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Immunology

A pertussis toxin-sensitive GTP-binding protein in the human neutrophil regulates multiple receptors, calcium mobilization, and lectin-induced capping

(stimulus–response coupling/chemotaxis/platelet activating factor/inflammation)

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ABSTRACT **Human neutrophils treated with pertussis toxin had decreased functional responses to several agents including zymosan-treated serum, heat-aggregated immunoglobulin, platelet-activating factor, and fMet-Leu-Phe. Responses affected include superoxide generation and release of lysozyme. The degree and type of inhibition was dependent on the individual receptor and the cellular response studied. Measurement of intracellular calcium levels with quin-2 showed that both fMet-Leu-Phe- and platelet-activating factor-mediated increases in quin-2 fluorescence were diminished as a result of pertussis toxin treatment. fMet-Leu-Phe-mediated calcium uptake was also inhibited. However, under conditions where fMet-Leu-Phe-mediated effects on cell function were completely abolished, only a partial inhibition of 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8) sensitive calcium uptake was observed. A study of the linked reactions of chemotaxis, capping, and shape change revealed that (*i*) chemotaxis was inhibited regardless of the chemoattractant utilized (zymosan-treated serum, fMet-Leu-Phe, and platelet-activating factor) and (*ii*) the associated reactions of Con A capping and fMet-Leu-Phe- or Con A-mediated shape change were reduced in pertussis toxin-treated cells. Our results suggest that multiple mediators of inflammation act through a pertussis toxin-sensitive GTP-binding protein that regulates the mobilization of internal calcium as well as calcium uptake and is, in addition, a key control element of shape change, capping, and chemotaxis.**

The human neutrophil carries out several complex cellular host-defense and inflammation reactions in response to diverse receptor-mediated signals (1–4). We have shown that the fMet-Leu-Phe (FMLP) receptor acts through Ni, a GTP-binding protein sensitive to modification by pertussis toxin (PT) (5). As a result of the modification of Ni, guanine nucleotide regulation of the receptor and multiple responses associated with this receptor are inhibited (5). By contrast, the prostaglandin E_1 and β-adrenergic receptors act through a cholera toxin-sensitive GTP-binding protein, Ns (6), to modulate the fMet-Leu-Phe-initiated reactions in a manner that is specific for the receptor occupied and the level of cyclic AMP generated (7–9).

We have explored whether a PT-sensitive substrate may participate in the regulation of human neutrophils and have investigated specifically its role in the action of several mediators of inflammation, calcium-related transduction mechanisms (10–13), and the linked cellular reactions of locomotion, capping, and shape change (14, 15). Our results indicate that the PT-sensitive Ni protein is central to the regulation of multiple reactions of the human neutrophil.

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MATERIALS AND METHODS

$^{45}CaCl_2$ was purchased from ICN. Con A was purchased from E-Y Laboratories (San Mateo, CA) and fluorescein isothiocyanate-Con A (FITC-Con A) was from Vector Laboratories (Burlingame, CA). Versilube F-50 was obtained from Carlsen (Greenwich, CT). PT, purified by the method of Sekura *et al.* (16), was from List Biologicals (Campbell, CA). All other purchases were as described (5). Aggregated immunoglobulin (17, 18) and zymosan-treated serum (ZTS) (19, 20) were prepared as previously stated (5).

Isolation of Neutrophils. Neutrophils were isolated by sequential centrifugation on dextran and Ficoll/Hypaque (21) and PT treatment of the cells was as described (5). PT-treated cells were viable by exclusion of trypan blue and by phorbol 12-myristate 13-acetate mediated superoxide generation, which was unaffected by the toxin.

Neutrophil Function Tests. Lysozyme release was measured (20). The percent lysozyme released was 35–40% of the total measured in the presence of 0.5% Triton X-100. Superoxide generation was monitored as the reduction of cytochrome *c* (22). Controls included cells incubated without substrate and cells incubated with fMet-Leu-Phe and superoxide dismutase. Chemotaxis was measured using the modified Boyden chamber assay (5, 23).

