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Modulation of Leukotriene Generation by Pertussis Toxin

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The purpose of our study was to characterize the properties of the interaction of pertussis toxin with human polymorphonuclear leukocytes for the modulation of leukotriene generation and metabolism. The cells were stimulated with either the Ca ionophore A23187, opsonized zymosan, or the bacterial peptide formyl-methionyl-leucyl phenylalanine. Incubation of the cells with pertussis toxin led to a rapid inhibition of LTB_4 generation when formyl-methionyl-leucyl phenylalanine was used as the stimulus, whereas there was no effect with the Ca ionophore and just a low effect with opsonized zymosan. The inhibition of leukotriene generation was dependent on the incubation time, temperature, and pertussis toxin concentration. The effect was not dependent on the presence of calcium. Incubation of the cells with guanosine 5′-*O*-(3-thiotriphosphate) the stable analog of GTP, led to a time-dependent increase in leukotriene generation induced by formyl-methionyl-leucyl phenylalanine which was abolished by the simultaneous addition of pertussis toxin. Our data suggest that the formyl-methionyl-leucyl phenylalanine-induced generation of leukotrienes is dependent on a GTP-binding protein. The participation of the various G proteins has yet to be elucidated.

The ability of leukocytes to participate in the inflammatory response is regulated by second-messenger systems such as the calcium-mediated activation pathway and the cyclic AMP system (31). Activation of the leukocyte is initiated by the binding of chemoattractants to specific receptors on the cell surface. These events lead to the rapid formation of inositol phosphates and diacylglycerol and to a release of calcium from intracellular stores, suggesting that receptors for chemoattractants activate a phospholipase C.

The process of coupling activated receptor molecules to their effector enzymes is mediated by a class of G proteins (GTP-binding proteins). Different subclasses of G proteins have been described, but all of them conform to a common design (12). They are generally characterized by an alpha-, beta-, and gamma-heterotrimeric structure and act by binding and hydrolyzing guanine nucleotides.

Pertussis toxin (PT), an oligomeric AB-type toxin, can induce an ADP ribosylation of the G-protein alpha subunit of the adenylate cyclase complex in eucaryotic cells (9). It has been suggested that among the various substrates, G_i is a possible substrate for PT, which may transduce inhibitory hormonal messages to adenylate cyclase (14, 33). In addition, PT can have effects on cells by activation of second-messenger pathways independent of its ability to ADP ribosylate G proteins. For the mitogenic effects of PT on T lymphocytes a specific receptor has been suggested (10, 29, 32).

Activation of G proteins occurs when, during ligand-receptor interaction, the bound GDP is exchanged by GTP. The alpha subunit [G(α)-GTP] dissociates from the beta-gamma chains [G(β, γ)] and modifies the activity of the effector enzymes. Nonhydrolyzable nucleotide analogs of GTP such as guanosine 5′-*O*-(3-thiotriphosphate) (GTP-gamma-S) produce persistent activation of alpha subunits (12). The chemotactic peptide formyl-methionyl-leucyl phenylalanine (fMLP) interacts with specific binding sites on polymorphonuclear leukocytes (PMN) (1) and induces cellular events such as the respiratory burst (4), cell adhesion (26), chemotaxis (30), release of arachidonic acid (5) and granular enzymes (20), and stimulation of the 5-lipoxygenase pathway (13, 25).

Arachidonic acid serves as a substrate for the formation of the lipoxygenase-derived leukotrienes and hydroxyeicosatetiaenoic acids (HETEs), which play an important role as mediators of inflammation and allergy (7). In addition to the slow-reacting substances of anaphylaxis (LTC_4, LTD_4, and LTE_4), the chemotactically active LTB_4 plays a central role in host defense against infection. It leads to chemotaxis of neutrophils and eosinophils and release of lysosomal enzymes and enhances the vascular permeability (18). Inactivation of LTB_4 by human PMN occurs by specific hydroxylation at the C-20 position. The omega oxidation products were identified as 20-OH-LTB_4 and 20-COOH-LTB_4 (21), which reveal diminished biological activity as compared with LTB_4 (11).

It was the purpose of our studies to analyze the role of PT on leukotriene generation and metabolism from human PMNs in the presence of fMLP, opsonized zymosan, and the Ca ionophore A23187.

