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Studies of human peripheral blood neutrophils (PMNs) demonstrated that botulinum neurotoxin D (BT-D) ADP-ribosylates a 22-kDa PMN G protein (G_{22k}) and inhibits the exocytosis of both specific and azurophilic granules stimulated by FMLP. Furthermore, this inhibition of PMN exocytosis by BT-D was found to be correlated with the degree of irreversible ADP-ribosylation of G_{22k} by BT-D and to require modification of at least 85% of PMN G_{22k} before significant inhibition of secretion is observed. Although both pertussis toxin and BT-D inhibited exocytosis in FMLP-stimulated PMNs, the inhibitory effects of the two toxins were found to be additive. Pertussis toxin and BT-D also inhibited Ca^{2+}/GTP/GTPγS-induced secretion in digitonin-permeabilized PMNs, but there were distinct differences between the inhibitory effects of the two toxins. In contrast to BT-D, the exotoxin botulinum C3 was found to ADP-ribosylate primarily a 24- to 25-kDa PMN protein, and it was not found to inhibit Ca^{2+}- and GTP-induced secretion in permeabilized PMNs. Ultrastructural studies of BT-D-treated PMNs showed an accumulation of distinct membrane-bound organelles in the periphery of the cells after FMLP stimulation, suggestive of a toxin-induced block in organelle-plasma membrane fusion. Taken together, these findings indicate that BT-D-sensitive G_{22k} has a functional role in stimulated exocytosis of PMNs. *Journal of Immunology*, 1994, 152: 1370.

Phagocytic cells, such as PMNs,[3] synthesize proteins during maturation in the bone marrow (1), and store them in cytoplasmic granules (2) until the cells are appropriately stimulated to release the contents of these granules into an endocytic vacuole, e.g., during phagocytosis of bacteria (2, 3), or to the exterior of the cell after fusion with the plasma membrane, e.g., during exocytosis (4, 5). Exocytosis of PMN granules is a highly regulated event that occurs within seconds of cell activation by soluble or particulate stimuli (2–4). As in most secretory cells (4, 5), an increase in the intracellular concentration of free Ca^{2+} has been shown to be a second messenger for PMN exocytosis (6, 7), although experimental evidence indicates that the two major granule types of mature neutrophils, the specific (or "secondary") and azurophilic (or "primary") granules (1), are differentially regulated by calcium during both exocytosis and intracellular degranulation (7–9). Other molecular components involved in stimulus-response coupling for PMN exocytosis and degranulation have been less well characterized.

In recent years, efforts to elucidate the biochemical and molecular mechanisms involved in cellular membrane traffic and organelle transport have focused both on ras-related low m.w. GTP-binding proteins (i.e., small G proteins or GTPases) with molecular masses between 20 and 30 kDa (10–12) and on the larger, heterotrimeric G proteins that have been linked to receptor protein complexes and receptor-mediated signal transduction (13, 14). The most definitive evidence for a functional role for small G proteins in intracellular membrane and organelle transport comes from genetic studies in yeast, in which mutations in the *sec4* or *ypt1* genes, which encode ras-related small G proteins, were found to inhibit fusion of secretory vesicles with the plasma membrane resulting in secretory defects (15, 16). Although a precise role for small G proteins in the secretory functions of higher eukaryotic cells has not been defined, there is substantial experimental evidence, obtained primarily from studies conducted with cell-free reconstituted systems, that membrane-flow and membrane-fusion events during constitutive and regulated secretion are

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[3] Abbreviations used in this paper: PMN, peripheral blood polymorphonuclear leukocyte; PT, pertussis toxin; BT-D, botulinum neurotoxin D; BT-C 3, botulinum toxin C 3; PB, permeabilization buffer; Vit B_{12}-BP, vitamin B_{12}-binding protein.

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regulated in part by the cycling of small G proteins between a membrane-bound and free state (17–19).

PMNs are the principal effector cells in host defenses against bacterial and fungal infection and are capable of responding to a wide variety of molecular signals that trigger functional responses (20). Among these functional responses, secretory events are critical to the microbicidal and inflammatory activity of these cells (2, 3, 6). Studies conducted in several different laboratories have shown that PMN chemoattractant receptors are coupled to a PT-sensitive heterotrimeric G protein (20), and PT-induced inhibition of PMN inflammatory responses stimulated by the peptide chemoattractant FMLP has provided valuable insights into the transduction of signals via these receptors (20). However, PT-insensitive G proteins have also been described in PMNs (21), and there is evidence for a regulatory role of these G proteins in signal-response coupling in PMNs (22).

Recently, BT-D was reported to ADP-ribosylate a ras-related 22-kDa G protein, both in rabbit peritoneal neutrophils (23) and in human PMNs (24). Botulinum neurotoxins are known to block neurotransmitter release at peripheral cholinergic synapses producing muscular weakness and paralysis (25), and BT-D has also been shown to inhibit secretory responses in bovine adrenal medullary cells at a step distal to the stimulus-induced increases in intracellular Ca^{2+} (26). Hence, it was of interest to us to examine the effects of BT-D on stimulated exocytosis of human PMNs. Our interest in the possibility that BT-sensitive, ras-related, G proteins might have a role in stimulated PMN exocytosis was also prompted by the observation that guanine nucleotides stimulate exocytotic secretion in the absence of Ca^{2+} in a variety of permeabilized secretory cells (27–29) including PMNs (30).