FITC-Con A Capping and Shape Change. Neutrophils [2×10^7 cells per ml in modified Hanks' solution (HBSS)] were exposed to FITC-Con A (10 μg/ml). The reaction was terminated by the addition of 2% (wt/vol) paraformaldehyde. Wet mounts were examined for capping as described (24, 25). Shape change was determined after exposure of the cells to fMet-Leu-Phe (0.10 μM) or Con A (10 μg/ml) by scoring cells as round or with fully developed protuberances. At least 200 cells were counted for each condition in each experiment.

Calcium Uptake. Calcium uptake was quantitated as described (15, 16). Neutrophils (350 μl containing 2×10^7 cells per ml) were suspended in buffer A [150 mM NaCl/5 mM KCl/1.3 mM $CaCl_2$/1.2 mM $MgCl_2$/10 mM Hepes (pH 7.4)] and preincubated for 10 min, at 37°C with cytochalasin B (10 μg/ml) (15). At zero time, 50 μl of $^{45}Ca^{2+}$ (10^4 cpm/μl; 30 mCi/mg; 1 Ci = 37 GBq) and 50 μl of ligand were added (final volume, 500 μl). After 5 min the incubation was terminated with cold 100 mM EGTA (100 μl). The cell suspension was immediately layered onto 600 μl of Versilube F-50 in a 1.5-ml microcentrifuge tube and centrifuged in a Beckman Microfuge 12. The supernatant was aspirated, and the pellet was resuspended in NCS tissue solubilizer (500 μl) and was incubated at 60°C for 1 hr. The mixture was transferred to a scintillation vial, and the tubes were washed with an additional 500 μl of NCS. Omnifluor (15 ml) was added, and ^{45}Ca

Abbreviations: ZTS, zymosan-treated serum; PT, pertussis toxin; PAF, platelet-activating factor; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester; FITC, fluorescein isothiocyanate.

was quantitated in a Beckman LS-7500 counter. Counts in the absence of cells were ≈50 cpm. The basal level of calcium uptake refers to the amount of uptake observed with buffer alone.

Measurement of Intracellular Calcium Using Quin-2 Fluorescence. The measurement of intracellular calcium was done as described (15, 16). Fluorescence of quin-2-loaded cells was measured by the method of Tsien *et al.* (27). Neutrophils (10^8 cells per ml) incubated for 30 min at 37°C with quin-2 acetomethoxyester (50 μM) in buffer A. The suspension was then diluted 1:10 with buffer A and incubated for an additional 30 min. The cells were centrifuged at 250 × *g* for 15 min and resuspended in buffer A at a concentration of 10^7 cells per ml at room temperature. The assay was as follows: 1 ml of cell suspension was mixed with 2.5 ml of buffer A (15), incubated at 37°C for 5 min, and then transferred to a 1-cm cuvette. Fluorescence intensities were measured in a Perkin-Elmer LS-5 fluorescence spectrophotometer using an excitation wave length of 339 nm and an emission wave length of 492 nm. Zero suppression and an enhanced sensitivity setting (FS = 30) were utilized for optimal readings. The excitation slit was set to 5 nm and the emission slit to 20 nm to enhance sensitivity. A measure of the total quin-2 present in the cells was estimated by lysing the cells in the presence of 0.1% Triton X-100. No difference was noted in the amount of quin-2-loaded in PT-treated and control cells. The instrument was autozeroed after the reading was stabilized. The ligand (35 μl) was added, and the fluorescence was followed for 100 to 500 sec. The level of intracellular calcium was computed from a standard curve using quin-2/KCl/Ca^{2+}. The PT treatment was carried out by first incubating the cell suspension (1 × 10^8 cells per ml) with PT (15 μg/ml) for 30 min at 37°C, then immediately incubating with quin-2 as described above.