MATERIALS AND METHODS

Material. The reagents used were from the following sources: Ficoll 400 was obtained from Pharmacia, Uppsala, Sweden; Macrodex (6%, wt/vol) was from Knoll, Ludwigshafen; sodium metrizoate solution (75%, wt/vol) from Nyegaard, Oslo; Zymosan A, fMLP, the Ca ionophore A23187, cytochalasin B, GTP-gamma-S, and heparin were obtained from Sigma Munich, Federal Republic of Germany; acetonitrile (high-performance liquid chromatography [HPLC] grade) was purchased from Baker Chemicals, Gross-Gerau, Federal Republic of Germany; and methanol, EDTA, dipotassium hydrogenphosphate, and phosphoric acid were from Riedel de Haën, Seelze, Federal Republic of Germany.

PT was purified as described previously (22). In addition, the commercially available PT from List Biological Laboratories, Inc., was analyzed. Both toxins behaved in a similar manner. A stock solution of PT was prepared by reconsti-

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tuting 50 μg of the toxin with 500 μl of buffer containing 0.1 M sodium phosphate and 0.5 M NaCl (pH 7.0); a stock concentration of 100 μg/ml was obtained. The stock solution was stored at 4°C and diluted in phosphate-buffered saline (PBS).

Synthetic leukotrienes C_4, D_4, E_4, B_4, 20-OH-LTB_4, and 20-COOH-LTB_4 were a generous gift from J. Rokach (Merck, Frosst Pointe Claire, Québec, Canada).

Buffer. The buffer used throughout all experiments consisted of 0.137 M NaCl, 8 mM Na_2HPO_4, 2.7 mM KH_2PO_4, and 2.7 mM KCl (pH 7.4) (modified Dulbecco PBS; referred to as PBS).

Preparation of the cells. Human PMN were obtained from 200 ml of heparinized blood (15 U/ml) of healthy donors separated on a Ficoll-metrizoate gradient followed by dextran sedimentation (6). This method leads to more than 95% pure and intact PMN. The PMN were suspended to a final concentration of 2×10^7 cells per ml in PBS (unless stated otherwise).

Preparation of opsonized zymosan. Zymosan A (2 mg/tube) was suspended in PBS (2 ml) and boiled for 5 min, PBS was added, and the suspension was centrifuged for 10 min at 4°C at $1,900 \times g$ (Heraeus Christ Cryofuge 6-4). The pellet was washed twice with PBS, suspended in human serum (5 mg/ml of human serum), and incubated for 30 min at 37°C. After centrifugation the pellet was washed with PBS and suspended to a final concentration of 40 mg of opsonized zymosan per ml in PBS.

Stimulation of PMN. PMN (10^7/500 μl) were incubated for 2 h (unless stated otherwise) with 500 ng of PT, GTP-gamma-S (100 μM), or the same volume of PBS (50 μl) at 37°C. Then the stimulus (either the Ca ionophore A23187, fMLP, opsonized zymosan, or the same volume of PBS in the presence of calcium [2 mM] and magnesium [1 mM]) was added to the cell suspension, and incubation proceeded for an additional 20 min (unless stated otherwise).

To induce leukotriene generation and to prevent receptor internalization, cytochalasin B was added with fMLP as a stimulus. Incubation with fMLP started 2 min after the addition of cytochalasin B (5 μg/ml). The reaction was terminated by the addition of 2 ml of methanol-acetonitrile (50:50, vol/vol). Samples receiving PBS instead of the toxin served as controls to determine the modulatory effects of the toxin with regard to the subsequent leukotriene generation. The release of lactate dehydrogenase, which is a marker enzyme for cell viability, was determined as previously described (17). The extracellular release of LDH was calculated as a percentage of the total enzyme activity available after sonication of unstimulated cells (10^7/500 μl).

Metabolism of exogenously added LTB_4. PMN (10^7/500 μl) were preincubated with the toxin or PBS for 2 h at 37°C. After preincubation, LTB_4 (100 ng in 50 μl) or the same volume of PBS was added to the cell suspension, and the incubation proceeded in the presence of calcium and magnesium for an additional 15 min. Samples receiving PBS instead of LTB_4 served as controls. The reaction was stopped by the addition of methanol-acetonitrile (50:50, vol/vol).