Results of our present studies indicate that BT-D ADP-ribosylates a 22-kDa G protein (G_{22k}) in human PMNs and significantly inhibits exocytosis of both specific and azurophilic granules of PMNs stimulated by the peptide chemoattractant FMLP. In addition, BT-D is shown to significantly inhibit the secretory responses of digitonin-permeabilized PMNs, when stimulated with Ca^{2+}, Ca^{2+} + GTP, and/or GTPγS alone. These studies also demonstrate a correlation between the degree of irreversible ADP-ribosylation of G_{22k} and inhibition of PMN exocytosis by BT-D. Ultrastructural changes in BT-D-treated PMNs, which are consistent with an inhibitory effect of BT-D on PMN exocytosis, are also defined. These studies contrast the effects of BT-D with those of the exoenzyme BT-C3 (31), which primarily ADP-ribosylates 24- to 25-kDa PMN proteins that appear to be distinct from the 22-kDa PMN G protein modified by BT-D. Unlike BT-D, the exoenzyme BT-C3 does not inhibit Ca^{2+} and/or GTP-induced exocytosis of PMN granules in digitonin-permeabilized cells.

Experimental Procedures

Preparation of human PMNs

PMNs were isolated from freshly drawn venous blood of healthy volunteers by centrifugation through Ficoll-Hypaque followed by dextran sedimentation (32). Residual erythrocytes were removed by hypotonic lysis, and >95% pure PMNs were resuspended in HBSS without Ca^{2+} and Mg^{2+}, pH 7.4.

Toxin treatment of PMNs

In our preliminary studies with BT-D, we used incubation conditions described for previous studies with rabbit neutrophils (23). However, when freshly prepared human PMNs were incubated for 60 min at 37°C with 500 ng/ml of BT-D (Wako Chemicals, Richmond, VA), we did not observe any inhibition of FMLP-induced functional responses after the toxin treatment. In subsequent studies when PMNs at 5×10^6/ml were incubated with higher concentrations (1–5 μg/ml) of BT-D for 90 to 120 min at 37°C, significant inhibitory effects of the toxin were observed. Therefore, for all studies described here (except where indicated otherwise, as in Fig. 4), PMNs at 5×10^6/ml in HBSS without Ca^{2+} and Mg^{2+}, were incubated for 120 min at 37°C, in the absence or presence of 1 to 5 μg/ml of BT-D. After the incubation, both control and toxin-treated PMNs were centrifuged at 1000 × g and then resuspended in HBSS containing Ca^{2+} and Mg^{2+} for assessment of FMLP-induced exocytosis in intact PMNs, or, in a PB containing 100 mM KCl, 20 mM NaCl, 1 mM EGTA, 30 mM HEPES, at pH 7.0, for assessment of Ca^{2+}/GTP/GTPγS-induced secretion after digitonin permeabilization of the cells (33). The BT-D stock solution (1.0 mg/ml, in 0.2 M NaCl with 0.05 M sodium acetate, pH 6.5) was used in 1:200 to 1:2000 dilutions to achieve the required toxin concentrations used in this study, and that did not affect cell viability as assessed by trypan blue exclusion and lactic dehydrogenase release.

Digitonin permeabilization of PMNs

Permeabilization of PMNs was achieved by a slight modification of a procedure described by Smolen et al. (33). Briefly, PMNs at 25×10^6/ml in PB were prewarmed to 37°C and digitonin was added at 10 μg/ml, from a freshly prepared stock solution of 1.0 mg/ml (maintained at 37°C). Permeabilization was performed for 15 min at 37°C, and the PMNs were then immediately diluted to 5×10^6/ml with PB. As reported by Smolen et al. (33), these digitonin-permeabilized cells maintained Ca^{2+}-induced secretory responses of both granule types, even after the release of 30% to 40% of cytoplasmic lactic dehydrogenase during permeabilization. Consistent with the previous report (33), extracellular release of β-glucuronidase (a marker for azurophilic granule content) and lactoferrin or Vit B_{12}-BP (markers for specific granule contents) in Ca^{2+}-free PB was <5% of the total, during 15 min incubation with 10 μg/ml digitonin (data not shown). In our hands, incubation with 10 μg/ml digitonin for 15 min at 37°C was found to be the most optimal permeabilization condition for studying Ca^{2+}-induced exocytosis in PMNs.

