Pertussis Toxin Produces Differential Inhibitory Effects on Basal, \(P_2\)-purinergic, and Chemotactic Peptide-stimulated Inositol Phospholipid Breakdown in HL-60 Cells and HL-60 Cell Membranes*

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Daniel S. Cowen‡§, Barbara Baker¶, and George R. Dubyak||

From the Departments of Physiology and Biophysics and Pharmacology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

\(P_2\)-purinergic receptor agonists (UTP) and formylated peptide receptor agonists (FMLP) were found to be equally efficacious in eliciting rapid 6-7-fold increases in inositol phosphate accumulation in differentiated HL-60 granulocytes. The activation of this response by either agonist was substantially but incompletely inhibited in cells treated with pertussis toxin. Thus, in cells containing only 1-10% of the control level of non-ADP-ribosylated \(G_{12/13}\), UTP induced rapid 2-fold increases in inositol polyphosphate accumulation whereas smaller 50% increases were observed in FMLP-stimulated cells. Washed membranes prepared from control and toxin-treated HL-60 cells were used to characterize this toxin-insensitive activation of phospholipase C further. The agonist-independent stimulation of phospholipase C by either millimolar \(Ca^{2+}\) or the nonhydrolyzable GTP analog guanosine 5'-O-(3-thiotriphosphate) (GTP\(\gamma\)S) was only modestly attenuated by toxin treatment. There was a 70-80% decrease in the rate and extent of phospholipase C activity stimulated by GTP per se in the absence of receptor agonists. The rate and extent of FMLP-induced potentiation of GTP-dependent phospholipase C activity were also inhibited by >80% in toxin-treated membranes. Conversely, the potency and efficacy characterizing UTP-induced potentiation of GTP-dependent phospholipase C activity were only modestly attenuated (<20% inhibition). The results indicate that \(P_2\)-purinergic receptors (and perhaps other \(Ca^{2+}\)-mobilizing receptors) activate both pertussis toxin-sensitive and toxin-insensitive pathways for phospholipase C regulation in phagocytic leukocytes.

The ability of pertussis toxin to inhibit formylated peptide receptor-activated GTP-dependent inositol phospholipid breakdown in phagocytic leukocytes such as neutrophils and differentiated HL-60 cells has been studied and described extensively (1-4). Considerable effort has been directed toward characterizing the regulation of phospholipase C effector enzymes by those G-proteins that are: 1) known to be expressed by phagocytic leukocytes, and 2) are substrates for pertussis-catalyzed ADP-ribosylation. Polakis et al. (5) have reported that the FMLP-receptor co-purifies with a 40-kDa GTP-binding protein that is the major pertussis toxin substrate in neutrophils and HL-60 cells (5). Recent immunological and molecular cloning studies from several laboratories have identified this protein as the \(a_1\) subunit of the \(G \alpha_\text{superfamily}\) of regulatory proteins (6,7). These and other studies have demonstrated that neutrophils and HL-60 cells also express \(G_{12/13}\) but no readily detectable amounts of \(G_{11}\) or \(G_{16}\). Thus, \(G_{12/13}\) and \(G_{16}\) appear to be the only pertussis toxin-sensitive G-proteins expressed in HL-60 cells. Recent studies by Gierschik et al. (8) have indicated that the FMLP receptor can interact functionally with \(G_{15}\) in addition to \(G_{12/13}\) in membranes from differentiated HL-60 cells.

Despite the considerable evidence indicating that formylated peptide receptors are coupled to \(G_{12/13}\), important questions concerning the role of specific G-proteins in the regulation of GTP-dependent inositol phospholipid breakdown in phagocytic leukocytes remain unresolved. Kikuchi et al. (9) reported that both rat brain \(G_{1}\) (isotype unspecified) and \(G_{15}\) could reconstitute FMLP receptor-phospholipase C coupling when added to HL-60 granulocyte membranes wherein the native \(G_{15}\)-type proteins were ADP-ribosylated by pertussis toxin. However, there is no direct evidence as to whether native \(G_{15}\) or \(G_{15}\) (or both) mediates activation of the inositol phospholipid specific phospholipase C isoform(s) expressed in these cells. Moreover, the mechanism(s) whereby pertussis toxin interferes with agonist-induced activation of the phospholipase is only partially understood. Although pertussis toxin treatment is known to uncouple \(G_\alpha\) from agonist-occupied FMLP receptors significantly (9), it has not been ascertained whether interaction of the G-protein with uncoupled receptors, guanine nucleotides, or the phospholipase effector may also be attenuated by ADP-ribosylation. Finally, several studies have indicated that \(Ca^{2+}\)-mobilizing agonists might activate inositol phospholipid hydrolysis or other \(Ca^{2+}\)-dependent signaling events in neutrophils and related cells via pertussis toxin-insensitive mechanisms. Vergehese et al. (10) reported that pertussis toxin treatment of neutrophils only partially inhibited the \(Ca^{2+}\) mobilization elicited by leukotriene B\(_4\) or platelet-activating factor. Similarly, Saussy et al. (11) found that substantial leukotriene D\(_4\)-induced \(IP_3\) accumulation and \(Ca^{2+}\) mobilization could be observed in pertussis toxin treated U937 cells differentiated along the monocytic pathway. Thus, phagocytic leukocytes, like most other cell types, may express both pertussis toxin-sensitive

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§ Recipient of a medical scientist training program award.

¶ Established Investigator of the American Heart Association. To whom all correspondence and reprint requests should be addressed.