Analysis of leukotrienes. For reversed-phase HPLC analysis of leukotriene generation, the reaction mixture was stopped and deproteinized by the addition of 2 ml of methanol-acetonitrile (50:50, vol/vol), overlayered with argon, and frozen at −70°C for 12 h. After centrifugation at $1,900 \times g$ for 10 min at 4°C, the supernatants were evaporated to dryness by lyophilization (EF 4 Modulyo; Edwards-Kniese, Marburg, Federal Republic of Germany). The residues were dissolved in 600 μl of methanol-water (30:70, vol/vol), covered with argon, and left at −20°C for 2 h. Centrifugation was performed at $9,700 \times g$ for 2 min at room temperature (Eppendorf Centrifuge 3200); the supernatants were then applied to HPLC. HPLC analysis was performed on reversed-phase columns (250 by 4.6 mm, packed with Nucleosil 5 C18 [Machery Nagel, Düren, Federal Republic of Germany]) with a mixture of phosphate buffer (17 mM dipotassium hydrogenphosphate, containing EDTA, adjusted to pH 5.0 with phosphoric acid), acetonitrile, and methanol (50:30:20, vol/vol) as the effluent with a flow rate of 1 ml/min at 40°C (15). The absorbance of the column effluent was monitored using a variable UV detector (LDC-Milton Roy/Spectromonitor D) adjusted to 280 nm at 0.005 absorbance units, full scale. The peak areas were integrated and calculated by using the program of Nelson analytical chromatography software, AS-Analysensystem, Wuppertal. The recorded HPLC peaks were identified by calculating their α factors as described previously (16). For analysis of leukotriene generation, LTB_4 served as a reference substance to calculate the α factors of the other substances. The calculated α factors were 0.15 to 0.17 for 20-COOH-LTB_4, 0.19 to 0.21 for 20-OH-LTB_4, 0.52 to 0.54 for LTC_4, 0.62 to 0.64 for LTD_4, 0.69 to 0.71 for LTE_4, 0.80 to 0.82 for 6-trans-LTB_4, 0.90 to 0.92 for 12-epi-6-trans-LTB_4, and 1.12 to 1.14 for 5S,12S-di-HETE. The quantification of identified leukotrienes was performed by area integration of the absorption peaks. LTB_4 generation was calculated as combined amounts of LTB_4 and the LTB_4-omega-oxidation products (20-OH-LTB_4 and 20-COOH-LTB_4). With the extraction procedure, the recovery rates of leukotrienes from the cell suspension were 75 to 85% for the cysteinyl leukotrienes and 90 to 95% for LTB_4 and the omega oxidation products.

LTB_4- and fMLP-binding assay. LTB_4-binding studies were carried out as described previously (8, 24). The assays were performed with 96-well filtration plates with 5-μm-pore-size polyvinylidene fluoride membranes (Millipore, Eschborn, Federal Republic of Germany). Each well contained 2.3 nM [^{3}H]LTB_4 (0.9 KBq) and 125 μg of bovine serum albumin. All samples were carried out as triplicates with variation coefficients ranging from 7 to 12%. Specific binding was expressed as total binding minus nonspecific binding; nonspecific binding was determined in the presence of 220 nM unlabeled LTB_4. To determine the specific binding of LTB_4 to stimulated PMN, cells (10^7) in 1.2 ml of buffer were incubated for 2 h with PT. Incubation was terminated by centrifugation for 5 s at $9,700 \times g$ (Eppendorf centrifuge 3200); the cell pellets were suspended with 1.2 ml of PBS. Values for total and nonspecific binding were calculated as the means of triplicate determinations; thus one-sixth (200 μl) of the cell suspension was obtained and assayed.

fMLP-binding studies were carried out as for LTB_4. Each well contained 20 nM [^{3}H]fMLP (21 kBq); for determination of nonspecific binding, 100 μM unlabeled fMLP was added.

Statistical analysis. Data from different experiments with different donor cells were combined and reported as the mean ± standard deviation. The Student t test for independent means was used to provide a statistical analysis ($P > 0.05$ was considered as not significant).

