 determinations. $[Ca^{2+}]_i$ was lowered in parallel to the effect on IP_3 generation. Similar to human neutrophils, calcium transients in calcium-free medium were slightly more affected than in calcium medium (not shown). In Table II, the values of inositol-phosphates and phosphoinositides are given. In addition to its effects on IP_3, pertussis toxin inhibited the generation of IP_2 and IP_1 due to FMLP. While degranulation and superoxide production are more sensitive to pertussis toxin inhibition than IP_3 generation and $[Ca^{2+}]_i$ rises, the two latter are equally sensitive to toxin treatment of HL-60 cells.

Discussion

Two aspects of the present study should be emphasized. First, the inhibition of IP_3 generation demonstrates directly that pertussis toxin interferes with the FMLP induced activation of phospholipase C. Second, modulation and monitoring of IP_3 revealed parallel, although quantitatively different inhibition of IP_3 generation, calcium rises and functional responses.

Two cell types that possess specific chemotactic peptide FMLP receptors were used for this investigations: human neutrophils and the differentiated human promyelocytic cell line HL-60, and for both cell types the same pattern of results was observed. Neutrophils possess the advantage of being mature and functional cells, but they have the disadvantage of a short life span, allowing less prolonged labeling periods with [^{3}H]inositol (18 h). Under these conditions, however, the cells exhibit reduced functional responses (3). In addition, large quantities of cells are necessary (12×10^6 cells per determination).

Table II. Effect of Different Concentrations of Pertussis Toxin on Levels of Inositolphosphates and Phosphoinositides on Unstimulated HL-60 Cells and FMLP-stimulated HL-60 Cells

		Pertussis toxin			
		ng/ml	*ng/ml*	*ng/ml*	*ng/ml*
		0	30	100	250
	Basal	100±5	91±8	85±6	79±6
IP_3	Stimulated	318±16	228±3	158±10	112±15
	Basal	100±4	76±4	75±6	63±8
IP_2	Stimulated	297±24	241±70	152±9	106±5
	Basal	100±10	100±4	93±4	83±22
IP_1	Stimulated	139±9	124±5	109±5	90±13
	Basal	100±9	103±17	108±7	88±11
PIP_2	Stimulated	100±5	101±8	122±19	106±9
	Basal	100±13	79±21	87±6	66±6
PIP_1	Stimulated	101±8	96±11	106±8	88±6
	Basal	100±1	97±12	100±11	90±14
PI	Stimulated	108±3	100±6	96±4	97±3

HL-60 cells were cultured in the presence of myo-[2-^{3}H]inositol (1 μCi/ml) for 48 h and with pertussis toxin at the indicated concentrations for 2 h 1 μM FMLP (stimulated) or 0.1% DMSO (basal) was added. The reaction was stopped after 30 s by adding trichloracetic acid. Inositolphosphates were determined in the supernatant, and phosphoinositides in the precipitate. The values are given as percentage of basal values of control cells (cells incubated without pertussis toxin). Basal values of control cells were 122±6, 140±6, and 350±36 dpm for IP_3, IP_2, and IP_1, respectively, and 671±64, 1,027±130, and 1,287±237 dpm for PIP_2, PIP_1, and PI, respectively. Values are mean±SD of three determinations.

Working with HL-60 cells has the advantage that phosphoinositides can be labeled to isotopic equilibrium (48 h), such that alterations in label reflect quantitative changes and that less cells (3×10^6) are required for each determination. HL-60 cells remain fully responsive to the various stimuli after the labeling, allowing quantitative comparison between effects on intracellular signals and functional responses. The effect of pertussis toxin on human neutrophils is quite similar to that on HL-60 cells: The FMLP-induced generation of IP3, rises in intracellular calcium, degranulation, and superoxide production are inhibited in a differential concentration dependent manner by pertussis toxin. Calcium changes and functions tested in response to stimuli bypassing the receptor, such as the calcium ionophore ionomycin and the phorbolester PMA, are not inhibited. Thus, our data demonstrate, that the action of pertussis toxin on the chemotactic peptide receptor is proximal to the IP_3 rise, and that the regulation of calcium rise, superoxide production, degranulation, and membrane depolarization is distal to the site of inhibition of the toxin.

Elevation of $[Ca^{2+}]_i$ upon chemotactic peptide activation in neutrophils has been shown to consist of two components: release of Ca^{2+} from intracellular stores and influx across the plasmalemma from the extracellular medium (1). Although the release of calcium from the stores appears to be mediated by IP_3 (6), not much is known about the mechanism that induces calcium influx from the extracellular medium. Our data show parallel inhibition of Ca^{2+} transients both in the presence and absence of extracellular Ca^{2+}; thus, both release from internal stores and Ca^{2+} influx are affected by pertussis toxin. The inhibition of $[Ca^{2+}]_i$ rises is slightly more pronounced in calcium-free medium, possibly because of a different sensitivity of influx and release to the inhibition by pertussis toxin. Alternatively, phospholipase C might be more sensitive to inhibition by pertussis toxin in the absence of extracellular calcium.