FMLP-induced secretion in intact PMNs

For studies of FMLP-induced exocytosis of PMN granules, PMNs at 5×10^6/ml in HBSS containing Ca^{2+} and Mg^{2+} were warmed to 37°C in round-bottom 12 × 75 mm polypropylene tubes, and the PMNs were treated with 5 μg/ml cytochalsin B for 10 min at 37°C. Secretion was initiated by the addition of 10^{-6} M FMLP, and the PMNs were incubated for additional 10 min at 37°C. The cells were then chilled on ice and KCl was added to a final concentration of 0.1 M to minimize adsorption of exocytosed granule proteins. The samples were then transferred to Eppendorf centrifuge tubes and centrifuged at 4°C for 2 min at 10,000 × g, in an Eppendorf centrifuge. Supernatants were carefully aspirated, placed on ice, and immediately analyzed for β-glucuronidase and Vit B_{12}-BP. The remainder of the samples were stored overnight at 4°C before measurement of lactoferrin by ELISA (34). Total cellular contents of the various granule marker proteins were measured in parallel from an identical PMN sample in HBSS, after disruption by sonication in the presence of 1% Triton X-100. Furthermore, in each experiment, appropriate controls with buffer, cytochalasin B, or FMLP alone, were assessed routinely for any exocytosis of PMN granules that could have occurred under

nonstimulatory conditions. In studies conducted with toxin-treated PMNs, both control and experimental samples were subjected to identical incubation conditions in the presence or absence of the toxin(s), before FMLP-induced secretion was studied as described above.

Ca^{2+}-induced exocytosis in digitonin-permeabilized PMNs

Digitonin-permeabilized PMNs at 5×10^6/ml in a Ca^{2+}-free PB were warmed to 37°C and secretion was induced by addition of 1.5 mM Ca^{2+}. Where indicated, GTP or GTPγS was added at 0.1 mM. The concentration of Ca^{2+} required for optimal secretory responses was determined from a series of preliminary dose-response studies. It is noteworthy that under our experimental conditions, preexisting intracellular free Ca^{2+}, $[Ca^{2+}]_i$, was not detectable in these PMNs when measured by a sensitive indo-1 method. $[Ca^{2+}]_i$ measurements were also made after the addition of extracellular Ca^{2+} to digitonin-permeabilized PMNs in PB (containing 1 mM EGTA). Addition of 1.5 mM Ca^{2+}, which triggered optimal responses in the secretion studies, resulted in a $[Ca^{2+}]_i$ of about 1.30 μM. It is worthwhile to mention here that although Mg/ATP has been reported to enhance stimulated exocytosis in permeabilized PMNs, we did not observe any significant effect of Mg/ATP on Ca^{2+}-induced secretory responses of digitonin-permeabilized PMNs in our preliminary studies. In view of this observation, Mg/ATP was not added to the external medium during induction of Ca^{2+}-induced exocytosis in digitonin-permeabilized PMNs.

ADP-ribosylation with botulinum toxin D or C3

Botulinum toxin-mediated ADP-ribosylation of PMN G proteins was performed according to procedures described previously (24). PMNs were sonicated in a medium containing 0.1 M Tris-buffered sucrose, pH 7.2, 2 mM EDTA, and 1 mM DTT, in the presence of a mixture of proteolytic inhibitors (containing 1 μg/ml each of PMSF, aprotinin, and leupeptin) at 4°C and centrifuged at 1000 × *g* for 5 min to remove unbroken cells. For the enzyme assay, protein concentration was adjusted so as to add 1 to 2 mg PMN protein per ml of the reaction mixture. ADP-ribosylation was conducted in a final reaction volume of 100 μl, containing 100 mM Tris · HCl, pH 7.4, 1 mM EDTA, 10 mM thymidine, 5 mM $MgCl_2$, 0.5 mM ATP, 1 μM ^{32}P-labeled nicotinamide adenine dinucleotide (1–2 μCi) and 1 to 2 mg/ml PMN protein. BT-D (Wako Chemicals) was activated with 20 mM DTT for 10 min at 30°C, and was added at 25 μg/ml to initiate ADP-ribosylation (23). This concentration of the toxin was chosen from previously reported values (23). When ADP-ribosylation was conducted with exoenzyme BT-C3, 0.1 μg/ml of a partially purified preparation of C3 (a generous gift of Dr. Leonard Smith, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD) was used (34). All other experimental conditions were identical. The [^{32}P]ADP-ribosylation of PMN G proteins by BT-D and BT-C3, as shown in Figure 1, *A* and *B*, were conducted with two different PMN sonicates, in parallel studies. The reaction was performed for 60 min at 37°C and was stopped with 10% ice-cold TCA (final concentration). Samples were chilled on ice for an additional 15 min, and the protein precipitates were collected by centrifugation and washed two more times with ice-cold 10% TCA. The precipitated proteins were finally washed with ether and the samples were immediately processed for SDS-PAGE on 11% to 23% gradient gels (Amersham, Phorcast). The gels were dried under high vacuum and the [^{32}P]ADP-ribosylated protein bands were detected by autoradiography, using Kodak X-Omat films.

Assay of PMN granule marker enzymes

In our present studies, β-glucuronidase was used as a marker for the azurophilic granules and Vit B_{12}-BP and lactoferrin were used as markers for specific granule contents (1–3). All assays were conducted in duplicate using standard procedures, the details of which have been described (35). β-Glucuronidase was assayed by the phenolphthalein-glucuronic acid method and spectrophotometrically quantitated at 540 nm. Vit B_{12}-BP was measured by the charcoal binding assay using cyanocobalamin ^{57}Co, and lactoferrin was quantitated by ELISA using a purified human lactoferrin (Cappel) as the standard (35). The *p* values were calculated by Student's *t* test.