RESULTS

PT pretreatment of human neutrophils resulted in pronounced inhibition of superoxide generation (Fig. 1*A*). Receptors affected by the toxin treatment include fMet-Leu-Phe, ZTS (C5a), and platelet-activating factor (PAF). The time course of pretreatment suggests that incubations 1–2 hr in length are required before toxin inhibition becomes evident, although variations in sensitivity to toxin are noted in the receptors examined. Such a time course is consistent with the entry of toxin into the cell (5, 41). In contrast to the pronounced effects on these receptors, the effect of phorbol 12-myristate 13-acetate on superoxide generation was completely unaffected by the pretreatment. The concentration dependence of the ligands for superoxide generation and enzyme release was then examined in neutrophils that had been preincubated with PT for 90 min. A pronounced inhibition was observed for fMet-Leu-Phe, ZTS, PAF, and IgG for superoxide generation and enzyme release (Figs. 1*B* and *C*), and different degrees of inhibition were noted within the receptors tested.

A measure of calcium mobilization is obtained from the fluorescent dye quin-2 that detects changes in the concentration of free intracellular calcium (27). As shown in Fig. 2, FMLP and PAF caused a concentration-dependent increase in the fluorescence intensity of quin-2 loaded cells. PT pretreatment diminished this increase in fluorescence by >80% for both receptors.

fMet-Leu-Phe caused a dose-dependent enhancement in calcium uptake. The reaction was linear up to 5 min for fMet-Leu-Phe (Fig. 3 *Inset*) and PAF (data not shown). The basal levels of $^{45}Ca^{2+}$ uptake are slightly enhanced after toxin treatment. A substantial toxin-mediated inhibition of the fMet-Leu-Phe-mediated calcium uptake is observed. Inhibition is noted in both time course and dose–response studies at 1–10 μM fMet-Leu-Phe. A consistent feature of the inhibition by the toxin is that under conditions where fMet-Leu-Phe-mediated effects on neutrophil function are completely suppressed, only partial inhibition of calcium uptake is observed. To test whether receptor-mediated calcium uptake is in fact relevant to cell function, another agent, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8), a ubiquitous inhibitor (28, 29) of neutrophil functions and calcium uptake was examined

