Regulation of Leukotriene B₄ Generation from Human Polymorphonuclear Granulocytes after Stimulation with Formyl-Methionyl-Leucyl Phenylalanine: Effects of Pertussis and Cholera Toxins

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The effects of holotoxins and toxin subunits from Bordetella pertussis and Vibrio cholerae strains on intact and digitonin-permeabilized human polymorphonuclear neutrophils were studied. Our data clearly demonstrate that formyl-methionyl-leucyl-phenylalanine (fMLP)-induced generation of chemotactic active leukotriene B₄ was inhibited by both holotoxins as well as by their isolated enzymatic A protomers. In contrast, the respective binding components (B oligomers) did not affect leukotriene formation. Priming of digitonin-permeabilized neutrophils with either guanylylimidodiphosphate or inositol trisphosphate increased subsequent stimulation with fMLP. In contrast, diacylglycerol decreased fMLP-induced leukotriene B₄ formation, but inositol trisphosphate and diacylglycerol had no effect on inhibition mediated by the toxins. In addition, pertussis and cholera toxins reduced the specific binding of [³H]fMLP. Scatchard plot analysis revealed that the observed decrease of peptide binding was due to a reduced number of receptor sites. The fMLP-stimulated [³H]guanylylimidodiphosphate binding and GTPase activity used as parameters for the activation of G proteins were decreased in parallel. These results suggest altered chemotactic receptor numbers and G-protein functions responsible for the toxin-dependent suppression of fMLP-mediated response for neutrophils.

Phagocytic cells such as polymorphonuclear neutrophils (PMN) play a crucial role in host defense against bacterial infections (17). N-formylated peptides (e.g., formyl-methionyl-leucyl-phenylalanine [fMLP]), which are produced by bacteria, are highly potent chemotactic agents and interact with specific binding sites on the cell surface of PMN (2). GTP-binding proteins (G proteins) serve as transducers for signal processing linking extracellularly oriented receptors to membrane-bound effector systems (11). By GDP-GTP exchange they bind to distinct effector proteins, which are subsequently activated. Activation of the G protein itself is terminated by hydrolysis of bound GTP to GDP. Thus, nonhydrolyzable nucleotide analogs of GTP such as guanylylimidodiphosphate [Gpp(NH)p] lead to a persistent activation of G proteins. Receptor coupling to phospholipase C (PLC) as an effector protein has been suggested as one important G-protein-mediated process (14). Activation of PLC promotes hydrolysis of membrane-bound phosphoinositides such as phosphatidylinositol 4,5-bisphosphate to inositol trisphosphate (IP₃) and diacylglycerol (DAG) (5). IP₃ enhances cytosolic calcium concentration by mobilizing calcium from internal stores. DAG directly activates calcium- and phospholipid-dependent protein kinase C (PKC) (5, 21). These biochemical reactions parallel phospholipase A₂ (PLA₂) activation (3), which leads to the release of free arachidonic acid and the subsequent conversion of the acid, e.g., via 5-lipoxygenase, to leukotrienes (17, 26). Leukotrienes are potent mediators which induce inflammatory and allergic reactions (7). Leukotriene B₄ (LTB₄) leads to the chemotaxis of neutrophils and eosinophils and lysosomal enzyme release and enhances the vascular permeability (17). Human PMN metabolize LTB₄ by specific hydroxylase at position C-20 (omega-oxidation), resulting in the formation of 20-hydroxy-LTB₄ and 20-carboxy-LTB₄, which reveal diminished biological activities compared with that of LTB₄ (8, 13).

As was reported elsewhere, pertussis toxin (PT) inhibited receptor-mediated LTB₄ generation from PMN (15). Many of the physiological and cellular effects of the toxins generated by Bordetella pertussis and Vibrio cholerae (CT) result from their abilities to affect G proteins by ADP-ribosylation of the G-protein alpha subunit (4, 22, 29). Sensitivity toward CT or PT has often been used to distinguish between different types of G proteins (Gi, Gs), especially for the adenylate-cyclase system (11). PT and CT are known as AB toxins. The A protomer exhibits ADP-ribosyltransferase activity. The B oligomer serves as an anchor for the binding of the toxin to the cell surface and assists in the transport of the A protomer through the membrane (29).