RESULTS

Effect of PT on leukotriene formation. In previous publications (8, 27, 28) we demonstrated that stimuli such as the Ca ionophore A23187, opsonized zymosan, and fMLP induce the generation and release of leukotrienes from human

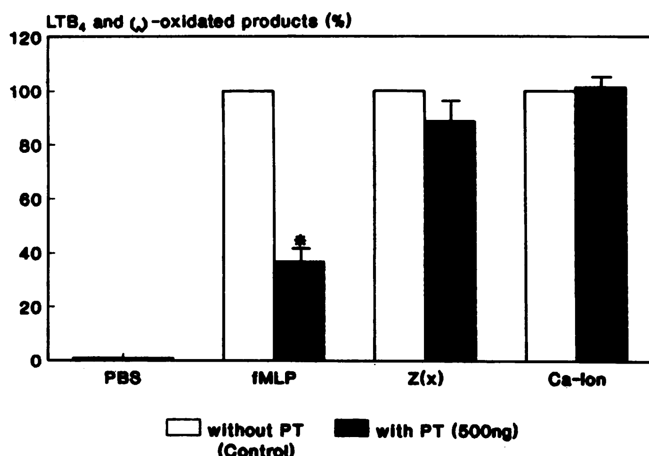

FIG. 1. Treatment of human granulocytes (10^7) with PT (500 ng) or PBS for 2 h at 37°C followed by incubation with fMLP (7 μM), opsonized zymosan (2 mg), the Ca ionophore A23187 (6.3 μM), or PBS for another 20 min at 37°C. Each value represents the mean ± standard deviation of three independent experiments with different donor cells (*, $P < 0.01$; 100% values were 10 ± 4 ng for fMLP, 50 ± 7.5 ng for opsonized zymosan, and 400 ± 75 ng for the Ca ionophore).

granulocytes in a time- and dose-dependent manner. Maximal release of LTB_4 after stimulation with the Ca ionophore A23187 occurred after 5 to 10 min, with a rapid decline at later time intervals. This decline was followed by the generation of the omega-oxidation products of LTB_4, 20-OH-LTB_4 and 20-COOH-LTB_4. Stimulation of human PMN with opsonized zymosan or fMLP induced the same pattern of leukotriene generation, but the amounts of leukotrienes generated on stimulation with the latter two stimuli comprised only 10 to 15% of that induced by the Ca ionophore.

Incubation of human granulocytes with PT (500 ng per 10^7 PMN) for 2 h before the addition of the Ca ionophore, opsonized zymosan, or fMLP modulated the aforementioned pattern of leukotriene generation. PT alone, in the absence of any additional stimulus, was not sufficient to induce leukotriene generation. Cytotoxicity was examined by lactate dehydrogenase, but insignificant amounts (less than 7%) were detected (data not shown). Figure 1 demonstrates the results of three separate experiments, in which cells were incubated with PT or PBS and subsequently activated with the indicated stimuli for 20 min. The values represent the combined amounts of LTB_4 and LTB_4 omega oxidation products (20-OH-LTB_4 and 20-COOH-LTB_4), reflecting the result of de novo synthesis and metabolism. The values for LTB_4 generation induced by the indicated stimuli in the absence of PT (PBS control) were expressed as 100%, and the amount of LTB_4 generation in the presence of PT was calculated as a percentage of the 100% control value.

Treatment of the cells with PT led to a significant decrease in LTB_4 generation (63% inhibition) induced by fMLP as compared with that in the control (Fig. 1). The decrease included LTB_4 as well as the omega oxidation products; the ratio of LTB_4, 20-OH-LTB_4, and 20-COOH-LTB_4 was not affected as compared with that in the control without PT. In contrast to these results, PT treatment had no effect on the Ca ionophore A23187-induced leukotriene generation. With opsonized zymosan as the subsequent stimulus, the inhibitory effects of PT were much lower than those obtained after stimulation with fMLP.

In another series of experiments, the temperature dependency for the effect of PT was investigated. Therefore, PMN (10^7) were incubated with PT (500 ng) or PBS (as a control) for 2 h at 37 or 4°C; the incubation then proceeded in the presence of calcium (2 mM) and magnesium (1 mM) with the various stimuli (Ca ionophore A23187, opsonized zymosan, or fMLP) for an additional 20 min at 37°C.

The results obtained by HPLC analysis are shown in Fig. 2. Incubation of PMN with PT at 4°C had no effect on the subsequent leukotriene generation induced by fMLP, opsonized zymosan, and the Ca ionophore. In contrast to PT