† Established Investigator of the American Heart Association. To whom all correspondence and reprint requests should be addressed.

1 The abbreviations used are: FMLP, formylated peptide receptor agonist; \(IP_3\), inositol 1,4,5-triphosphate; \(IP_4\), inositol 4,5-bisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BGGTA, [ethylendiaminetetraacetic acid]; SDS, sodium dodecyl sulfate; GTP\(\gamma\)S, guanosine 5'-O-(3-thiotriphosphate).
and toxin-insensitive G-proteins that can interact with receptors for Ca\(^{2+}\)-mobilizing agonists.

In previous studies (12-14), we have described the activation of inositol phospholipid hydrolysis and Ca\(^{2+}\) mobilization by P\(_2\)-purinergic receptors (for extracellular ATP) in neutrophils, HL-60 cells, and other human phagocytic leukocytes. The activation of both inositol polyphosphate accumulation and Ca\(^{2+}\) mobilization by P\(_2\)-purinergic agonists was significantly, but not completely, inhibited in undifferentiated and differentiated HL-60 cells pretreated with pertussis toxin (15). This contrasted with the more pronounced inhibition of FMLP-stimulated phospholipase D activity and Ca\(^{2+}\) mobilization measured in the same pertussis toxin-treated cells. Thus, although P\(_2\)-purinergic receptors and FMLP receptors are equally efficacious in activating inositol phospholipid breakdown and Ca\(^{2+}\) mobilization, there may be significant differences in the mechanisms whereby these receptors are coupled to effector enzymes via G-proteins. It should be noted that P\(_2\)-purinergic agonists, like leukotriene B\(_4\) and platelets-activating factor (10), are much less efficacious than FMLP in activating certain integrated biological responses, such as supernoxide generation and granule release, in neutrophils (15, 16) and HL-60 granulocytes (17). In a recent study utilizing washed membranes isolated from I-110 cells prelabelled with myo-[\(^{3}H\)]inositol, we demonstrated that P\(_2\)-purinergic activation of phospholipase C was strictly dependent on the presence of guanine nucleotides (14). In the present study, we have used a similar membrane system, prepared from HL-60 granulocytes, to examine further the effects of pertussis toxin treatment on basal and agonist-stimulated GTP-dependent phospholipase C activity and to correlate these functional effects with the degree of toxin-catalyzed ADP-ribosylation of the relevant G-protein \(\alpha\)-subunits.

**EXPERIMENTAL PROCEDURES**

**Cells**—HL-60 cells (obtained from the American Tissue Type Culture Collection) were routinely cultured in Iscove's medium (GIBCO) supplemented with 25 mM HEPES and 10% calf bovine serum (IvyClone Laboratories, Logan, UT). The cells were maintained in a humidified atmosphere of 92.5% air, 7.5% CO\(_2\) Three days before isotopic labeling, cells were transferred to serum-free Iscove's medium supplemented with 25 mM HEPES, 5 \(\mu\)g/ml transferrin, 5 \(\mu\)g/ml insulin, and 5 ng/ml sodium selenite. It should be noted that these experiments were performed over an 18-month period using HL-60 cells derived from four different frozen stocks. The cells were washed two times with an ice-cold solution containing 125 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 5 mM glucose, 50 mM HEPES (pH 7.4), 1 mM EGTA, and 2 mM EDTA (unlabeled), 2-5 \(\mu\)g [\(^32\)P]NAD, and 10 ng/ml activated pertussis toxin. Unless otherwise indicated, the reaction mixture also contained 0.05% Triton X-100.

**In Vitro Ribosylation Studies**—Membranes were isolated from control or pertussis toxin-treated cells as described above. In some experiments, the cells were pretreated with 3 mM diisopropylfluorophosphate for 20 min at 4°C prior to lysis. 4-10 \(\mu\)l of stock membranes (10-15 \(\mu\)g of protein) were incubated for 10 min at 37°C in a 100-\(\mu\)l reaction volume containing (final concentrations) 25 mM Tris (pH 8), 2 mM EDTA, 2 mM EGTA, 20 mM isonicotinic acid, 1 mM ADP-ribose, 10 mM thymidine, 0.1 mM ATP, 10 mM diethiothreitol, 50 \(\mu\)g/ml leupeptin, 125 kallikrein inhibiting units/ml aprotinin, 2.5 \(\mu\)g/ml leupeptin, and 2 mM EDTA. The reaction was stopped by the addition of 0.9 ml of 11% trichloroacetic acid. After incubation on ice for 30 min, the precipitated protein was collected by centrifugation and washed three times with 1 ml of diethyl ether. The protein pellets were dissolved in a standard SDS/mercaptoethanol extraction buffer and subjected to SDS-polyacrylamide gel electrophoresis. After autoradiography of the dried, Coomassie Blue-stained gels for 20-70 h, selected bands were excised and extracted at 50°C for 18 h in 200 \(\mu\)l of 30% H\(_2\)O\(_2\) and then analyzed by liquid scintillation counting.

**Materials**—GTP-S was purchased from Boehringer Mannheim. All other nucleotides and nucleotide analogs were purchased from Sigma.