The present study was addressed to clarify the mechanisms by which PT and CT inhibit the generation of receptor-mediated LTB₄ from PMN. For this purpose, distinct elements of the signal transduction cascade were analyzed.

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MATERIALS AND METHODS

Materials. Ficoll 400 was obtained from Pharmacia, Uppsala, Sweden; Macrodex (6%, wt/vol) was from Knoll, Ludwigshafen, Germany; and sodium metrizoate solution (75%, wt/vol) was from Nycomed, Oslo, Norway. The cell stimuli Ca ionophore A23187 and fMLP as well as App(NH)p, ATP, cytochalasin B, diacylglycerol (DAG), dithiothreitol, EDTA, EGTA [ethylene glycol-bis[β-aminoethy] ether]-N,N,N',N'-tetraacetic acid], Gpp(NH)p, heparin,

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IP₃, leupeptin, and ouabain were purchased from Sigma, Munich, Germany. Acetonitrile (high-performance liquid chromatography [HPLC] grade) was provided by Baker Chemicals (Gross-Gerau, Germany), and methanol, dipotassium hydrogen phosphate, and phosphoric acid were from Riedel de Haën (Seelze, Germany). All other chemicals were from Merck, Darmstadt, Germany.

PT, CT, and their corresponding subunits were purchased from List Biological Laboratories, Inc., Campbell, Calif. Stock solutions were made by the reconstitution of lyophilized powder in a sterile buffer containing 0.1 M sodium phosphate and 0.5 M NaCl (pH 7.0). The stock solutions were stored at 4°C and diluted in phosphate-buffered saline (PBS).

[^3H]fMLP (specific activity, 2.0 TBq/mmol), [^3H]Gpp(NH)p (666 GBq/mmol) and [γ-[^3P]GTP (222 TBq/mmol) were supplied by New England Nuclear, Dreieich, Germany.

Leukotriene standards (20-COOH-LTB₄, 20-OH-LTB₄, and LTB₄) were generously provided by Merck Frosst, Pointe Claire-Dorval, Quebec, Canada.

Buffer. All experiments were carried out in Dulbecco’s PBS (0.137 M NaCl, 8 mM Na₂HPO₄, 2.7 mM KH₂PO₄, 2.7 mM KCl; pH 7.4).

**Purification of human neutrophils.** Human PMN were isolated from heparinized (15 U/ml) peripheral blood of healthy donors by using a Ficoll-metrizoate gradient, and subsequent dextran sedimentation was done as described elsewhere (6). The purified cell fraction contained more than 95% pure and intact PMN. The PMN were diluted to a final concentration of 2 × 10⁷ cells per ml in PBS.

**Cell permeabilization.** Cell permeabilization was performed as described elsewhere (23). A 100-fold-concentrated solution of digitonin dissolved in water was added at a final concentration of 10 µM. The incubation proceeded at 37°C for 8 min. The neutrophils were then washed twice (300 x g for 10 min) at 4°C in PBS and finally suspended at a concentration of 2 x 10⁷ cells per ml and kept on ice until use.

**Incubation conditions.** The PMN were pretreated for the indicated times either with PBS as control or with the toxins (CT and PT), toxin subunits (CTA, CTB, PT(A), and PT(B), or G-MAN, or IP₃, or DAG) at 37°C. Subsequently, stimuli were added to the cell suspensions in the presence of calcium (2 mM) and magnesium (1 mM), and the incubation proceeded for an additional 20 min. In the case of fMLP stimulation, cytchalasin B (5 µg/ml) was added 2 min prior to the addition of fMLP.

**Cell viability.** To ascertain a possible degranulation or cytotoxic reaction by the used compounds, the release of the cytoplasmic enzyme lactate dehydrogenase (a marker for cytotoxic effects), lysozyme, and β-glucuronidase (markers for the azurophilic and/or specific granules) was measured. The release of lactate dehydrogenase after pretreatment of cells with the indicated compounds never exceeded 4.8% ± 2.2% of the total cellular lactate dehydrogenase content (except for digitonin-permeabilized PMN [21.0% ± 3.8%]). Enzyme activities were calculated as the percentage of the total enzyme activity available after sonication of unstimulated PMN. Cytotoxicity was also assayed microscopically by the trypan blue exclusion test (7.1% ± 4.4% for the pretreatment of intact PMN; 29.4% ± 7.8% for digitonin-permeabilized cells). The release of β-glucuronidase did not exceed 1.0% ± 0.1% (permeabilized PMN, 5.5% ± 0.6%), and the release of lysozyme was 15.4% ± 7.9% (permeabilized PMN, 21.3% ± 12.3%).