The correlation of the generation of IP_3 with the rise in $[Ca^{2+}]_i$ measured in intact cells confirms and extends the findings that IP_3 mobilizes Ca^{2+} as assessed in permeabilized neutrophils (6). Although our studies cannot define whether IP_3 is involved in calcium influx across the plasmalemma, they do suggest its dependency on a signal created distally to the site of action of pertussis toxin. In another study, in which the quin2 method has been applied to rabbit neutrophils, it was concluded that pertussis toxin selectively inhibited FMLP-stimulated calcium influx without affecting calcium mobilization (14). Although interspecies differences might account for these conflicting results, they are hard to reconcile with the knowledge that in a variety of other cells, IP_3 has been shown to release calcium from intracellular stores (24, 25).

In human neutrophils, $[Ca^{2+}]_i$ transients and secondary granule releases were less sensitive to pertussis toxin action than superoxide production, depolarization, and primary granule release. In HL-60 cells, comparison of IP_3 production with the functional responses and the Ca^{2+} transients in response to FMLP could be performed under identical experimental conditions. The inhibition of superoxide production and degranulation required lower concentration of pertussis toxin than the inhibition

of Ca^{2+} transients and IP_3 production. These results indicate a tighter relationship between IP_3 and $[Ca^{2+}]_i$ than IP_3 and functional responses. It is known that FMLP receptor occupancy generates another potent stimulatory signal (1), presumably diacylglycerol, which also is produced by phospholipase C. This compound is generally believed to activate protein kinase C and synergise with Ca^{2+} to generate functional responses. Although this remains to be shown, the more marked inhibition of functions could be explained by concomitant inhibition of both IP_3 and diacylglycerol. In previous studies it was reported that FMLP transiently decreased PIP_2 levels, assessed after short-term labeling with $^{32}P_i$, and that pertussis toxin abolished this response. PIP_2 can be decreased both by the action of phosphomonoesterase or phospholipase C; the data presented above showing a block of FMLP-induced IP_3 production by pertussis toxin now provides evidence that it is indeed the activation of phospholipase C that is impaired by the toxin. In addition, in HL-60 cells labeled to isotopic equilibrium, no significant decrease of PIP_2 was observed at 30 s, indicating that resynthesis of phosphoinositides maintains constant PIP_2 levels, except for times very early after stimulation as suggested by the ^{32}P-labeling experiments (15, 16).

The diminished FMLP-induced IP_3 rise after preincubation with pertussis toxin does not seem to be a consequence of an accelerated degradation, as it is associated with decreased levels of its degradation products, IP_2 and IP. The essentially unchanged levels of membrane phospholipids argue against a diminished concentration of phospholipase C substrate as a cause of the inhibition of IP_3 production. A reduction of FMLP binding sites as well as substantial alterations of cAMP levels by pertussis toxin have been excluded by previous studies (27, 28). Our data suggest an inhibition of the receptor-mediated phospholipase C activation by pertussis toxin. What could be the molecular mechanism of this event? A direct effect of pertussis toxin on phospholipase C is possible, however there is no positive evidence for this. The most likely site of action is the known substrate of pertussis toxin, an N protein. Several lines of evidence suggest this: (*a*) N proteins are guanosine-triphosphate (GTP)-dependent proteins, and GTP has been shown to influence the affinity of chemotactic peptide receptors of neutrophils (29) and the stimulus-response coupling in mast cells (10); (*b*) the phospholipase C of human neutrophil plasma membranes can be activated simply by adding GTP analogues (11); (*c*) the role of GTP-dependent proteins in the signal transduction from the stimulus-receptor complex to the catalytic unit of the adenylate cyclase is well known (7, 8); (*d*) an N protein that can be ADP-ribosylated by pertussis toxin has been demonstrated in neutrophil plasma membranes (27, 28); and (*e*) nicotinamide, an inhibitor of ADP-ribosyltransferase, attenuates the effect of pertussis toxin on neutrophils (27).