Electron microscopy

For electron microscopic analysis of toxin-treated and untreated PMNs, the cells were subjected to identical experimental conditions as described for studying FMLP-stimulated exocytosis (see above). For electron microscopy, 1 to 2×10^7 PMNs were used for each experimental condition, and PMNs were incubated in HBSS without Ca^{2+} and Mg^{2+}, in the presence or absence of 5 μg/ml of BT-D, for 120 min at 37°C. PMNs were then centrifuged and resuspended in HBSS containing Ca^{2+} and Mg^{2+} with PMN concentration at 5×10^6/ml. FMLP-stimulated exocytosis was induced in toxin-treated and untreated PMNs after cytochalasin b treatment, as described for Figures 1 and 2. The PMNs were centrifuged at 1000 × *g* for 10 min and fixed for 1 h in a mixture of 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The fixed PMNs were kept overnight in 0.1 M cacodylate buffer (pH 7.4) at 4°C, and postfixed in 1% osmium tetroxide for 1 h at room temperature. The samples were then dehydrated and processed for electron microscopy. The details of the procedure have been described previously (36). The sections were poststained with aqueous solutions of 5% uranyl acetate and lead citrate and the specimens were examined and photographed in a Zeiss EM109 transmission electron microscope at 80 kV.

Results

In view of prior reports of the intrinsic ADP-ribosyltransferase activity of BT-D (23, 24, 37) and its effect on secretion (26), as well as reports of the ability of some botulinum toxins (C2 and C3) to ADP-ribosylate cellular G actin selectively (38) and to block the polymerization of G actin to F actin (39), it was essential to determine the nature of the ADP-ribosyltransferase activity of the BT-D preparation used in our present studies. As shown in Figure 1*A* and previously reported (23, 24), BT-D was found to ADP-ribosylate a 22-kDa G protein in PMN sonicates. However, the possibility remains that more than one PMN G protein, of similar molecular masses, are substrates for BT-D-mediated ADP-ribosylation. Results identical with those shown in Figure 1*A* were obtained in three separate experiments with different PMN preparations. Figure 1*B* illustrates the comparative ADP-ribosyltransferase activities of BT-D and a partially purified preparation of exoenzyme BT-C3 (34) in the same PMN homogenate. It should be noted that the [^{32}P]ADP-ribosylated PMN G proteins, shown in Figure 1, were separated by electrophoresis on 11% to 23% polyacrylamide gradient gels, which gave the best one-dimensional separation of 20- to 30-kDa range proteins in our hands. A 42-kDa [^{32}P]ADP-ribosylated protein was detected only when the reaction was conducted with BT-C3 preparation and not with BT-D. This 42-kDa [^{32}P]ADP-ribosylated PMN protein is most likely actin (38), and it raises the possibility that a contaminating C2-type toxin could also be present in the C3 preparation. The major 24- to 25-kDa PMN protein that was modified by BT-C3, appeared to have a distinctly different mobility from that of the G_{22k} which was ADP-ribosylated by the BT-D. This has been a reproducible finding in our hands. As is also shown in Figure 1*B*, BT-C3 ADP-ribosylated additional PMN proteins of 20 to 30 kDa range to a minor degree and the possibility remains that the G_{22k} is one of these proteins. It should be noted that because the dynamics of actin polymerization (and depolymerization) play a

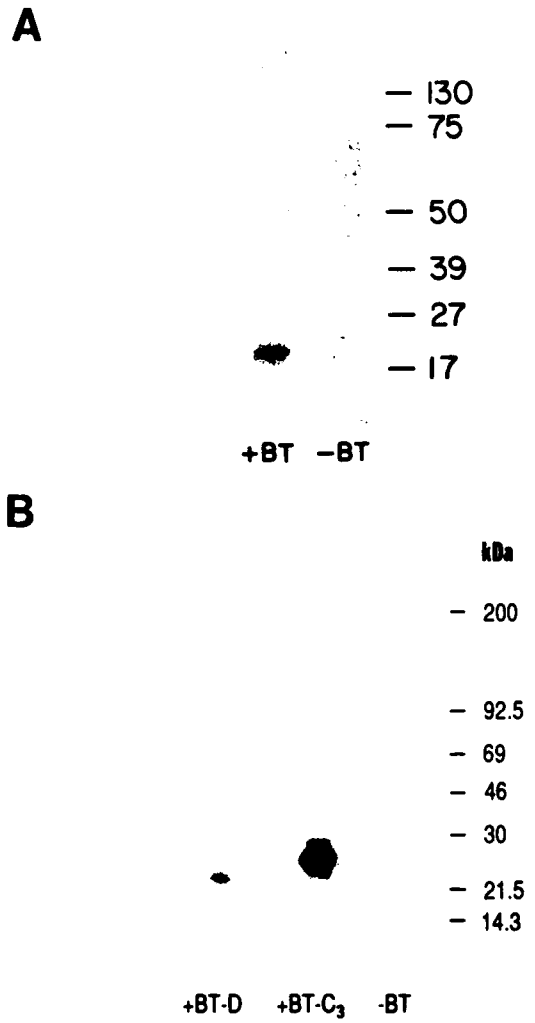

FIGURE 1. Botulinum toxin-mediated [^{32}P]ADP-ribosylation of small G proteins in PMN homogenates. *A*, ADP-ribosylation of a single G_{22k} by BT-D; *B*, BT-D and BT-C3-mediated ADP-ribosylation of distinctly different G proteins in a PMN homogenate. The [^{32}P]ADP-ribosylated proteins were electrophoresed on a 11% to 23% polyacrylamide gradient gel. Other experimental details are given in the text. The results are representative of three separate experiments.