**RESULTS**

Effects of Pertussis Toxin Treatment on P\(_2\)-purinergic Versus FMLP-stimulated Inositol Polyphosphate Formation in Intact HL-60 Cells—UTP is an agonist with a potency and efficacy equal to that of ATP in stimulating inositol phospholipid hydrolysis and Ca\(^{2+}\) mobilization in human neutrophil...
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and monocyte cell types (12, 14). As an agonist for in vitro studies of P2-purinergic receptor effects on the phosphatidylinositol phospholipase C, UTP has the advantage of being a poor substrate for membrane-associated lipid kinases (14). As shown in Fig. 1, maximally activating concentrations of UTP or FMLP were equally efficacious in stimulating rapid accumulation of IP3 (4-fold increases within 15 s) and IP2 (6-fold increases in 15 s) in intact HL-60 granulocytes. The EC50 for UTP stimulation was approximately 3 μM whereas that for FMLP was 30 nM.

Treatment of HL-60 granulocytes with high concentrations of pertussis toxin for 3 h significantly reduced the efficacies of both FMLP and UTP in stimulating inositol polyphosphate accumulation. However, the maximal extent of the pertussis toxin-induced inhibition was invariably smaller when UTP (or ATP) rather than FMLP was the agonist. This differential sensitivity of P2-purinergic receptors versus FMLP receptors to inhibition by pertussis toxin was very reproducible. It is important to note that varying the concentration of pertussis toxin (250–1000 ng/ml) used to treat the cells during the 3-h incubation had little effect on the magnitude of inhibition of the response to FMLP or UTP. Cells treated with this range of toxin concentrations were characterized by a 91 ± 1% (mean ± S.E., n = 7) inhibition of maximal FMLP-induced inositol polyphosphate accumulation. In these same cells there was an average 78 ± 2% (n = 8) inhibition of the maximal UTP-induced response.

Quantitation of Pertussis Toxin Substrates in Membranes Isolated from HL-60 Granulocytes—Given the previous results, it was important to determine the residual content of unribosylated Gi.2/3 in HL granulocytes treated with pertussis toxin under a variety of incubation conditions. However, precise quantitation of the residual substrates for in vitro toxin-catalyzed ADP-ribosylation requires that the activated toxin have unimpaired access to the relevant membrane-associated protein substrates. Membranes are routinely prepared from neutrophils and HL-60 cells lysed by nitrogen cavitation (4, 5, 14, 19). The plasma membranes of cells lysed by this method re-form vesicles that may not be permeated readily by the catalytically active 26-kDa A subunit of pertussis toxin. Consistent with this possibility, we found that the rate and extent of toxin-catalyzed incorporation of [32P]ADP-ribose into the major 40–41-kDa substrate of freshly isolated HL-60 membranes are increased by a greater than 10-fold factor if certain detergents such as Triton X-100 and digitonin are also effective, data not shown) are included in the in vitro ADP-ribosylation reaction medium (Fig. 2). This is in agreement with the studies of Ribeiro-Neto et al. (22) who reported that Triton X-100 increased pertussis toxin-catalyzed ADP-ribosylation of thyroid membranes. These investigators also noted that inclusion of detergent produced a 2–3-fold potentiation of the ADP-ribosylation of purified Gt (derived from human erythrocytes); this latter result suggests that detergent may additionally affect the conformational states of the toxin and/or the G-protein substrate. Although the use of detergents increased accessibility of the HL-60 membrane G-proteins to the toxin, it also increased the accessibility to endogenous proteases that are particularly abundant in the crude membrane preparations used in the present studies (Fig. 2). In the presence of detergent, there was a very rapid incorporation of ADP-ribose into the major 40–41-kDa substrate after an initial 2-min lag. This reaction approached >75% completion within 5 min (Fig. 2B); there was only a minor 5–10% increase when incubations were extended from 10 to 15 min (data not shown). However, with incubation times longer than 10 min, three additional 32P labeled bands appeared; these had molecular masses of 30, 27, and <10 kDa (migration with dye front). With longer incubation times, there was a progressive loss of radioactivity in the major 40–41-kDa substrate which was quantitatively matched by increased 32P counts associated with the lower molecular mass species (Fig. 2B). The rate of this apparent proteolysis of the 40–41-kDa substrate could be reduced but not eliminated by 1) preincubation of the cells with diisopropylfluorophosphate prior to lysis; and 2) inclusion of divalent cation chelators and leupeptin in the reaction medium. Presumably, this proteolysis might also be reduced by separating the protease-containing azurophilic granules from the plasma membrane fraction by gradient centrifugation (19); however, this is not practical when dealing with the relatively low numbers of cells pretreated with pertussis toxin. Thus, we routinely employed 10-min incubations in the presence of 0.05% Triton to ensure maximal incorporation of [32P]ADP ribose into the 40–41-kDa substrate (combined αt2 and αt3) but minimal proteolytic degradation of these G-protein α-subunits.