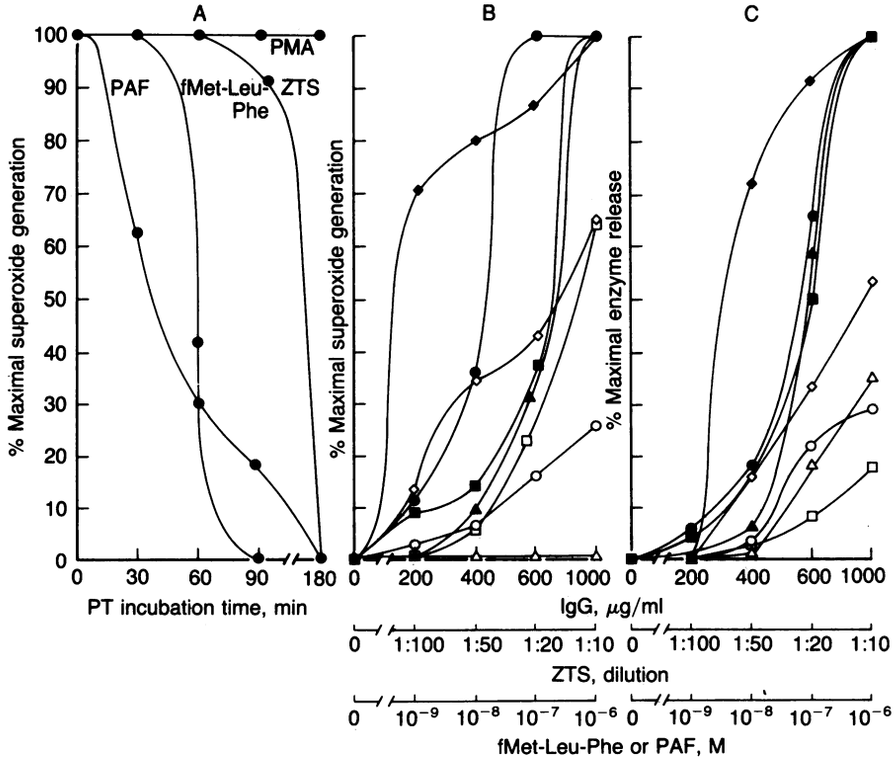

FIG. 1. Effects of pretreatment with PT on receptor-mediated functional responses. Neutrophils were incubated in the presence or absence of pertussis toxin (15 μg/ml). The cells were then pelleted, resuspended in modified HBSS, and assayed for superoxide generation and enzyme release in the presence of various agents. (*A*) The concentrations of the ligands were 0.10 μM PAF, 0.10 μM fMet-Leu-Phe, ZTS (1:10 dilution) and PMA (10 μg/ml) (panel A). The preincubation time was as indicated. (*B* and *C*) The preincubation time was 90 min for various ligands at the concentration indicated. Symbols: fMet-Leu-Phe, circles; ZTS, squares; PAF, triangles; IgG, diamonds. Filled symbols represent the control incubation; open symbols represent the PT-treated cell. For each ligand, the maximal responses for superoxide generation (nmol O_2 per 5 min per 10^7 cells) were as follows: fMet-Leu-Phe, 32.2; ZTS, 17.0; PMA, 55.2; and IgG, 34.5. The maximal responses for enzyme release were as follows: fMet-Leu-Phe, 20.4; PAF 18.5; and ZTS 7.8 lysozyme μg equivalents per min per 10^7 cells; and IgG 20.6 lysozyme μg equivalents per hr per 10^7 cells.