However, this effect of pertussis toxin appears to be a selective event which does not inhibit all calcium mobilizing receptor agonists. Whereas these agonists are inhibited in neutrophils, HL-60 cells, and mast cells, the calcium mobilization by the carbamylcholine receptor of the insulin secreting cell line RINmF5 and the thyrotropin releasing hormone receptor of the pituitary cell line GH_3, for instance, are not inhibited even by high doses (up to 1 μg/ml) of pertussis toxin (Wollheim C. B., and W. Schlegel, unpublished data), although both receptor types are thought to work by phospholipase C activation, IP_3 production, and consecutive calcium rise, just as the FMLP-receptor of human neutrophils (25), and in beta cells and GH_3 cells, an N protein that can be ADP-ribosylated by pertussis toxin has been demonstrated in the plasma membrane (30, 31). Interestingly, in human neutrophils, the concanavalin A–induced rise in $[Ca^{2+}]_i$ is not inhibited by pertussis toxin (15). Taken together, these findings suggest the existence of differences in transduction mechanisms between calcium mobilizing agonists.

Since submission of this manuscript, it has been reported that pertussis toxin inhibits exocytosis and IP_3 formation in response to FMLP and LTB4 in rabbit neutrophils and HL-60 cells (32–34).

Acknowledgments

We are most grateful to Antoinette Monod for her skillful and devoted technical assistance, and to Dr. Tullio Pozzan for helpful discussion.

This work was supported by grants Nr. 3.840.083, 3.990.0.84, and 3.246.082 from the Swiss National Foundation.

References

1. Pozzan, T., P. D. Lew, C. B. Wollheim, and R. Y. Tsien. 1983. Is cytosolic free calcium regulating neutrophil activation? *Science (Wash. DC).* 221:1413–1415.

2. Lew, P. D., C. B. Wollheim, F. A. Waldvogel, and T. Pozzan. 1984. Modulation of cytosolic-free calcium transients by changes in intracellular calcium-buffering capacity: correlation with exocytosis and O_2^- production in human neutrophils. *J. Cell Biol.* 99:1212–1220.

3. Di Virgilio, F., L. M. Vicentini, S. Treves, G. Riz, and T. Pozzan. 1985. Inositol phosphate formation in FMet-Leu-Phe stimulated human neutrophils does not require an increase of the cytosolic free Ca^{2+} concentration. *Biochem. J.* 229:361–367.

4. Bradford, P. G., and R. P. Rubin. 1985. Characterization of formylmethionyl-leucyl-phenylalanine stimulation of inositol triphosphate accumulation in rabbit neutrophils. *Mol. Pharmacol.* 27:74–78.

5. Dougherty, R. W., P. P. Godfrey, P. C. Hoyle, J. W. Putney, Jr., and R. J. Freer. 1984. Secretatogue induced phosphoinositide metabolism in human leukocytes. *Biochem. J.* 222:307–314.

6. Prentki, M., C. B. Wollheim, and P. D. Lew. 1984. Ca^{2+} homeostasis in permeabilized human neutrophils: characterization of Ca^{2+}-sequestering pools and the action of Inositol 1,4,5-Trisphosphate. *J. Biol. Chem.* 259:13777–13782.

7. Rodbell, M. 1980. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature (Lond.).* 284:17–22.

8. Gilman, A. G. 1984. G-proteins and dual control of adenylate cyclase. *Cell.* 36:577–579.

9. Haslam, R. J., and M. M. L. Davidson. 1984. Guanine nucleotides decrease free $[Ca^{2+}]$ required for secretion from permeabilized blood platelets. Evidence of a role for a GTP-binding protein in platelet activation. *FEBS Fed. Eur. Biochem. Soc. Letts.* 174:90–95.

10. Gomperts, B. D. 1983. Involvement of guanine nucleotide-binding protein in the gating of Ca^{2+} by receptors. *Nature (Lond.).* 306:64–66.

11. Cockcroft, S., and B. D. Gomperts. 1985. Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. *Nature (Lond.).* 314:934–936.

12. Katada, T., and M. Ui. 1981. Islet activating protein. *J. Biol. Chem.* 256:8310–8317.

13. Katada, T., G. M. Bokoch, J. K. Northup, M. Ui, and A. G. Gilman. 1984. The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. *J. Biol. Chem.* 259:3568–3577.

14. Molski, T. F. P., P. H. Naccache, M. L. Marsh, J. Kermode, E. L. Becker, and R. I. Sha'afi. 1984. Pertussis toxin inhibits the rise in the intracellular concentration of free calcium that is induced by chemotactic factors in rabbit neutrophils: possible role of the "G proteins"

in calcium mobilization. *Biochem. Biophys. Res. Commun.* 124:644–650.

15. Verghese, M. W., C. D. Smith, and R. Snyderman. 1985. Potential role for a guanine nucleotide regulatory protein in chemoattractant receptor mediated polyphosphoinositide metabolism, Ca^{2+} mobilization and cellular responses by leukocytes. *Biochem. Biophys. Res. Commun.* 127:450–457.