Correlation between ADP-ribosylation of Gi.2/3 and Toxin Effects on UTP- and FMLP-stimulated Inositol Polyphosphate Accumulation—Additional experiments were designed to correlate the apparent pertussis toxin-resistant component of agonist-induced phospholipase C activity with the residual content of nonribosylated Gi.2/3. Parallel cultures of unlabeled and [3H]inositol-labeled HL-60 granulocytes were incubated with various concentrations of toxin for 3 or 15 h. Membranes prepared from the unlabeled cells were assayed for unmodified Gi.2/3 content using the in vitro ADP ribosylation protocol described above (Fig. 3). Cells treated for 3 h with 0.1 μg/ml toxin contained retained 30% of the normal level of unmodified 40-kDa substrate. Three-h exposures to 0.5 or 1 μg/ml toxin or a 15-h incubation with 0.1 μg/ml produced approximately equivalent 80–90% decrements in ADP-ribosylation of Gi.2/3. Incubation of the cells for 15 h with 1 μg/ml toxin virtually eliminated subsequent in vitro incorporation of ADP-ribose; results from five different cell preparations treated with 1 μg/ml toxin for 15 h indicated a 98.5 ± 0.3% decrease in ADP-ribosylation. Parallel measurements of inositol polyphosphate accumulation revealed that the additional
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Fig. 2. In vitro ADP-ribosylation of membranes isolated from HL-60 granulocytes: kinetics and effect of detergent. Membranes isolated from HL-60 granulocytes were used as substrate for pertussis toxin-catalyzed ADP-ribosylation as described under "Experimental Procedures." ADP-ribosylation was assayed in standard reaction medium in either the absence (−) or presence (+) of 0.05% Thesit detergent. Reactions were initiated by the addition of membranes (10 μg of protein/assay) and were stopped at the indicated times by the addition of trichloroacetic acid. Labeled proteins were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography (panel A) and liquid scintillation counting (panel B). Closed symbols (●) represent data from membranes assayed in the absence of detergent; open symbols (○) represent data from detergent-containing assays. Circles represent 32P incorporation into the 40-41-kDa substrate; squares illustrate 32P incorporation into protein (<10 kDa) that runs at or near the dye front. These data are representative of three similar experiments.

Fig. 3. In vitro ADP-ribosylation of G12/13 in membranes from HL-60 granulocytes: effects of pretreatment of intact cells with varying concentrations of pertussis toxin (PTX). Parallel samples of HL-60 granulocytes were pretreated with the indicated concentrations of pertussis toxin for 3 or 15 h. Membranes isolated from these cells were used as substrate for pertussis toxin-catalyzed ADP-ribosylation of G12/13 as described under "Experimental Procedures" and in the Fig. 2 legend. ADP-ribosylation was assayed in standard reaction medium containing 0.05% Thesit detergent. Reactions were initiated by the addition of membranes (15 μg of protein/assay) and were stopped after 10 min by the addition of trichloroacetic acid. Panel A shows the autoradiograms from two separate experiments; panel B details average ± range of the relative (control = 100%) 32P cpm incorporated into the 40-41-kDa substrate.

decrease in unribosylated G-protein observed in cells treated with toxin for prolonged times was not matched by an additional inhibition of agonist-induced inositol polyphosphate accumulation (Fig. 4). Cells containing only 1–2% of the control level of nonribosylated G12/13 exhibited a similar relative inhibition of UTP-induced accumulation (81% versus 75–80%) as was observed in cells containing 10–15% of the normal, unmodified G-protein content. Likewise, similar 87–91% inhibitions of FMLP-induced action were measured in cells wherein ADP-ribosylation of G12/13 ranged from 85 to 99%.

Effects of Pertussis Toxin on Basal, P2-purinergic, and FMLP-activated GTP-dependent Inositol Polyphosphate Formation in Isolated HL-60 Cell Membranes—In previous studies of GTP-dependent phospholipase C activity in membranes from neutrophils and differentiated HL-60 cells, ATP was routinely present in either the membrane isolation media or in the incubation media used for the phospholipase assays (1–
FIG. 4. UTP- and FMLP-activated inositol polyphosphate accumulation in differentiated HL-60 granulocytes: effects of pretreatment of intact cells with varying concentrations of pertussis toxin (PTx). Parallel samples of [3H]inositol-labeled HL-60 granulocytes were pretreated with the indicated concentrations of pertussis toxin for 3 or 15 h. The accumulation of [3H]IP plus IP; in response to a 15-s incubation with either 100 μM UTP (panel A) or 10 μM FMLP (panel B) was quantified as described under “Experimental Procedures.” Solid blocks indicate the basal levels of inositol polyphosphate; open blocks show the agonist-induced increments. Values represent the average ± range of the data from two separate experiments. Each incubation condition was assayed in duplicate in the individual experiments.

FIG. 5. Differential inhibitory effects of pertussis toxin (PTx) treatment on FMLP- and UTP-induced potentiation of GTP-dependent inositol polyphosphate accumulation in membranes isolated from HL-60 granulocytes: effects on the kinetics of accumulation. Membranes isolated from [3H]inositol-labeled HL-60 granulocytes were incubated for the indicated times at 37 °C in basic assay medium (described under “Experimental Procedures”) containing 350 nM free Ca2+ (first panel from left). Incubation media additionally contained 1 μM GTP (second panel), 1 μM GTP plus 30 μM UTP (third panel), or 1 μM GTP plus 10 μM FMLP (fourth panel). Circles (●) represent data from membranes of control cells; squares (■) represent data from membranes of cells treated for 3 h with 500 ng/ml pertussis toxin. Accumulation of [3H]inositol polyphosphates was quantified as described under “Experimental Procedures.” Data points represent the average of duplicate determinations from a single experiment. These data are representative of three separate experiments.

FIG. 6. Differential inhibitory effects of pertussis toxin (PTx) treatment on FMLP- and UTP-induced potentiation of GTP-dependent inositol polyphosphate accumulation in membranes isolated from HL-60 granulocytes: dose-response relationships. Membranes isolated from [3H]inositol-labeled HL-60 granulocytes were incubated for 9 min at 37 °C in basic assay medium (described under “Experimental Procedures”) containing 350 nM free Ca2+ and 1 μM GTP. Incubation media additionally contained the indicated concentrations of UTP (○, left panel) or FMLP (□, right panel). Filled symbols (●) represent data from membranes of control cells; open symbols (○) represent data from membranes of cells treated for 4 h with 1 μg/ml pertussis. Accumulation of [3H]inositol polyphosphates was quantified as described under “Experimental Procedures.” Data points represent the average ± range of duplicate determinations from a single experiment. These data are representative of three separate experiments.