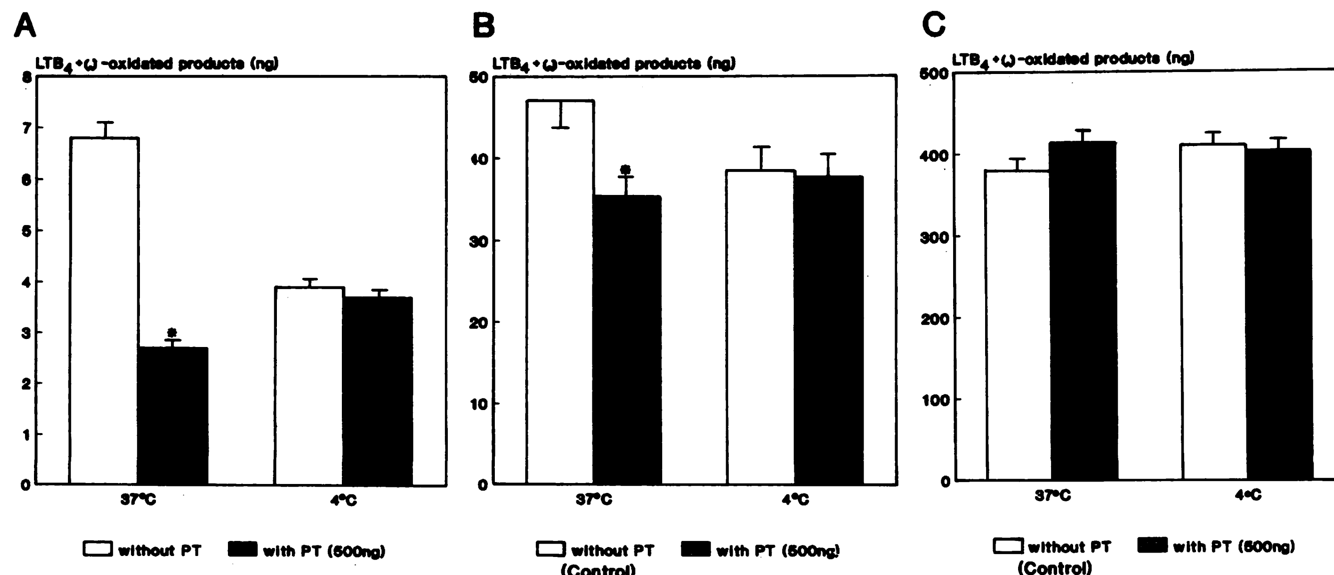

FIG. 2. Temperature-dependent effects of treatment with PT. PMN (10^7) were treated with PT (500 ng) or PBS (control) for 2 h at 37 or 4°C; the incubation was continued with fMLP (7 μM) (A), opsonized zymosan (2 mg) (B), or the Ca ionophore A23187 (6.3 μM) (C) for another 20 min at 37°C. Results represent mean values of triplicates obtained from one typical experiment out of three independent experiments (*, $P < 0.01$).

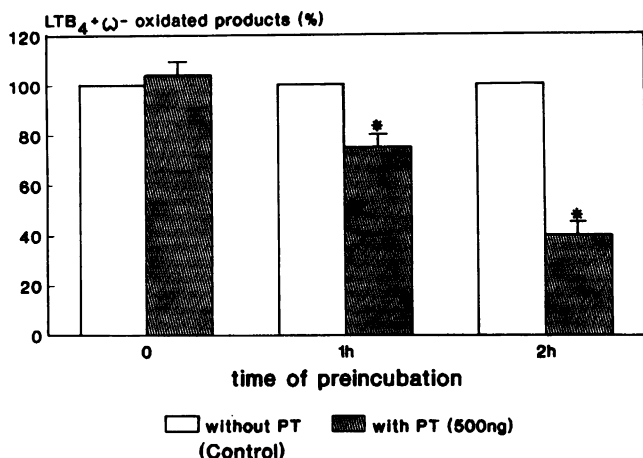

FIG. 3. Time-dependent effects of incubation with PT. PMN (10^7) were incubated with PT (500 ng) or PBS (control) for different times at 37°C, followed by incubation with fMLP (7 μM) for another 20 min. Each value represents the mean ± standard deviation of three independent experiments with different donor cells (*, $P < 0.01$; the 100% value was 10 ± 4 ng).