**Analysis of leukotriene generation.** After the incubation period, 2 ml of methanol-acetonitrile (50:50; vol/vol) was added. The vials were overlaid with argon and frozen at −70°C for 12 h. After centrifugation at 1,900 × g for 10 min (Cryo)fuge 6-4 Heraeus Christ), the supernatants were removed and evaporated to dryness by lyophilization (EF 4 Modulo; Edwards-Knie, Marburg, Germany). The precipitate was dissolved in 600 µl of methanol-water (30:70, vol/vol), and centrifugation was performed at 9,600 × g for 4 min for further purification. Aliquots (200 µl) were subjected to reversed-phase HPLC analysis. The HPLC equipment consisted of a CM 4000 pump and an SM 4000 detector (Laboratory Data Control/Milton Roy, Hasselroth, Germany) and the automatic sample injector WISP 710 B (Waters, Eschborn, Germany). The reversed-phase column (4.6 by 250 mm) was packed with Nucleosil 5 C₈ (pore size, 5 µM; Macherey-Nagel, Düren, Germany). Isocratic elution for leukotriene analysis was carried out by using a solvent system consisting of water-acetonitrile-methanol (50:30:20, vol/vol) and including 0.04% EDTA and 0.15% K₂HPO₄. The pH was adjusted to 5.0 by the addition of phosphoric acid. The flow rate was maintained at 0.9 ml/min. All solvents were degassed before use and constantly stirred during HPLC analysis. The A₂₈₀ of the column effluent was determined (16). Quantification and identification of leukotrienes were performed with synthetic standard solutions.

**LTB₄ generation was calculated as the combined amounts of LTB₄ and the LTB₄ omega-oxidation products (20-hydroxy-LTB₄ and 20-carboxy-LTB₄).**

**Specific binding of [^3H]fMLP.** PMN were pretreated in the presence or absence of PT (1 µg/ml) or CT (10 µg/ml) for 2 h at 37°C. The binding studies were carried out using 96-well filtration plates with 5-µm-pore-size polyvinylidene fluoride membranes (Millipore, Eschborn, Germany). Each well contained 4 × 10⁶ PMN, 20 nM [^3H]fMLP, and 37.5 µg of bovine serum albumin. Nonspecific binding was assessed in the presence of 10 µM unlabeled fMLP. After 45 min of incubation at 4°C, the unbound [^3H]fMLP was removed by rapid filtration with a millifiliter vacuum holder. The filters were transferred to scintillation vials, methanol (0.5 ml) and Rotiszit 2211 (4 ml) were added, and the radioactivity was measured by liquid scintillation counting (Rack beta 1209; LKB, Turku, Finland). Specific binding was expressed as total binding minus nonspecific binding. Total and nonspecific binding were calculated as values from triplicate determinations. Scatchard plot analysis was performed with concentrations of [^3H]fMLP between 0.25 and 256 nM.

**Specific binding of Gpp(NH)p.** PMN were pretreated in the presence or absence of PT (1 µg/ml) or CT (10 µg/ml) for 2 h at 37°C. The cell suspensions were washed and suspended in Tris (0.05 M)-sucrose (0.34 M) buffer (pH 7.5), and then EGTA (1 mM), diithiothreitol (1 mM), and leupeptin (100 µg/ml) were added. Cell disruption was carried out by sonication (sonifier 250; Branson Power Company, Danbury, Conn.). The sonicate extracts were centrifuged at 300 × g for 10 min, and the resulting postnuclease supernatant was further differentiated by centrifugation at 10,000 × g for 20 min (J2-21 centrifuge with a JA-20 rotor; Beckman, Palo Alto, Calif.) and at 100,000 × g for 60 min (L8-70 ultracentrifuge with an SW60 Ti rotor; Beckman). The pellet produced by 100,000 × g centrifugation was suspended in PBS buffer, assayed for protein content according to the method of Lowry et al. (19), and stored at −70°C.