FMLP-stimulated inositol polyphosphate accumulation in membranes isolated from either control cells or pertussis toxin-treated (500–2000 ng/ml, 3–4 h) cells. In control membranes, 1 μM GTP alone elicited a mean 5.4-fold increase in the rate of inositol polyphosphate accumulation measured over 1–4-min incubation periods (Fig. 5 and Table I). The inclusion of either 30 μM UTP or 10 μM FMLP produced an additional 1.5–2.5-fold increase in the rate of this GTP-dependent phospholipase C activity. We have verified previously that this effect of UTP (or other P2-purinergic agonists) is not due to elevation of membrane polyphosphoinositide levels or to inhibition of GTP catabolism by membrane-associated phosphatases/nucleotidases (14). The threshold for UTP action was approximately 100 nM, the EC50 was about 1.8 μM, and near maximal activation was obtained with 30–100 μM. These values are similar to those characterizing the effects of UTP on intact cells (Fig. 1). Conversely, the FMLP dose-response relationship in control membranes was markedly different from that observed in intact cells. The threshold was 30 nM whereas the EC50 was at least 300 nM; a plateau in activation was not observed even with 10 μM FMLP. The data presented in Fig. 6 and Table I also demonstrate that efficacy of FMLP (mean 1.8-fold stimulation) was invariably less than that of UTP (mean 2.5-fold stimulation) in this in vitro phospholipase C assay system.

Parallel studies performed on membranes isolated from cells pretreated with pertussis toxin revealed several differences. First, as shown in Figs. 5 and 6 and Table I, the “basal” rate of GTP-dependent inositol polyphosphate formation (i.e., measured in the absence of receptor agonists) was significantly inhibited (mean inhibition = 72%) in the toxin-treated membranes. It should be emphasized that pertussis toxin treatment did not alter the levels of the various membrane [3H]inositol phospholipids (data not shown); nor did it significantly alter activation of the membrane-associated phospho-
lipase C by 1 mM free Ca^{2+} (Table I). As reported in previous studies (1–3, 9), the ability of FMLP to potentiate GTP-dependent inositol phosphate accumulation was markedly reduced in membranes prepared from cells that had been pretreated with pertussis toxin (Figs. 5 and 6 and Table I). Time course studies (Fig. 5) revealed that both the total extent of (FMLP + GTP)-stimulated inositol phosphate accumulation and the differential ((FMLP + GTP) - GTP) rate of accumulation were markedly depressed in the membranes from the toxin-treated cells. In contrast, although the total extent of UTP-stimulated GTP-dependent inositol phosphate accumulation was modestly depressed in the same toxin-treated membranes, the differential ((UTP + GTP) - GTP) rate of agonist-induced accumulation was not inhibited significantly (Fig. 5). The dose-response relationships (Fig. 6) characterizing the effects of FMLP and UTP underscored further the differential abilities of these agonists to potentiate GTP-dependent phospholipase C activity in membranes from toxin-treated cells. Both the potency and efficacy of FMLP were significantly reduced (Fig. 6B). Conversely, the UTP dose-response curve for the toxin-treated membranes paralleled that for the control membranes (Fig. 6A). The threshold (<300 nM UTP) and EC_{50} (approximately 1.8 μM UTP) for UTP stimulation of GTP-dependent activity were identical in membranes prepared from control or pertussis toxin-treated cells; the efficacy of UTP stimulation was reduced by only 15–20% in the toxin-treated membranes.

As summarized in Table I, toxin-treated membranes were characterized by a mean 72% decrease in the GTP-dependent activity measured in the absence of receptor agonists. Likewise, there was a mean 71% decrease in the total FMLP + GTP-stimulated inositol phosphate accumulation. Conversely, the total phospholipase C activity stimulated by UTP + GTP was inhibited by only 36%. This difference in the inhibition of inositol phosphate release elicited by the two receptor agonists was even more striking when the differential ((agonist + GTP) - GTP) activities were compared. The additional GTP-dependent activity observed in the presence of UTP was inhibited by only 14% compared with the 82% reduction in FMLP-induced action.

The above data were obtained using membranes prepared from cells treated for 3–4 h with 500 ng/ml pertussis toxin. As noted previously, such membranes retain only 10–15% of the normal content of nonribosylated G_{i,213}. It is possible, however, that in the presence of high numbers of occupied P_{2}-purinergic receptors, this residual amount of unmodified G-protein was sufficient to mediate the appreciable stimulation of GTP-dependent phospholipase C observed in the membrane experiments. To test this possibility, similar experiments were performed using membranes isolated from cells treated with pertussis toxin for 15 h. In the experiment illustrated in Fig. 7A, such membranes contained only 1% of the normal content of unmodified G_{i,213} as compared with 11% content measured in a membrane derived from a parallel culture of cells treated for 4 h (Fig. 7A). Despite the differences in unmodified G_{i,213} content, essentially similar patterns of altered phospholipase C regulation were observed. In both sets of toxin-treated membranes, the FMLP-induced potentiation of GTP-dependent activity was reduced by 65–70% whereas the potentiation elicited by UTP was decreased by only 10–12%. In both types of toxin-treated membranes, the GTP-dependent activity measured in the absence of receptor agonists was decreased by 50–65%. Likewise, the Ca^{2+}-stimulated phospholipase C activities measured in both sets of toxin-treated membranes were not significantly different from those observed in control membranes. The only functional difference noted in membranes from cells treated with toxin for prolonged times was a 38 ± 7% (n = 4) reduction in the maximal GTP_{7S}-stimulated phospholipase C activity; a much smaller 12 ± 7% inhibition (n = 5) in this parameter was observed when membranes were prepared from cells treated with toxin for 3–4 h. This latter observation suggests that prolonged exposure to pertussis toxin may down-regulate the total amount of G-protein(s) that is functionally coupled to phospholipase C effector enzymes.