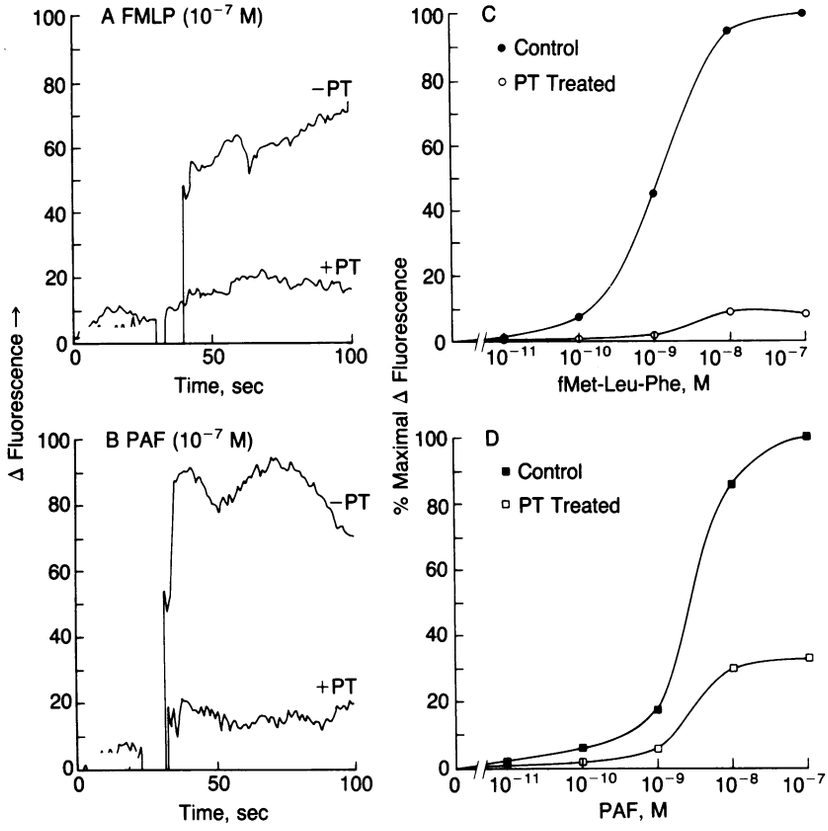

FIG. 2. Receptor-mediated changes in the fluorescence of quin-2-loaded cells. Neutrophils were treated with PT and loaded with quin-2. The cells were then exposed to (*A*) 0.1 μM fMet-Leu-Phe and (*B*) 0.1 μM PAF and the changes in fluorescence were monitored. From a precalibration curve 100 units of fluorescence corresponds to 355 nmol of Ca^{2+}. (*C* and *D*) Fluorescence readings at 100 sec for different concentrations of the two ligands. The fluorescence change noted with 0.10 μM fMet-Leu-Phe was 30% of that observed for cells treated with 0.1% Triton. These results are representative of four experiments carried out on different normal donors. The break in fluorescence signal in Fig. 2*A* and *B* corresponds to the addition of ligand.

(Fig. 4). Complete inhibition by TMB-8 of calcium uptake was noted in control cells. In PT-treated cells the "basal" level of calcium uptake was reduced by about 50%, and the component of calcium uptake that was insensitive to toxin was further inhibited by TMB-8 with a concentration dependence similar to that noted for control cells.

Since chemotaxis, shape change, and capping are regarded as closely related morphologic events, these phenomena were also examined. Inhibition of chemotaxis by PT was observed regardless of the chemoattractant utilized. Thus, the effects of PAF, fMet-Leu-Phe, and ZTS were inhibited after toxin pretreatment (Table 1). The capping reaction was then investigated by using FITC-Con A as the agent to crosslink the cell surface-binding sites. The typical cap is shown in Fig. 5. The predominant structures were either random clusters (*A*) or caps (*B*). The process of cap formation was found to be dependent on temperature and was enhanced 9-fold by colchicine [93 ± 1 capped cells per field (10 μM colchicine, 10 min, 37°C) vs. 11 ± 2 capped cells per field (buffer, 10 min, 37°C)]. No effect of lumicolchicine was noted

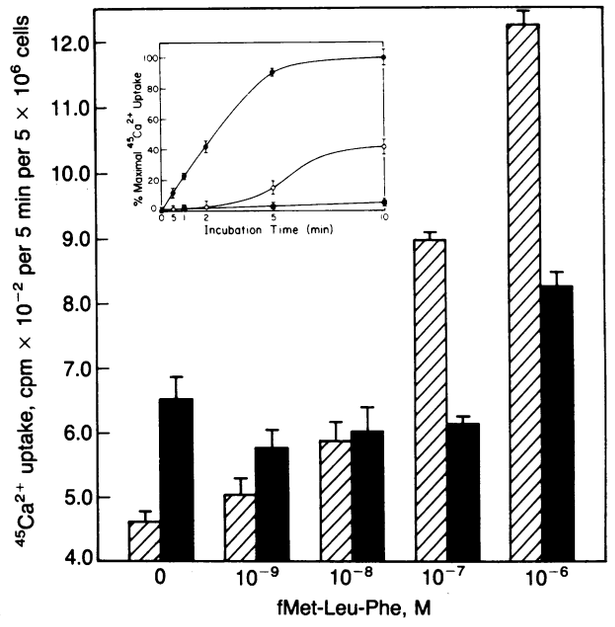

FIG. 3. Inhibition of fMet-Leu-Phe-mediated calcium uptake by PT pretreatment. For the dose-response histographs cells were suspended in buffer A (2×10^7 cells per ml) and preincubated for 10 min at 37° C as described in the legend to Fig. 1. At time zero, 50 μl of $^{45}Ca^{2+}$(10^4 cpm/μl) and 50 μl of fMet-Leu-Phe at the concentrations indicated were added (final volume of reaction mixture, 500 μl), and $^{45}Ca^{2+}$ uptake was measured. Hatched bars, control-treated cells; solid bars, PT-treated cells. (*Inset*) Time course of $^{45}Ca^{2+}$ uptake. (■) Buffer alone; (●) fMet-Leu-Phe-treated cells; (○) PT/fMet-Leu-Phe-treated cells. Error bars represent the SEM of determinations in quadruplicate from five experiments.