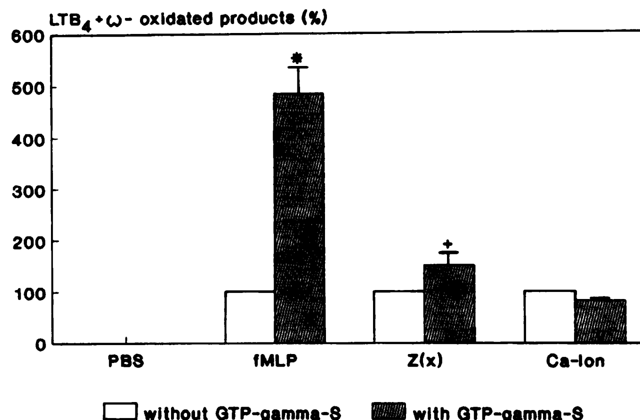

FIG. 4. Effects of GTP-gamma-S treatment on leukotriene generation. PMN (10^7) were incubated with GTP-gamma-S (100 μM) or PBS (as 100% control) for 2 h at 37°C; the incubation was then continued with fMLP (7 μM), opsonized zymosan (2 mg), the Ca ionophore A23187 (6.3 μM), or PBS for another 20 min at 37°C. The data represent the means ± standard deviations of three independent experiments (*, $P < 0.01$; +, $P < 0.05$; the 100% values were 10 ± 4 ng for fMLP, 50 ± 7.5 ng opsonized zymosan, and 400 ± 75 ng for the Ca ionophore).

treatment at 37°C, no inhibition of the leukotriene generation induced by fMLP was observed when the cells were incubated with PT at 4°C.

Experiments were then carried out to analyze the time dependency of the effects. For this purpose, PMN (10^7) were incubated with PT (500 ng) or PBS (control value) at 37°C for 0, 1, and 2 h; the incubation was then continued in the presence of calcium and magnesium with fMLP for another 20 min at 37°C. The results obtained by HPLC analysis for LTB_4 generation from three independent experiments are shown in Fig. 3. No inhibition of LTB_4 generation was observed when the cells were incubated with PT and fMLP simultaneously for 20 min. A prolonged incubation of cells with PT resulted in decreasing amounts of LTB_4 and LTB_4 omega oxidation products after fMLP stimulation.

To determine the metabolism of exogenously added LTB_4, PMN (10^7) were incubated with PT (500 ng) or with PBS for 2 h at 37°C. Then LTB_4 was added, and the incubation proceeded for an additional 15 min at 37°C. The metabolism of LTB_4 to 20-OH-LTB_4 and 20-COOH-LTB_4 was calculated by HPLC. Treatment of PMN with the PT had no effect on the metabolism of exogenously added LTB_4 (data not shown).

Preliminary experiments were then directed to determine the influence of PT on the specific binding of LTB_4 and fMLP to PMN. For this purpose, the binding of radiolabeled LTB_4 and fMLP to PMN treated with PT was studied. Our data indicate that treatment of PMN with PT (500 ng) decreased the specific binding of LTB_4 and fMLP by nearly 60% (mean of two independent determinations; data not shown).

Modulation of leukotriene formation by GTP-gamma-S. It has been established that GTP-gamma-S modulates G-protein functions in permeabilized cells (23). Experiments were performed to analyze the effects of GTP-gamma-S pretreatment on leukotriene generation induced with the indicated stimuli. PMN (10^7) were incubated with GTP-gamma-S (100 μM) or PBS (as a control) for 2 h at 37°C. The incubation was then continued in the presence of calcium and magnesium with the various stimuli (fMLP, opsonized zymosan, and Ca ionophore) for an additional 20 min at 37°C. The data obtained by HPLC analysis from three independent experiments are shown in Fig. 4. GTP-gamma-S alone, in the absence of any additional stimuli, was not sufficient to induce leukotriene generation. Incubation of PMN with GTP-gamma-S had no effect on the leukotriene generation induced by the Ca ionophore and revealed a low increase after stimulation with opsonized zymosan. In contrast, the GTP-gamma-S treatment of PMN significantly enhanced the subsequent leukotriene generation induced by fMLP.

In another series of experiments the time-dependent effects of incubation with GTP-gamma-S were investigated. For this purpose, PMN (10^7) were stimulated with GTP-gamma-S (100 μM) or PBS (as a control) at 37°C for 0, 15, 30, 60, and 120 min; the incubation was then continued after the addition of fMLP for another 20 min at 37°C. The simultaneous addition of GTP-gamma-S and fMLP (time point 0 min) enhanced the leukotriene generation as compared with that in PBS-treated cells (Fig. 5). A prolongation of the incubation time with GTP-gamma-S up to 60 min resulted in increasing amounts of LTB_4 and LTB_4 omega oxidation