**Additive Stimulation of GTP-dependent Phospholipase C Activity in HL-60 Membranes by P_{2}-purinergic Agonists and Formylated Peptides**—As described in the previous experiments, membranes prepared from pertussis toxin-pretreated cells exhibited significantly (70%) reduced rates of GTP-dependent inositol phosphate release in the absence of receptor agonists. Other investigators have reported that basal GTP-dependent activation of phospholipase C is unaltered in membranes prepared from neutrophils (9, 3) and differentiated HL-60 cells pretreated with pertussis toxin (1, 4, 9). However, the membranes used in these studies were prepared and stored in the presence of millimolar ATP. Thus, the subsequently assayed phospholipase activity was measured in the presence of 0.05 mM ATP. Under these conditions, the basal GTP-stimulated inositol phosphate formation re-
purinergic agonists (either 1 mM ATP or 30 PM UTP) induced this is significant given the relative pertussis toxin insensitivity of P2-purinergic-induced phospholipase C activation of (FMLP + GTP)-induced activity were actually measured. Similarly, the measurements reported in such studies was actually a measurement of (GTP + ATP)-stimulated phospholipase C activity due to the unintentional occupation of P2-purinergic receptors. This is significant given the growing number of cell types in which extracellular ATP has been reported to activate transmembrane signaling processes known (or hypothesized) to involve mediation by GTP-binding regulatory proteins.

Functional effects of pertussis toxin (PTX) treatment on the regulation of inositol polyphosphate accumulation in membranes isolated from HL-60 granulocytes: comparative effects in membranes containing 10% versus 1% of the normal content of unribosylated G_{i,z,s}. Parallel samples of unlabeled and \[^{3}H\]inositol-labeled HL-60 granulocytes were pretreated with 1 µg/ml pertussis toxin for either 4 h (panel A) or 15 h (panel B). Membranes were prepared as described previously. Unlabeled membranes were then assayed in vitro for pertussis toxin-catalyzed \[^{32}P\]ADP-ribosylation of G_{i,z,s} (40-41-kDa substrate) as described in Fig. 3. Lanes 1, 2, and 3 of each autoradiogram illustrate \[^{32}P\] incorporation in control membranes measured at 5, 10, and 20 min, respectively. Lanes 4, 5, and 6 represent the corresponding time points in membranes isolated from toxin-treated cells. H-Labeled membranes were assayed for inositol polyphosphate release during 1-min incubations (57 °C) in basic assay medium (350 nM free Ca\(^{2+}\)) containing either 1 µM GTP, 1 µM GTP plus 30 µM UTP, 1 µM ATP plus 10 µM FMLP, 10 µM GTP-S, or 1 mM free Ca\(^{2+}\). Open bars (□) represent data from control membranes; cross-hatched bars (■) represent data from pertussis toxin-treated membranes. Inositol polyphosphate accumulation has been normalized relative to the total content of \[^{3}H\]inositol phospholipid in each membrane preparation. Data points show the average ± range of duplicate measurements from single experiments. Each experiment is representative of results obtained from two separate experiments.

**FIG. 7.** Effects of pertussis toxin (PTX) treatment on the regulation of inositol polyphosphate accumulation in membranes isolated from HL-60 granulocytes: comparative effects in membranes containing 10% versus 1% of the normal content of unribosylated G_{i,z,s}. Parallel samples of unlabeled and \[^{3}H\]inositol-labeled HL-60 granulocytes were pretreated with 1 µg/ml pertussis toxin for either 4 h (panel A) or 15 h (panel B). Membranes were prepared as described previously. Unlabeled membranes were then assayed in vitro for pertussis toxin-catalyzed \[^{32}P\]ADP-ribosylation of G_{i,z,s} (40-41-kDa substrate) as described in Fig. 3. Lanes 1, 2, and 3 of each autoradiogram illustrate \[^{32}P\] incorporation in control membranes measured at 5, 10, and 20 min, respectively. Lanes 4, 5, and 6 represent the corresponding time points in membranes isolated from toxin-treated cells. H-Labeled membranes were assayed for inositol polyphosphate release during 1-min incubations (57 °C) in basic assay medium (350 nM free Ca\(^{2+}\)) containing either 1 µM GTP, 1 µM GTP plus 30 µM UTP, 1 µM ATP plus 10 µM FMLP, 10 µM GTP-S, or 1 mM free Ca\(^{2+}\). Open bars (□) represent data from control membranes; cross-hatched bars (■) represent data from pertussis toxin-treated membranes. Inositol polyphosphate accumulation has been normalized relative to the total content of \[^{3}H\]inositol phospholipid in each membrane preparation. Data points show the average ± range of duplicate measurements from single experiments. Each experiment is representative of results obtained from two separate experiments.