The binding of [^3H]Gpp(NH)p was determined by a modification of the procedure of Matsumoto et al. (20). The membrane fraction (10 µg of protein in the reaction mixture)
was incubated in 20 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 5 mM MgCl₂, 0.1 mM EGTA, 1.14 mM ATP, 0.5 mM App(NH)p, 0.25 mM ouabain, and 1 μM [³H]Gpp(NH)p in the presence or absence of fMLP. Nonspecific binding was defined as the amount of [³H]Gpp(NH)p bound in the presence of 10 μM nonlabeled Gpp(NH)p. Incubations were terminated after 60 min at room temperature by rapid filtration through cellulose ester membranes with a pore size of 0.45 μm (millititer HA filtration plates; Millipore). The filters were washed four times with 20 mM Tris buffer (pH 7.5) containing 0.25 mM MgCl₂ and 50 μM EGTA. The dried filters were assayed for radioactivity by liquid scintillation counting. The amount of specific binding of [³H]Gpp(NH)p was calculated as total binding minus nonspecific binding.

GTPase activity. PMN were pretreated in the presence or absence of PT (1 μg/ml) or CT (10 μg/ml) for 2 h at 37°C. The membrane fraction was prepared as described above. GTPase activity was determined by measuring the liberation of ³²P from [γ-³²P]GTP by a modification of the procedure of Matsumoto et al. (20). The final assay mixture contained 10 μg of protein, 150 mM KCl, 20 mM Tris buffer (pH 7.5), 5 mM MgCl₂, 0.1 mM EGTA, 1.14 mM ATP, 0.5 mM App(NH)p, 0.25 mM ouabain, 0.375 μM GTP, and 0.125 μM [γ-³²P]GTP as well as the stimulus or fMLP for the control. The reaction proceeded for 60 min at 37°C and was terminated by adding 0.5 ml of a 5% charcoal mixture containing 0.1% dextran and 0.5% bovine serum albumin in 20 mM phosphate buffer (pH 7.5). The tubes were then centrifuged to sediment the charcoal. The supernatants were removed, and free ³²P was determined by liquid scintillation.

Statistics. If not stated otherwise, data from at least three independent experiments with different donor cells were combined and expressed as mean ± standard deviation. The paired Student’s t test was used to provide statistical analysis.

RESULTS

Effects of PT and CT on LTB₄ generation induced by fMLP and Ca ionophore. Pretreatment of human neutrophils (10⁷ in 0.5 ml of PBS) with PT (1 μg/ml) or CT (10 μg/ml) for 2 h at 37°C decreased the formation of LTB₄ depending on the stimulus. The combined amounts of LTB₄ and LTB₄ omega-oxidated products generated after stimulation with fMLP were 27% (PT) and 33% (CT) of those generated by control cells without toxin exposure (Fig. 1a). The metabolism of LTB₄ to its omega-oxidated products was not affected by PT or CT exposure. These results were confirmed by experiments in which exogenously added LTB₄ was not detected (data not shown). The inhibitory pattern indicates that receptor-mediated generation of LTB₄ by fMLP was sensitive to both toxins. Control stimulations of neutrophils with the Ca ionophore were not affected by PT and CT. The toxins alone, in the absence of any additional stimulus, did not induce LTB₄ generation. The PT- or CT-induced effects were obtained under noncytotoxic conditions, as assessed by the failure of lactate dehydrogenase release, indicating that the cells remained intact during the incubation and stimulation periods. Half-maximal inhibition of LTB₄ generation occurred at concentrations of 100 ng/ml for PT and 10 μg/ml for CT. In subsequent experiments, the time dependency of the toxin effects was studied. CT-mediated inhibition reached a maximum after 1 h, while 2 h were required to obtain similar effects with PT.