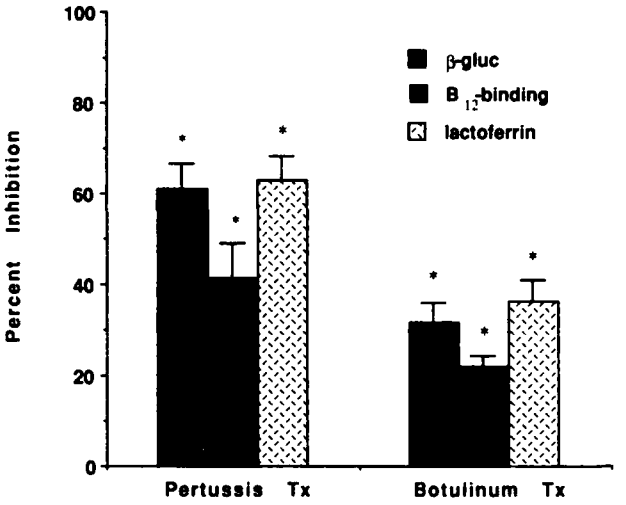

FIGURE 2. Inhibition of FMLP-induced secretion by BT and PT in intact PMNs. PMNs were incubated for 10 min at 37°C with 5 μg/ml cytochalasin b before stimulating with 10^{-6} M FMLP. In control PMNs (n = 10), FMLP-induced secretion varied between 20% and 50% for B_{12}-binding protein, between 50% and 70% for lactoferrin, and between 35% and 75% for β-glucuronidase (*β-gluc*). *p value < 0.0001.

critical role in modulating PMN inflammatory responses (40) including exocytosis (2–4), it was particularly important to rule out the possibility of ADP-ribosylation of PMN actin by the BT-D preparation that was to be used for examining its effects on PMN secretory responses.

Having established the nature of the ADP-ribosylating activity of the BT-D preparation, we next examined the effect of the toxin on exocytotic secretion in PMNs, as induced by the chemotactic peptide FMLP. As a positive control, we also studied the effect of PT, which is known to inhibit FMLP-induced secretory responses in PMNs (41). As shown in Figure 2, a significant inhibition of exocytosis was observed in BT-treated PMNs. The data represent values obtained from 10 different experiments and are highly significant ($p < 0.0001$). In studies conducted in parallel, with the same PMN preparations, we also observed a significant inhibition of PMN secretion by PT, which varied between 40% and 70%. Interestingly, we never obtained a complete inhibition of FMLP-induced secretion by PT, suggesting a PT-insensitive component of the response. In view of the results shown in Figure 2, we examined the secretory responses of PMNs that were treated with both PT and BT-D. As shown in Figure 3, an additive inhibition of FMLP-induced secretion was observed in PMNs incubated with both toxins, and this result suggests that the two toxins act at different steps in the signal-transduction cascade for FMLP-stimulated exocytosis in PMNs. As indicated in the legend to Figure 2, the Vit B_{12}-BP release values were generally lower than the lactoferrin values. This could have been due to the known susceptibility of Vit B_{12}-BP to oxidative inactivation.

In preliminary studies, conducted to establish the optimal incubation conditions with BT-D (see *Experimental Procedures*), it was apparent to us that an incubation time of 90 to 120 min, with 1 to 5 μg/ml BT-D, was necessary to obtain a significant inhibition of PMN secretory responses by BT-D. In an effort to correlate inhibition of secretion by BT-D with the degree of irreversible ADP-ribosylation of PMN G_{22k} by the toxin, we incubated PMNs with different concentrations of BT-D for different periods of time at 37°C, and measured the time- and dose-dependent inhibition of in vitro [^{32}P]ADP-ribosylation of G_{22k} by BT-D in those PMN sonicates to quantitate the

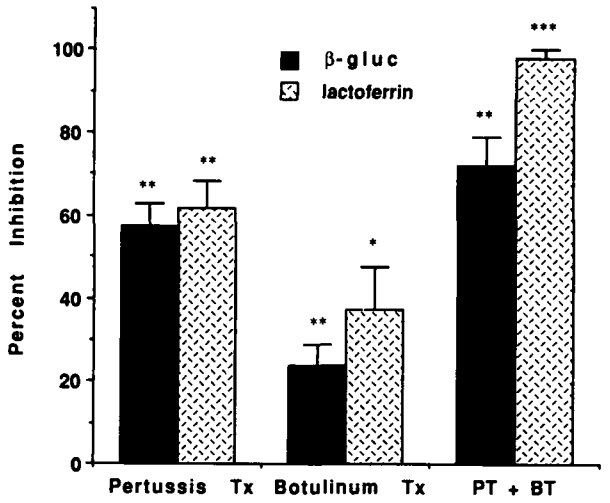

FIGURE 3. Additive inhibition of FMLP-induced secretion in PMNs by PT and BT. PMNs were incubated for 10 min at 37°C with 5 μg/ml cytochalasin b before stimulating with 10^{-6} M FMLP. In control PMNs (n = 3), FMLP-induced secretion varied between 50% and 70% for lactoferrin and 35% and 75% for β-glucuronidase. *p value < 0.07; **p value < 0.05; ***p value < 0.0005.