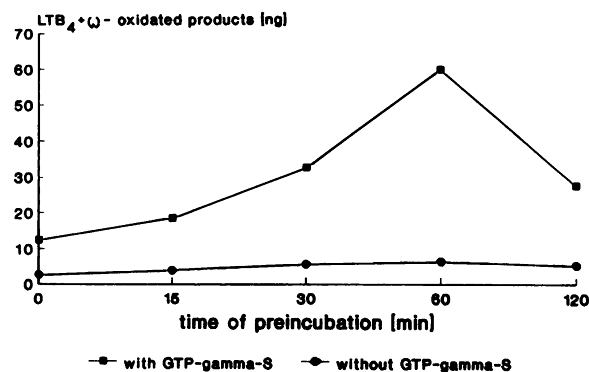

FIG. 5. Time-dependent effects of GTP-gamma-S on the leukotriene generation induced by fMLP. PMN (10^7) were pretreated with GTP-gamma-S (100 μM) or PBS (control) at 37°C over various times; the incubation was then continued by the addition of fMLP (7 μM) for another 20 min at 37°C. Results represent values obtained from one typical experiment out of three independent experiments.

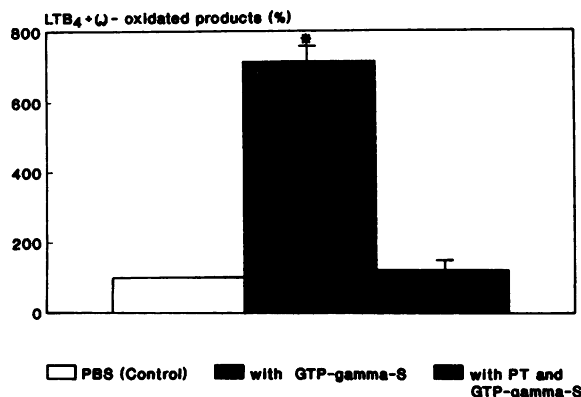

FIG. 6. Effects of simultaneous treatment of PMN with GTP-gamma-S and PT on leukotriene generation. PMN (10^7) were incubated for 2 h at 37°C with GTP-gamma-S (100 μM) and PT (500 ng) or PBS, followed by leukotriene formation induced by fMLP (7 μM). The control value (100%) was determined in the absence of GTP-gamma-S and PT. The data represent the mean LTB_4 generation from three different donors (*, $P < 0.01$; the 100% value was 10 ± 4 ng).

products after stimulation with fMLP. A decrease in LTB_4 generation was obtained when the incubation periods exceeded 60 min.

Our data clearly demonstrate that the incubation of cells with PT significantly decreased the leukotriene generation induced by fMLP, whereas the incubation of cells with GTP-gamma-S significantly enhanced the leukotriene generation induced by fMLP. Our subsequent experiments were then addressed to study the treatment of PMN in the presence of GTP-gamma-S and PT on leukotriene generation. For this purpose PMN (10^7) were incubated for 2 h at 37°C simultaneously with GTP-gamma-S (100 μM) and PT (500 ng). Cells that were treated with either compound or PBS served as controls. The leukotriene generation was then initiated after incubation of the cells with fMLP for 20 min. Not only did PT prevent the enhancing effect of GTP-gamma-S on LTB_4 production, but also, in the presence of both PT and GTP-gamma-S, the fMLP response was abolished altogether (Fig. 6).

DISCUSSION

Our data demonstrate that incubation of the cells with PT modulates the subsequent leukotriene generation induced by various stimuli. A significant decrease in LTB_4 generation induced by fMLP was observed after treatment of the cells with PT. The effect of pertussis toxin was time and temperature dependent. In contrast, leukotriene generation induced by the Ca ionophore A23187 was not affected. With opsonized zymosan as a secondary stimulus, the inhibitory effects were much lower but significantly less than those observed when the cells were stimulated with fMLP. Furthermore the ratios of LTB_4, 20-OH-LTB_4, and 20-COOH-LTB_4 in the absence or presence of PT did not change, suggesting that PT does not affect the LTB_4-metabolizing enzymes.