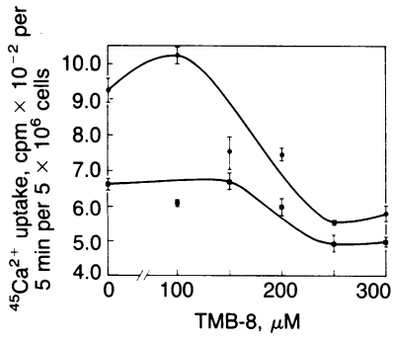

FIG. 4. Inhibition of fMet-Leu-Phe-mediated calcium uptake in control and PT-treated neutrophils by TMB-8. Neutrophils were pretreated with PT (■) or buffer (●) and then assayed for $^{45}Ca^{2+}$ uptake as in Fig. 3. Cells were preincubated for 10 min at 37°C with TMB-8 as indicated prior to the addition of $^{45}Ca^{2+}$ and 0.10 μM fMet-Leu-Phe. Error bars represent the SEM of triplicate determinations from three experiments.

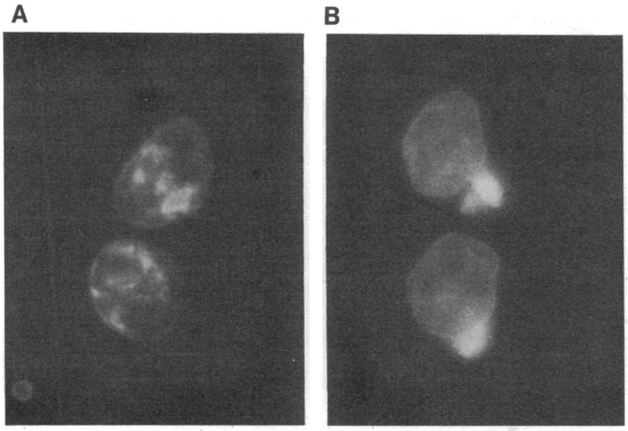

FIG. 5. Distributions of FITC-Con A in neutrophil capping. Human neutrophils were treated with FITC-Con A. Distributions of label included random clusters (*A*) and caps (*B*), a local intense concentration of label. A characteristic shape change (protuberance or bleb) was always observed in capped cells. PT-treated cells showed a round appearance with reduced protuberance formation (*A*).

[20 ± 2 capped cells per field (10 μM lumicolchicine, 10 min, 37°C)]. These observations are in agreement with those reported for the capping reaction in human neutrophils (24-26). PT treatment of the cells caused a complete abolition of capping in neutrophils in the presence and absence of colchicine only random clusters (*A*) being noted.

An observation made during the capping experiments was that the cells that show a characteristic shape change with attendant "bleb" formation are rarely observed after toxin treatment. The influence of toxin on receptor-induced shape change was, therefore, examined. Receptor-mediated shape change was substantially inhibited by the toxin. Only 14 ± 1% of the control cells changed shape in the absence of ligand. By contrast 80 ± 1% or 76 ± 2% of the cells treated with 10 μM fMet-Leu-Phe or Con-A (10 μg/ml), respectively, changed shape. After PT treatment only 10 ± 1% or 5 ± 1% cells treated with 10 μM fMet-Leu-Phe or Con-A showed a change in shape. A smaller component of spontaneous shape change was also observed, and was not sensitive to the effect of the toxin.

Table 1. Influence of PT on neutrophil chemotaxis

Ligand	Migrating cells	
	Control	PT
Buffer	8 ± 3*	10 ± 1*
fMet-Leu-Phe		
10^{-10}M	20 ± 1†	10 ± 1†
10^{-9}M	46 ± 1	16 ± 6
10^{-8}M	81 ± 3	35 ± 1
10^{-7}M	121 ± 8	48 ± 7
10^{-6}M	172 ± 1	43 ± 6
PAF		
10^{-10}M	9 ± 3	7 ± 4
10^{-9}M	76 ± 8	11 ± 1
10^{-8}M	90 ± 8	13 ± 3
10^{-7}M	169 ± 15	11 ± 5
10^{-6}M	181 ± 5	12 ± 4
ZTS		
1:100	15 ± 4	14 ± 2
1:50	38 ± 4	15 ± 1
1:20	56 ± 8	18 ± 3
1:10	58 ± 4	13 ± 1
1:5	97 ± 5	24 ± 5