degree of toxin-mediated, irreversible ADP-ribosylation during the various preincubation conditions. As shown in Figure 4, incubation of PMNs for 90 to 120 min with 1 to 5 μg/ml of BT-D was required to obtain ≥85% inhibition of subsequent in vitro [^{32}P]ADP-ribosylation of G_{22k}. These conditions were also those that were required to induce a significant inhibition of PMN exocytosis by BT-D. Hence, it would appear that at least 85% of G_{22k} must be ADP-ribosylated by the BT-D before a functional effect of the toxin-mediated modification on PMN secretion is detectable in FMLP-stimulated cells.

Because a link between GTP and Ca^{2+} has been observed in stimulated PMN exocytosis (21), in that GTP, or the nonhydrolyzable GTP analogue GTPγS, acts synergistically with Ca^{2+} in triggering secretion in permeabilized PMNs (21), and because there appears to be a Ca^{2+}-independent, GTPγS-inducible secretory component in exocytotic cells (27, 28, 42), it was of interest to study the effect of BT-D on Ca^{2+}-induced exocytosis in digitonin-permeabilized PMNs. As shown in Figure 5, there was significant inhibition of secretion of both primary and secondary granule contents when permeabilized PMNs treated with BT-D were stimulated with Ca^{2+}, Ca^{2+} + GTP, or GTPγS alone. Interestingly, extracellular release of β-glucuronidase, a marker for primary granules, appeared to be more

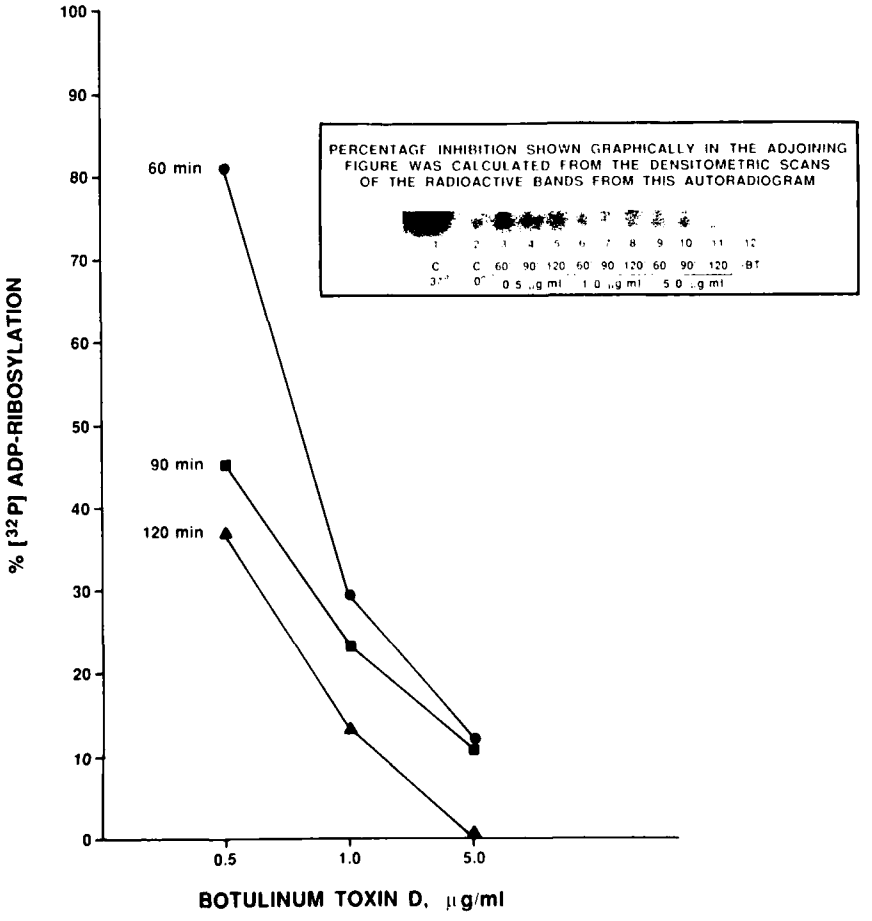

FIGURE 4. Time- and dose-dependent inhibition of in vitro [^{32}P]ADP-ribosylation of G_{22k} in a PMN homogenate previously incubated with BT-D. Details of the experimental procedure are given in the text.