Effects of PT and CT subunits on LTB₄ formation from digitonin-permeabilized neutrophils. Experiments were performed to study the influence of the isolated toxin subunits on inflammatory mediator release. As was observed for the holotoxins, the subunits alone did not induce leukotriene formation in human PMN. In subsequent experiments, studies on digitonin-permeabilized neutrophils were carried out. For this purpose, neutrophils were permeabilized by digitonin (10 μM) and pretreated with the holotoxins and their corresponding subunits separately for 2 h at 37°C. The cells were then stimulated with fMLP (7 μM) for an additional 20 min. Control incubations were performed in the absence of toxins or toxin fragments and defined as 100%. As is shown in Fig. 1b, pretreatment of PMN with the holotoxins of B. pertussis (1 μg/ml) and V. cholerae (10 μg/ml) or the same amounts of their corresponding A proteomers (PT(A) or CT(A)) decreased the formation of LTB₄ and its omega-oxidated products by more than 50%. The decrease included LTB₄ as well as its omega-oxidated products; the ratio of LTB₄, 20-hydroxy-LTB₄, and 20-carboxy-LTB₄ was not affected under these conditions. In contrast to these results, the binding components PT(B) and CT(B) did not exert significant effects on fMLP-induced LTB₄ generation (Fig. 1). Half-maximal inhibition of LTB₄ generation occurred at concentrations of 1 μg of PT(A) and 10 μg of CT(A) per ml (data not shown). As was shown for the holotoxin, PT(A)
exerted effects similar to those of CT(A) but at lower doses. The same amounts of PT(B) and CT(B) were unable to modulate LTB$_4$ generation. In subsequent experiments, we investigated the time dependency of PT(A) and CT(A) pre-treatments. The inhibitory effects of A protomers on digitonin-permeabilized cells became apparent without any preincubation time; only a marginal increase was observed with prolonged preincubation (data not shown).

**Effect of Gpp(NH)p, IP$_3$, and DAG on LTB$_4$ generation from intact and digitonin-permeabilized neutrophils.** We further investigated the mechanisms of transmembrane signaling involved in fMLP-induced inflammatory mediator release. Gpp(NH)p has been used to activate signal-transmitting G proteins, thus simulating receptor-mediated events. Digitonin-permeabilized human neutrophils (10$^7$ in 0.5 ml of PBS) were incubated with 100 μM Gpp(NH)p for 1 h at 37°C and then stimulated with fMLP for 20 min. As became apparent, the combined amounts of generated LTB$_4$ increased by 1.7-fold (Fig. 2a). No significant effect on leukotriene formation was demonstrated when intact PMN were studied. Gpp(NH)p alone in the absence of the fMLP stimulus was not sufficient to induce leukotriene generation in either intact or permeabilized PMN. The increase in mediator formation included LTB$_4$ as well as its omega-oxidated products; the ratio of LTB$_4$, 20-hydroxy-LTB$_4$, and 20-carboxy-LTB$_4$ was not affected under these conditions. The Gpp(NH)p-induced effect was time and concentration dependent (data not shown). A maximal increase in LTB$_4$ generation was obtained after a preincubation of 1 h with a Gpp(NH)p concentration of 10 μM.

The binding of diverse agonists to membrane-bound receptors activates PLC, which then transforms phosphatidylinositol bisphosphate into two second messengers, IP$_3$ and DAG (5). The phosphoinositide and DAG second-messenger systems have been shown to be important regulators for a variety of cell functions. As we observed, changes in the intracellular IP$_3$ content when digitonin-permeabilized PMN were used modulated the generation rate for LTB$_4$ (Fig. 2b).

Priming of permeabilized human neutrophils (10$^7$ in 0.5 ml of PBS) with 15 μM IP$_3$ for 1 h at 37°C increased the combined amounts of generated LTB$_4$ by 1.5-fold when cells were stimulated with fMLP (7 μM) for 20 min. No significant effects were shown when the same number of intact PMN were treated with IP$_3$ alone in the absence of fMLP stimulation, which was not sufficient to induce leukotriene generation from both intact and digitonin-permeabilized PMN. The increase in mediator generation included LTB$_4$ as well as its omega-oxidated products; the ratio of LTB$_4$, 20-OH-LTB$_4$, and 20-COOH-LTB$_4$ was not affected under these conditions. The priming effect by IP$_3$ was time and concentration dependent (data not shown). A maximal increase in LTB$_4$ generation was already obtained after 30 min of preincubation. An increase of 50% was obtained with 5 to 10 μM IP$_3$.