**DISCUSSION**

These studies provide several observations pertinent to understanding the role of GTP-binding regulatory proteins in the regulation of inositol phospholipid-specific phospholipase C effector enzymes in phagocytic leukocytes. Treatment of HL-60 granulocytes with pertussis toxin, under the conditions (500-2000 ng/ml; 3-4 h) routinely employed in our studies and those of other investigators (1-3, 9-11, 17), induced ADP-ribosylation of 85-90% of the major 40-41-kDa toxin subunit present in isolated HL-60 cell membranes (Fig. 3). Given the results of immunochemical measurements of G-protein expression in HL-60 cells (6, 7), this substrate presumably includes both the 40-kDa subunit of G_{i,z} and the 41-kDa \(\alpha\)-subunit of G_{i,z}. FMLP- and P2-purinergic-induced activation of phospholipase C in such toxin-treated cells was inhibited by 91 and 78%, respectively (Fig. 1). The magnitudes of these inhibitory effects suggest strongly that a substantial fraction of the particular G-protein species (G_{i,z}, G_{i,x}, or both) that interact functionally with these receptors has been modified by the toxin treatment. However, as noted in our previous studies, there is a small but significant quantitative difference in the efficiencies that characterize P2-purinergic- and FMLP-stimulated inositol polyphosphate accumulation in these toxin-treated cells. This residual phospholipase C activation is sufficient to produce a rapid 2-fold increase in IP3 levels. In turn, this elevation in IP3 produces near normal mobilization of Ca\(^{2+}\) stores by maximally active concentrations of UTP or ATP (data not shown).

Since 10-15% of the G_{i,z} pool in HL-60 cells treated with toxin for 3-4 h was not ADP-ribosylated (Fig. 3), it may be argued that additional blockade of P2-purinergic-induced sig-
TABLE II  
Pertussis Toxin Effects on GTP-dependent Phospholipase C Activity

| Agonist          | IP$_2$ + IP$_3$ Release Control | PTX | Change  | % |
|------------------|----------------------------------|-----|---------|---|
| Exp. 1           |                                  |     |         |   |
| None             | 104                              | 132 |         | -79|
| GTP              | 892                              | 296 |         |   |
| GTP/FMLP         | 2228                             | 512 |         | -82|
| GTP/ATP          | 2284                             | 1736|         | -26|
| GTP/ATP/FMLP     | 3116                             | 1984|         | -38|
| Exp. 2           |                                  |     |         |   |
| None             | 128                              | 108 |         | -18|
| GTP              | 440                              | 216 |         | -52|
| GTP/FMLP         | 904                              | 272 |         | -69|
| GTP/ATP          | 1332                             | 872 |         | -36|
| GTP/ATP/FMLP     | 1700                             | 1064|         | -40|
| GTP/UTP          | 1320                             | 844 |         | -39|
| GTP/UTP/FMLP     | 1988                             | 1224|         | -40|

The absence (control) or presence (PTX) of pertussis toxin for 3 h (500 ng/ml for Experiment 1; 400 ng/ml for Experiment 2). Membranes isolated from these cells were then incubated for 1 min (Experiment 2) or 2 min (Experiment 1) at 37 °C in the basic assay medium (described under ‘Experimental Procedures’) containing either 350 nM free Ca$^{2+}$ or 1 mM free Ca$^{2+}$. Parallel samples also contained (in addition to 350 nM free Ca$^{2+}$) no agonist (none); 1 μM GTP (GTP); 1 μM GTP plus 10 μM FMLP (GTP/FMLP); 1 μM GTP plus 1 μM Mg-ATP (GTP/ATP); 1 μM GTP plus 1 mM Mg-ATP plus 10 μM GTP (GTP/ATP/FMLP); 1 μM GTP plus 30 μM UTP (GTP/UTP); or 1 μM GTP plus 30 μM UTP plus 10 μM FMLP (GTP/UTP/FMLP). Accumulation of [3H]inositol phosphates was quantified as described under ‘Experimental Procedures.’ Each data point represents an average of duplicate determinations. Percentage changes in total activity are defined as 1 - [(agonist - none) / (agonist - none)$_{control}$].

Pertussis toxin treatment appears to attenuate some step in GTP-dependent inhibition of adenylate cyclase in S49 cyc- membranes. Thus, ADP-ribosylation of the relevant Gi-protein(s) also slows the GTP-dependent cycle of G-protein activation in the absence of agonist-occupied receptors. Similar inhibition has not been reported previously in studies of GTP-dependent phospholipase C activity in membranes derived from differentiated HL-60 cells or neutrophils (1-4). However, it is consistent with results from a recent study by McLeish et al. (32) showing that membranes isolated from pertussis toxin-treated HL-60 granulocytes exhibit a 65-70% reduction in the rate of high affinity GTPase and the rate of high affinity GTP binding measured in the absence of agonists. Similar findings have been reported in studies of neutrophil membranes (33-35).