16. Sheifcyk, J., R. Yassin, M. Volpi, T. F. P. Molski, P. H. Nacchache, J. J. Munoz, E. L. Becker, M. B. Feinstein, and R. I. Sha'afi. 1985. Pertussis but not Cholera toxin inhibits the stimulated increase in actin association with the cytoskeleton in rabbit neutrophils: role of the "G-proteins" in stimulus response coupling. *Biochem. Biophys. Res. Commun.* 126:1174–1181.

17. Boyum, A. 1976. Isolation of lymphocytes, granulocytes and macrophages. *Scand. J. Immunol.* 5:9–15.

18. Lew, P. D., S. Southwick, T. P. Stossel, J. C. Whitin, E. Simons, and H. J. Cohen. 1981. A variant of chronic granulomatous disease: deficient oxidative metabolism due to a low-affinity NADPH oxidase. *N. Engl. J. Med.* 305:1329–1333.

19. Dewald, B., U. Bretz, and M. Bagglioni. 1982. Release of gelatinase from a novel secretory compartment of human neutrophils. *J. Clin. Invest.* 70:518–525.

20. Lew, P. D., and T. P. Stossel. 1981. Effect of calcium on superoxide production by phagocytic vesicles from rabbit alveolar macrophages. *J. Clin. Invest.* 671:1–9.

21. Seligman, B. E., E. K. Gallin, D. L. Martin, W. Shain, and J. I. Gallin. 1980. Interaction of chemotactic factors with human polymorphonuclear leukocytes: studies using a membrane potential-sensitive cyanine dye. *J. Membr. Biol.* 53:257–272.

22. Jolles, J., K. W. A. Wirtz, P. Schotman, and W. H. Gipsen. 1979. $ACTH_{1-24}$ affects protein phosphorylation and polyphosphoinositide metabolism in rat brain. *Biochem. J.* 194:283–291.

23. Berridge, M. J., R. M. C. Dawson, C. P. Downes, J. P. Heslop, and R. F. Irvine. 1983. Changes in the levels of inositol phosphates after agonist dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* 212:473–482.

24. Berridge, M. J., and R. F. Irvine. 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature (Lond.).* 312:315–320.

25. Wollheim, C. B., T. J. Biden, P. D. Lew, and W. Schlegel. 1985. Calcium mobilization by inositol 1,4,5-trisphosphate during activation of islet, pituitary and myeloid cells. *Cardiovasc. Res.* In press.

26. Naccache, P. H., T. F. P. Molski, B. Spinelli, P. Borgeat, and C. N. Abboud. 1984. Development of calcium and secretory responses in the human promyelocytic leukemia cell line HL60. *J. Cell. Physiol.* 119:241–246.

27. Okajima, F., and M. Ui. 1984. ADP-ribosylation of the specific membrane protein by islet-activating protein, pertussis toxin, associated with inhibition of a chemotactic peptide-induced arachidonate release in neutrophils. *J. Biol. Chem.* 259:13863–13871.

28. Bokoch, G. M., and A. G. Gilman. 1984. Inhibition if receptor-mediated release of arachidonic acid by pertussis toxin. *Cell.* 39:301–308.

29. Koo, C., R. J. Lefkowitz, and R. Snyderman. 1983. Guanine nucleotides modulate the binding affinity of the oligopeptide chemoattractant receptor on human polymorphonuclear leukocytes. *J. Clin. Invest.* 72:748–753.

30. Katada, T., and M. Ui. 1982. Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. *Proc. Natl. Acad. Sci. USA.* 79: 3129–3133.

31. Wojcikiewicz, R. H., P. R. Dobson, L. R. Irons, A. Robinson, and Barry L. Brown. 1984. The relationship between pertussis-toxin induced ADP-ribosylation of a plasma-membrane protein and reversal of muscarinic inhibition of prolaction secretion in GH_3 cells. *Biochem. J.* 224:339–342.

32. Bradford, G. P., and R. P. Rubin. 1985. Pertussis toxin inhibits chemotactic factor-induced phosphilipase C stimulation and lysosomal enzyme secretion in rabbit neutrophils. *FEBS (Fed. Eur. Biochem. Soc.) Letts.* 183:317–320.

33. Brandt, S. J., R. W. Dougherty, E. G. Lapetina, and J. E. Niedel. 1985. Pertussis inhibits chemotactic peptide stimulated generation of inositol phosphates and lysosomal enzyme secretion in human leukemic (HL-60) cells. *Proc. Natl. Acad. Sci. USA.* 82:3277–3280.

34. Becker, E. L., J. C. Kermode, P. H. Naccache, R. Yassin, M. L. Marsh, J. J. Munoz, and R. I. Sha'afi. 1985. The inhibition of neutrophil granule enzyme secretion and chemotaxis by pertussis toxin. *J. Cell Biol.* 100:1641–1646.