DAG has been shown to be another important regulator for different cell functions resulting from PLC activation (5, 21). In order to investigate its role in inflammatory mediator release from human granulocytes, intact and digitonin-permeabilized neutrophils were incubated with 10 μM exogenously added DAG. As became apparent, DAG pretreatment inhibited the inflammatory mediator release induced by fMLP when both intact and permeabilized neutrophils were used (Fig. 2c). The inhibition amounted to 59% for intact and 70% for permeabilized PMN compared with levels for controls without DAG. Neither LTB$_4$ nor the LTB$_4$ omega-oxidated products were detected by reversed-phase HPLC when the neutrophils were stimulated with DAG alone. The inhibitory effect of DAG was dependent on a short preincubation time, which was optimal within 5 to 10 min. With prolonged preincubation, the DAG effect disappeared. No effects on leukotriene release were obtained when IP$_3$ was present during the preincubation time with DAG (106.4% ± 5.4% of control). Half-maximal inhibition of LTB$_4$ genera-

![FIG. 2. Generation of LTB$_4$ from Gpp(NH)p-, IP$_3$-, and DAG-treated leukocytes. Intact or digitonin-permeabilized PMN (10$^7$ cells per 0.5 ml of PBS) were stimulated for 20 min at 37°C with fMLP (7 μM) after pretreatment for different times with the indicated compounds. The combined amounts of LTB$_4$ and its omega-oxidated products are shown. The data are expressed as mean values ± standard deviations with different donor cells (P < 0.01 compared with controls). (a) Pretreatment with 100 μM Gpp(NH)p (●) for 1 h at 37°C. Control incubations (□) were performed in the absence of Gpp(NH)p and were defined as 100%. The 100% values for fMLP stimulation were 12.4 ± 3.7 ng for intact PMN and 38.4 ± 11.3 ng for permeabilized PMN (n = 4). (b) Pretreatment with 15 μM IP$_3$ (■) for 1 h at 37°C. Control incubations (□) were performed in the absence of IP$_3$ and were defined as 100%. The 100% values for fMLP stimulation were 11.6 ± 3.7 ng for intact PMN and 25.9 ± 7.8 ng for permeabilized PMN (n = 5). (c) Pretreatment with 100 μM DAG (●) for 10 min at 37°C. Control incubations (□) were performed in the absence of DAG and were defined as 100%. The 100% values for fMLP-stimulation were 12.9 ± 2.5 ng for intact PMN and 37.1 ± 14.5 ng for permeabilized PMN (n = 3).](http://iai.asm.org/Downloaded from)
tion was obtained at a concentration of 80 μM DAG (data not shown). Previous investigations have shown that DAG selectivity activates calcium-phospholipid-dependent PKC, which then phosphorylates several cell proteins (21). In order to study the activity of DAG on human PMN (10^7 in 0.5 ml of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), cells were prelabeled with ^32P, and stimulated with 100 μM DAG for different periods. An increased phosphorylation pattern for a 50-kDa protein could be detected after stimulations of up to 2 min (data not shown).

**Effects of Gpp(NH)p, IP3, and DAG on PT-, PT(A)-, CT-, and CT(A)-treated digitonin-permeabilized neutrophils.** We then studied whether the diminished cellular response of toxin-pretreated PMN was modulated by application of 100 μM Gpp(NH)p, 15 μM IP3, or 100 μM DAG. As was observed, there was no significant change when toxin-pretreated PMN were incubated with IP3 (30 min) or DAG (10 min) before they were stimulated with fMLP; combined incubation with IP3 and DAG did not alter the pattern and was therefore omitted (Fig. 3a). The inhibitions induced by the holotoxins as well as the enzymatic A subunits were not reversible. In contrast to these results, incubation with 100 μM Gpp(NH)p for a subsequent hour upregulated the decreased LTβ generation after PT, PT(A), CT, and CT(A) exposure compared with generation in controls without Gpp(NH)p (Fig. 3b). These results were more pronounced for PT-induced inhibition but were also significant for CT and the enzymatic A subunits.

**Effects of PT and CT on fMLP receptor expression and G-protein functions.** Experiments were carried out to analyze the effects of PT and CT on fMLP receptor expression as one basic requirement for cellular stimulation (Table 1). Control incubations were performed in the absence of PT and CT and were defined as 100%. Our data indicate that pretreatment of neutrophils with PT (1 μg/ml) or CT (10 μg/ml) reduced the specific binding of [3H]fMLP by 51 and 48%, respectively.