Cells were incubated with buffer or PT and assayed for chemotaxis. Data for migrating cells are given as cells per high power field ± SEM, migrating from 20 to 120 μm. ZTS is expressed in units of fold dilution. *P* values for the *t*-test were 0.01 for all samples except * ($P > .05$) and † ($P < .05$).

DISCUSSION

The effects of multiple receptor-directed agents on human neutrophils are inhibited by PT. This finding is important as it suggests a similar or common pathway for the action of protein (ZTS, fMet-Leu-Phe, and IgG) and lipid (PAF), mediators of inflammation. With our results (5), this study implies that the PT substrate in the neutrophil plasma membrane, a 41-kDa GTP-binding protein, Ni, may regulate multiple receptors not traditionally associated with GTP-binding proteins and suggests a central role for such a protein in the regulation of inflammation.

At the level of transduction, the major effect of fMet-Leu-Phe and PAF is apparently in the mobilization of calcium. Our studies using quin-2 fluorescence suggest that the pathway from the receptor–GTP-binding protein complex to a calcium-related event is affected by the toxin. At least one postulated sequence (30, 31, 41) involved in the mobilization of calcium is the rapid formation and subsequent hydrolysis of phosphoinositides with the resultant formation of inositol triphosphate that causes a release of intracellular calcium. Inhibition of calcium mobilization may entail the inhibition of the phosphodiesterase, that mediates the hydrolysis of L-α-phosphotidylinositol 4,5-bisphosphate hence preventing the formation of inositol triphosphate. The inhibition of calcium mobilization for PAF and fMet-Leu-Phe would imply that a GTP-binding protein may be involved in this and perhaps in other steps of phospholipid metabolism (30–33). The observation that calcium uptake is only partially inhibited under conditions of complete inhibition of calcium mobilization and cellular function implies that more than one component may contribute to the overall influx of calcium. The results obtained with TMB-8 suggest the presence of Ni-sensitive and insensitive components of calcium uptake. The Ni-insensitive component may represent either a calcium channel or an exchange of calcium from membrane bound sites (10, 11, 15, 34, 35). The effects observed on the basal levels of calcium uptake may indicate the involvement of calcium efflux perhaps through a Ca^{2+}/Mg^{2+}-ATPase. The relationship of the increase in intracellular calcium to calcium uptake is complex.

Events of shape change and capping are thought to be closely associated with cell locomotion (14, 15, 37–39). We have shown that in response to PT treatment (*i*) inhibition of chemotaxis occurs regardless of the receptor occupied; (*ii*) lectin induced capping is eliminated; and (*iii*) receptor induced shape changes are substantially decreased. Thus, the morphologic sequence of reactions leading up to and including cell locomotion seems to be under the control of a PT substrate that is probably the GTP-binding protein identified in the neutrophil plasma membrane (5). That the calcium related pathway is likely to be involved is suggested by the observation that TMB-8 and PT are inhibitors of the capping reaction. A pathway linked to Ns seems not to be involved, as multiple positive effectors of adenylate cyclase (prostaglandin E_1, β-adrenergic receptor agonists and cholera toxin), do not have any appreciable effect on the capping reaction. The exact step(s) at which PT acts to inhibit capping, chemotaxis, and shape change remains to be established, although an event relating N_i to the cytoskeleton (i.e., actin polymerization) seems likely (35-37). In summary, the results presented indicate an important role for a PT-sensitive substrate in the regulation of multiple aspects of the human neutrophil. Hypotheses concerning receptor regulation,

transduction events, and morphologic reactions should probably include a role for a PT-sensitive GTP-binding protein.

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