This observation suggests strongly that P$_2$-purinergic receptors might activate inositol phospholipid phospholipase C effector enzymes by mechanisms other than the primary pathway presumably mediated by unmodified Gi$_{12/3}$. In this regard, several studies have indicated that P$_2$-purinergic receptors can activate inositol phospholipid phospholipase C effector enzymes via both pertussis toxin-sensitive and -insensitive mechanisms (24-30). This differential toxin sensitivity can be explained in part by cell-specific expression of P$_2$-purinergic receptor subtypes with nucleotide selectivities different from those observed in HL-60 cells and neutrophils (24-28). However, receptors with a nucleotide selectivity identical to that observed in HL-60 cells have also been described in ovine and primate pituitary cells (29) and human fibroblasts (30). In the former cell types, activation of inositol phospholipase accumulation was completely insensitive to pertussis toxin treatment. In the toxin-treated fibroblasts, as in HL-60 cells, the effects of extracellular nucleotides were partially inhibited. The ability of a single G-protein-coupled receptor subtype to utilize multiple G-proteins (pertussis toxin sensitive and insensitive) for the activation of phospholipase C effectors has also been suggested by Askren et al. (31) who utilized Chinese hamster ovary cells stably transfected with DNA encoding distinct muscarinic receptor subtypes. Similar mechanisms may underlie the differential toxin sensitivity of P$_2$-purinergic versus FMLP-stimulated inositol phospholipid hydrolysis in HL-60 cells.
investigators observed no effects of ADP-ribosylation on GTPase activity or GDP release per se. They hypothesized that the toxin-induced inhibition of agonist-independent GTP-dependent signaling events observed in native cytosolic membranes may reflect G-protein activation by unoccupied receptors. Similar interaction of unoccupied receptors with G\(_{i1,2,3}\) in HL-60 membranes may explain the high rates of GTP-activated inositol polyphosphate release observed in the absence of added FMLP or P\(_2\)-purinergic agonist.

The absolute rate and extent of inositol polyphosphate release induced by (UTP + GTP) in toxin-treated membranes was reduced by about 20–35% relative to that observed in control membranes (Figs. 5–7; Tables I and II). However, we were surprised to observe that the ability of UTP (or ATP) to potentiate GTP-dependent phospholipase C activity was very similar in isolated membranes prepared from either control or pertussis toxin-treated cells. Thus, the dose-response curve characterizing UTP-induced activity in pertussis toxin-treated membranes, while negatively shifted along the y axis, paralleled that observed in control membranes. If it is assumed that P\(_2\)-purinergic receptors activate phospholipase C exclusively through the mediation of a toxin-sensitive G-protein(s) presumably G\(_{i1,2,3}\) or G\(_{o}\), our results would suggest that ADP-riboylation does not preclude interaction of this G-protein with occupied P\(_2\)-purinergic receptors. This interpretation would suggest that P\(_2\)-purinergic receptors, but not FMLP receptors, contain structural features that facilitate some degree of interaction with the ADP-riboylated \(\alpha\)-subunits of G\(_{i1,2,3}\) proteins. Alternatively, as discussed previously, P\(_2\)-purinergic receptors of the subtype expressed in HL-60 cells and neutrophils may have the capacity to activate phospholipase C effector enzymes via the mediation of a G-protein(s) that is not a pertussis toxin substrate.

It is also noteworthy that P\(_2\)-purinergic-induced activation of inositol phospholipid breakdown in intact HL-60 cells was largely (60%) sensitive to inhibition by pertussis toxin treatment whereas in HL-60 membranes this same parameter was largely insensitive to toxin treatment. Conversely, in both intact cells (Fig. 1) and membranes (Figs. 5–7 and Table I), FMLP-stimulated phospholipase C activity was predominately (70–90%) sensitive to toxin treatment. It should also be noted that although both receptor agonists were equally efficacious in stimulating inositol polyphosphate accumulation in intact cells, FMLP was invariably less efficacious than UTP-induced inositol phosphate accumulation observed in intact cells, one can speculate additionally that P\(_2\)-purinergic receptors, acting via G\(_{i1,2,3}\), also activate this phospholipase subtype. However, our results are consistent with the possibility that these receptors can also activate, via a pertussis toxin-insensitive mechanism, a distinct phospholipase C species that has relatively high avidity for the membrane. This putative P\(_2\)-purinergic-activated signaling pathway may predominate in studies of isolated membranes.

McLeish et al. (32) have suggested recently that differences in the activation of G\(_{i1,2,3}\) (or additional G-proteins) by FMLP receptors and leukotriene B\(_4\) receptors may be responsible for differential activation of various neutrophil responses by these agonists. Similarly, FMLP but not ATP (or UTP) is a much more efficacious stimulus for superoxide production and primary granule secretion in neutrophils and HL-60 granulocytes (15–17). The differential activation of G-protein-regulated phospholipase signaling pathways may explain in part the disparate effects of these two species of Ca\(^{2+}\)-mobilizing agonists on the function of phagocytic leukocytes.

REFERENCES

1. Anthes, J. C., Billah, M. M., Cali, A., Egan, R. W., and Siegel, M. I. (1987) Biochim. Biophys. Acta 1053, 195–200
2. Anthes, J. C., Billah, M. M., Cali, A., Egan, R. W., and Siegel, M. I. (1987) Biochim. Biophys. Acta 1053, 195–200
3. Anthes, J. C., Billah, M. M., Cali, A., Egan, R. W., and Siegel, M. I. (1987) Biochim. Biophys. Acta 1053, 195–200
4. Anthes, J. C., Billah, M. M., Cali, A., Egan, R. W., and Siegel, M. I. (1987) Biochim. Biophys. Acta 1053, 195–200
5. Anthes, J. C., Billah, M. M., Cali, A., Egan, R. W., and Siegel, M. I. (1987) Biochim. Biophys. Acta 1053, 195–200
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D S Cowen, B Baker and G R Dubyak

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