To determine whether these changes resulted from a change in receptor affinity or a change in receptor number, we performed Scatchard plot analyses of cells from different donors (Table 2). Under the conditions used, Scatchard plot analysis indicated one receptor population for fMLP on neutrophils treated with PBS, PT, or CT. The numbers of receptors were 5.5 × 10^6 per cell with PBS, 2.9 × 10^6 per cell with PT and 2.5 × 10^6 per cell with CT. The K_d values were 6.0 × 10^{-9} M with PBS, 1.9 × 10^{-8} M with PT, and 1.0 × 10^{-8} M with CT.

It is known that the alpha subunit of G proteins exchanges GDP with GTP after cellular stimulation. As was shown above, fMLP increased the Gpp(NH)p binding of neutrophil homogenates up to 2.6-fold. In order to analyze the effect of PT or CT treatment on this potential exchange, fMLP-induced binding of radiolabeled Gpp(NH)p, a nonhydrolyzable GTP-analog, to membrane preparations was studied (Table 1). Control stimulations were carried out in the absence of the toxins and were defined as 100%. As became apparent, treatment of PMN with PT (1 μg/ml) or CT (10 μg/ml) reduced fMLP-induced [3H]Gpp(NH)p binding by 53 and 50%, respectively.

Similar results were obtained when the GTPase activity of neutrophil membrane preparations was studied. fMLP in-
creased the GTPase activity of neutrophil homogenates up to 2.7-fold. Toxin treatment of PMN diminished fMLP-induced GTPase activity by 36% (PT) and 62% (CT).

The results provide evidence that fMLP receptor expression and G-protein functions are modulated by PT and CT. Thus, pretreatment with the toxins leads to a diminished binding capacity for the chemotactic factor fMLP and, further, to a diminished binding capacity for the GTP-analog Gpp(NH)p and a reduced GTPase activity of G proteins.

DISCUSSION

This study shows that exposure to PT and CT modulates the formation of inflammatory mediators generated by human neutrophils. After toxin treatment, the cellular response to the bacterial chemotaxin fMLP for the release of LTB4 was reduced. It is known that PT and CT downregulate cells without being cytotoxic (29). Previous investigations described the inhibitory effect of PT on diverse receptor-induced cellular functions (4). Our data confirm the effects of both toxins on receptor-mediated activation processes. Furthermore, when the Ca ionophore, which is known to bypass receptor-mediated membrane events, was used as a stimulus, the cellular response was not affected by the toxins. As is apparent, toxin exposure of neutrophils did not change the ratio of LTB4 and its omega-oxidated products, 20-hydroxy-LTB4 and 20-carboxy-LTB4, which reveal diminished biological activities compared with that of LTB4 (13). Therefore, we suggest that PT and CT do not affect the LTB4-omega-hydroxylase, a membrane-associated cytochrome P-450 complex (8). As a consequence, the capability of PMN to regulate the biological activity of their own inflammatory mediators remained unaffected, but the combined amounts of LTB4 and LTB4 omega-oxidated products, reflecting the results of de novo synthesis and metabolism, were suppressed after toxin exposure. In this regard, PT exerted its effects at lower concentrations than CT and achieved maximal inhibition after long-term exposure. Obviously, these latent periods may be due to the time required for the toxins to react with the surface, penetrate the cell membrane, and induce ADP-ribosylation (4, 29).

Sensitivity to CT or PT has often been used to distinguish between different types of G proteins (Gi, Gs), especially for the phospholipase C system (29, 30). As became apparent, the two bacterial toxins exerted similar effects on the generation of 5-lipoxygenase products from human polymorphonuclear granulocytes, which indicates that fMLP receptors are coupled to a different type of G protein (possibly Gp [4, 30]).

As it has been shown in previous investigations, both bacterial toxins exhibit multiple effects which are independent of their ADP-ribosyltransferase capacities. In this regard, the mitogenic effect of PT on T lymphocytes is mediated via the interaction of the B oligomer with CD3 (12, 24, 28). In order to exclude the modulatory capacity of the B oligomer on fMLP-induced mediator release, intact and digitonin-permeabilized neutrophils were separately pretreated with the A and B oligomer. Our previous studies on stimulus-response coupling took advantage of the availability of permeabilization techniques whereby putative second messengers, intracellular modulators, and toxin fragments were introduced into the cell interior. In agreement with previous investigations (23), we confirmed that digitonin preferentially interacts with the plasma membrane without significantly lysing the granules or releasing intact granules. Studies on digitonin-permeabilized neutrophils did not lead to mediator release per se but increased fMLP-induced LTB4 formation. Enhanced activity of permeabilized cells may be a general phenomenon, as it was also observed in at least one other cell line (1). Our results clearly demonstrate that the isolated A protomers of both toxins, unlike the binding components B, exerted inhibitory effects on digitonin-permeabilized granulocytes similar to those obtained with the holotoxins.