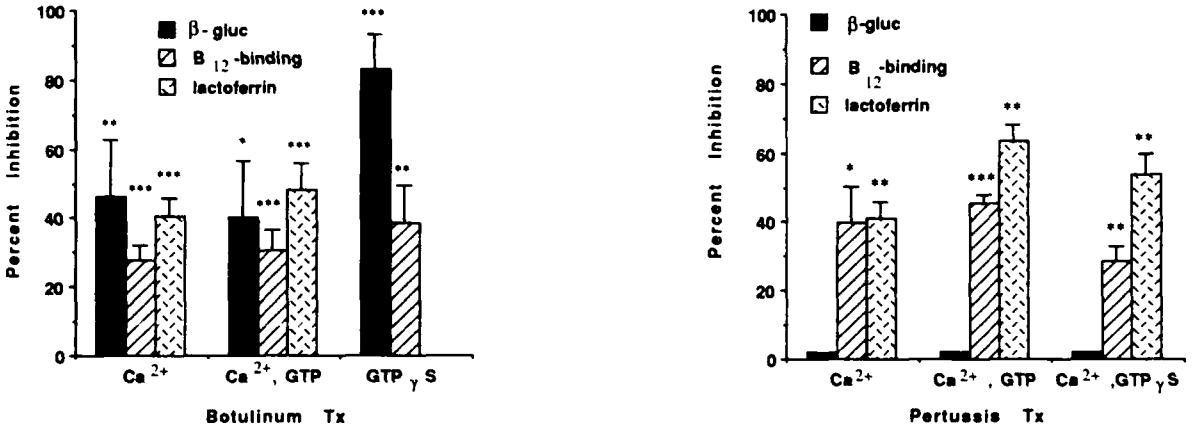

FIGURE 5. Effects of BT and PT on calcium and guanine nucleotide-induced secretion in digitonin-permeabilized PMNs. Digitonin-permeabilized PMNs (5×10^6/ml) were resuspended in a buffer containing 100 mM KCl, 20 mM NaCl, 1 mM EGTA, 30 mM HEPES, at pH 7.0. Secretion was induced by the addition of 1.5 mM Ca^{2+}, in the presence or absence of 0.1 mM GTP or GTPγS. Where indicated, GTPγS was also used to induce secretion in the absence of Ca^{2+}. Percentage inhibition of secretion was calculated from the values obtained with control PMNs. The secretion with Ca^{2+} alone ranged between 20% and 35% for β-glucuronidase, between 20% and 25% for B_{12}-binding protein and 20% to 30% for lactoferrin; with Ca^{2+} + GTP as the stimulus, these values were very similar to those obtained with Ca^{2+} alone; Ca^{2+} + GTPγS-induced secretion varied between 30% and 40% for β-glucuronidase, between 25% and 30% for B_{12}-binding protein and 30% and 35% for lactoferrin; GTPγS-induced, Ca^{2+}-independent exocytosis was only 10 to 12% above buffer control. *p value < 0.07; **p value < 0.05; ***p value < 0.0005.

sensitive to BT treatment than exocytosis of secondary granule contents. By contrast, in PT-treated permeabilized PMNs, a significant inhibition of exocytosis in response to Ca^{2+}, Ca^{2+} + GTP, or Ca^{2+} + GTPγS, was also observed, but only of secondary granule contents (Fig. 5). Furthermore, unlike BT-D which inhibited Ca^{2+}-independent, GTPγS-induced secretion, PT did not cause any significant inhibition of GTPγS-induced, Ca^{2+}-independent exocytosis in permeabilized PMNs (data not shown). These differences, illustrated in Figure 5, indicate the functional involvement of both BT-D-sensitive and PT-sensitive G proteins in the regulation of stimulated exocytosis in human PMNs. It is worthwhile to mention here that although the GTPγS-induced, Ca^{2+}-independent exocytosis was only about 10% above buffer controls, both secretion and its inhibition by BT-D were highly reproducible. These results also provide additional evidence for a differential regulation of the two major granule types of mature PMNs during stimulated exocytosis (7, 8). We also studied the effect of exoenzyme BT-C3 on Ca^{2+}-induced exocytosis in digitonin-permeabilized PMNs, but failed to observe any inhibition of exocytosis by BT-C3 (data not shown).

Ultrastructural studies of BD-treated PMNs were consistent with an inhibitory effect of the toxin on PMN secretory responses. As illustrated in Figure 6, transmission electron micrographs of BT-D-treated PMNs that were stimulated under conditions identical with those used for studying FMLP-induced secretion (Figs. 2 and 3) revealed an accumulation of membrane-bound empty organelles (*arrowheads*) and also of PMN granule-like structures (*arrows*) near the cell periphery, not seen in control, FMLP-stimulated PMNs not exposed to BT-D. Such accumulations of membrane-bound organelles near the cell periphery were not difficult to detect and were observed in >80% of BT-D-treated PMNs in any single preparation. Furthermore, as is also illustrated in Figure 6, FMLP-stimulated PMNs treated with BT-D were relatively smooth and rounded in shape compared with control FMLP-stimulated PMNs, which had characteristic pleomorphic shape changes and membrane ruffles (3, 6, 40).

Discussion

Models for stimulus-secretion coupling in a variety of secretory cells have included both receptor-mediated and nonreceptor-mediated activation mechanisms (43, 44), and in recent years, the role of cellular G proteins as components of these activation mechanisms in directed vesicular transport and membrane-fusion events has received substantial investigative attention (43, 44). Stimulated exocytosis of PMN granules plays a critical role in both the host-defense and inflammatory mechanisms of these cells, and the definition of cellular mechanisms that are involved in the regulation of exocytosis in activated PMNs remains an important challenge for research in leukocyte cell biology. In our present studies, we have examined in particular the role of BT-D-sensitive, small m.w. G proteins in stimulus-secretion coupling of human PMNs.