It is well established that the various stimuli interact with neutrophils via different membrane biochemical events. The Ca ionophore induces calcium influx, and opsonized zymosan interacts with the C3b and Fc receptors for binding and signal transduction. The chemotactic peptide fMLP interacts with specific binding sites on the cells. Our data indicate that PT modulates leukotriene generation induced by stimuli that interact with specific receptors on the cell surface. One may suggest that this decrease is due to the reduced binding of fMLP to its receptor. Indeed, treatment of the cells with PT also inhibited the binding of fMLP as well as of LTB_4. There is no information whether the change of the affinity state for the fMLP or LTB_4 receptor may affect the amount of leukotriene generation and metabolization. The unaltered stimulation with the Ca ionophore can be explained by the fact that it bypasses many of the initial events during receptor-mediated signal activation and thus acts independently of GTP-binding proteins.

The low changes in leukotriene generation observed with PT-treated cells stimulated by opsonized zymosan suggest that in this case PT-sensitive G proteins are only in part involved (2). Becker et al. (2) suggested that, in the same cell, a receptor to one agonist interacts with a PT-sensitive G protein, whereas a different receptor may interact with an insensitive G protein to activate a single effector enzyme, such as phospholipase C; which of the mechanisms is utilized depends on the stimulus. In addition to our data, Bokoch and Gilman (3) demonstrated that treatment of guinea pig neutrophils with PT inhibited the fMLP-mediated release of arachidonic acid as well as of granular enzymes.

PT pretreatment of cells also modulates histamine and LTC_4 release from human basophils (34) depending on the stimulus. Warner et al. demonstrated the inhibition of the mediator release from PT-treated basophils after stimulation with fMLP and the anaphylatoxin C5a; the toxin did not affect the immunoglobulin E-mediated mediator release. To what extent the immunoglobulin E-mediated signal proceeds via G proteins other than G_i or G_s has to be determined. It has been recently shown that PT can have effects on cells by activation of second messenger pathways independent of its ability to ADP ribosylate G proteins (29, 32). In this regard the mitogenic effect of PT holotoxin is mediated by the interaction of the B oligomer with CD3 (10). Furthermore, PT led to a rapid rise in cytosolic free Ca^{2+} from both intra- and extracellular sources, associated with an increase in the cellular diacylglycerol and inositol trisphosphate levels with a concomitant decrease in the levels of phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate. Whether similar events induced by PT also occur in neutrophils and affect leukotriene generation has to be clarified in future studies.

Our data clearly show that incubation of PMN with the nonhydrolyzable GTP analog GTP-gamma-S enhanced the LTB_4 generation after subsequent stimulation with fMLP in a time-dependent manner. The addition of GTP-gamma-S leads to a persistent activation of the alpha subunits of the G proteins (12), which is followed by an increase in leukotriene formation. The mechanism by which GTP-gamma-S acts in our system is still unclear and has to be elucidated in further studies. GTP-gamma-S has been shown to enter permeabilized cells (23). One may speculate that GTP-gamma-S also exerts an effect on neutrophils from the outside. Indeed, recent data also suggest that G proteins express recognition sites on the outside of the plasma membrane (FASEB program, Second messengers and signal transduction, New Orleans, 1989, p. 34). Since an incubation time of 1 h is required for an optimal effect, one may also suggest an uptake of GTP-gamma-S by the PMN. In any case such an uptake, if it occurs, is obviously not due to cytotoxic activity of the compound. The viability of the cells did not change under the experimental conditions; in addition, the enhanced

leukotriene formation and its inhibition by PT suggest that the cell is completely viable.

It has been demonstrated that the fMLP receptor on human neutrophils exists in both high- and low-affinity states and that the conversion of the affinity is catalyzed by GTP (19). Therefore it has to be analyzed whether the addition of GTP-gamma-S to the PMN converts the fMLP-receptor from the high- to the low-affinity state, which then results in an enhanced leukotriene formation.

The addition of GTP-gamma-S did not alter the Ca-ionophore-induced stimulation; these results indicate that the Ca ionophore bypasses the activation of G proteins. A minute enhancement of leukotriene formation was observed in GTP-gamma-S-treated PMN stimulated with opsonized zymosan. These results are suggestive that both PT-sensitive and -insensitive G proteins may be activated after stimulation with opsonized zymosan; it appears that they may be involved in the signal-transducing cascade to a different degree.

The simultaneous treatment of PMN with GTP-gamma-S and PT did not affect leukotriene generation with fMLP.

Our data indicate that the modulatory effect of GTP-gamma-S on leukotriene formation by fMLP and opsonized zymosan is suggestive of G-protein involvement. Further experiments are needed to analyze the signal transduction pathway for receptor-mediated leukotriene generation from phagocytes.

ACKNOWLEDGMENTS

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