One may suggest that the diminished generation of LTB4 caused by PT and CT exposure is due either to a reduced binding of fMLP to its receptor or to a loss of G-protein functions. Indeed, treatment of the cells with PT or CT inhibited the specific binding of fMLP. Scatchard plot analysis revealed that the observed decrease of peptide binding was due to a decreased number of fMLP receptors. Our results were due to reports suggesting only one subset of fMLP receptor populations on human neutrophils (2). As also became apparent, PT as well as CT reduced fMLP-induced Gpp(NH)p binding, which reflects an altered exchange of GDP by GTP. Similar inhibitory effects were noted by analyzing the toxin effect on the GTPase activity of membrane preparations obtained from human granulocytes. Our results are in agreement with those of former investigations, which showed that the receptor expression for formylpeptides was regulated by guanine nucleotides (18, 27) and that ligand binding induces GTP hydrolysis as well as guanine nucleotide exchange (20). In this regard, evidence was presented that priming of digitonin-permeabilized PMN with the nonhydrolyzable GTP analog Gpp(NH)p significantly enhanced fMLP-induced LTB4 generation.

Previous reports have shown that fMLP translocates and activates two different phospholipases, PLC and PLA2 (3, 14). Whether they act synergistically or antagonistically to evoke neutrophil responses is still unclear, because the mechanisms of such interaction are not entirely understood. Application of DAG or IP3 to digitonin-permeabilized cells revealed contradictory effects on fMLP-induced LTB4 formation, suggesting a potential counterregulatory mechanism. As we showed, DAG priming decreased fMLP-induced LTB4 generation of intact and digitonin-permeabilized PMN. Previous investigations have confirmed that 1,2-sn-DAG was fully permeable for intact cells (21). The DAG effects were transient because of the rapid inactivation by DAG kinases, as described elsewhere (21). Our findings suggest that DAG may serve as a termination signal for cellular activation processes. This effect may be explained by the observation of Conquer and Mahadevappa (10), who reported that inhibition of PKC by H-7 potentiated the release of arachidonic acid in Ca ionophore-stimulated human neutrophils. Protein phosphorylation induced by PKC is a likely key component of the mechanisms through which DAG mediates its effect on intact cells (21). Preliminary experiments have shown that DAG exerted transient effects on the phosphorylation of a 50-kDa membrane protein of PMN. In contrast, previous studies have reported the phosphorylation of a 47-kDa membrane protein in neutrophils (25). In addition, our results suggest that IP3 serves as a potent signal in fMLP-mediated events of both A and B oligomer.

However, IP3 and/or DAG priming of PT- PT(A)-, CT-, or CT(A)-pretreated neutrophils caused no change in fMLP-induced mediator release, suggesting that the toxins uncouple receptor-G-protein-PLA2 interaction. We therefore considered the possibility that LTB4 production and 5-lipoxygenase activation are subject to an fMLP-driven pathway that is different from the pathway leading to the generation of IP3 and DAG. Former investigations indeed showed
that fMLP separately activates PLA₂ and PLC (9). Our results provide indirect evidence that PLA₂ inhibition by PT and CT is the crucial component for the toxin-mediated inhibition of LTB₄ formation, because inhibition was not restored by exogenous IP₃ and/or DAG. Therefore one may propose a regulatory function of IP₃ and DAG in PLA₂-initiated mediator release. The capacity to generate LTB₄ was restored when permeabilized toxin-treated cells were subsequently incubated with the G-protein activator Gpp(NH)p, suggesting a possible mechanism of exchange.

The results emphasize the central role for the integrity of receptors to PT- and CT-sensitive G proteins as signal transducers for the inflammatory response. Further studies are required to elucidate the complex interactions of various G proteins affected by CT and PT.

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REFERENCES