There is convincing biochemical evidence for an ADP-ribosyltransferase activity of BT-D (37, 45, 46), and the enzyme activity has been associated with the toxic component of the progenitor toxin (46). Furthermore, a 21- to

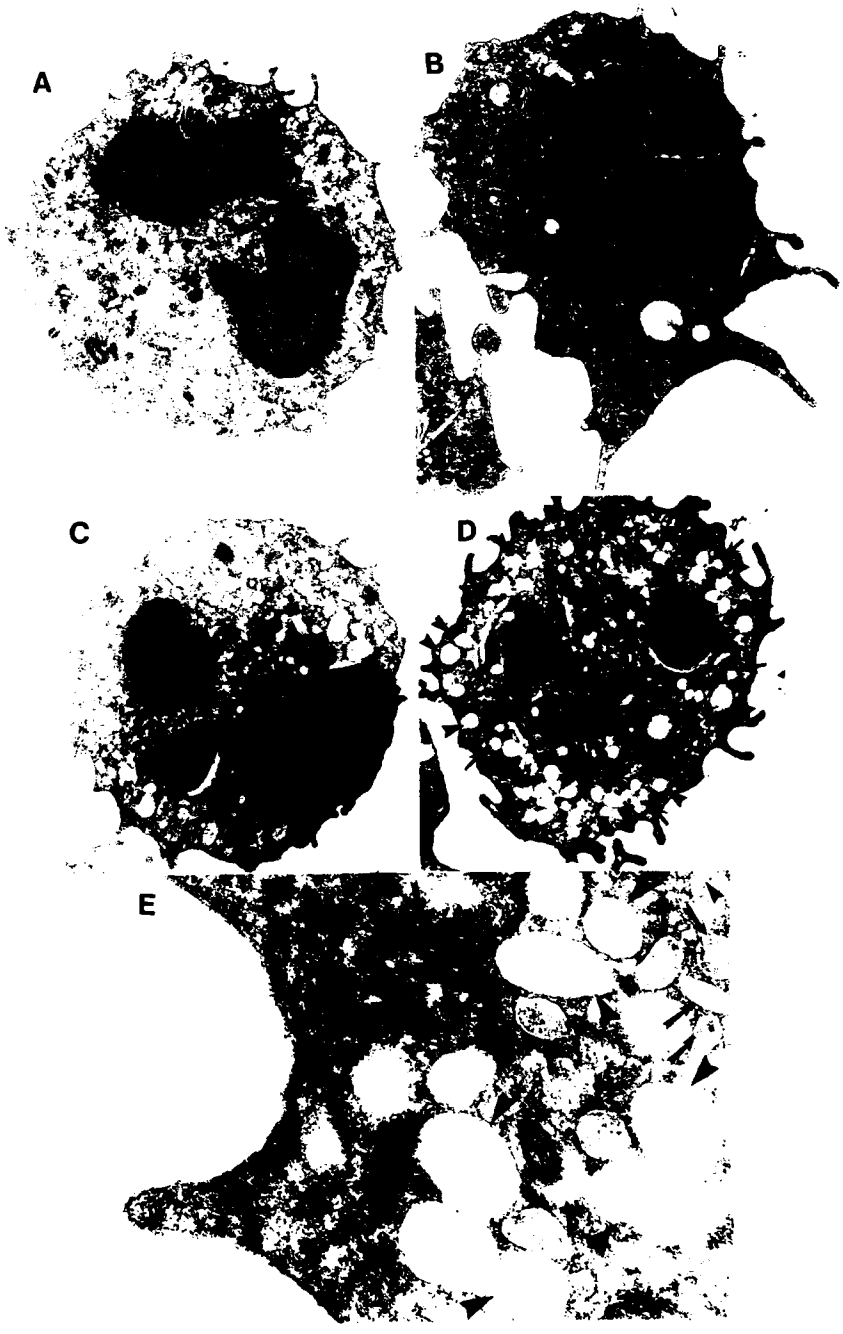

FIGURE 6. Ultrastructural effects of BT-D on exocytotic human PMNs. The PMNs were subjected to identical experimental conditions as described for FMLP-induced secretion (Fig. 2). The cells were then fixed and processed for electron-microscopy. Other details are described in the text: *A*, control; *B*, control + FMLP; *C*, BT-D alone; *D*, BT-D + FMLP (magnification × 4400); *E*, BT-D + FMLP-treated PMN at a higher magnification (× 87,000).

22-kDa G protein, distinct from ras G proteins, has been shown to be ADP-ribosylated specifically by BT-D in membrane and cytosolic fractions obtained from bovine adrenal gland (37, 45, 46). A BT-sensitive G_{22k} has also been detected in mouse brain, pancreas, and pituitary gland (45, 46). In addition, Knight et al. has reported an inhibition of stimulus-induced catecholamine release by BT-D in bovine adrenal medullary cells, and the toxin-induced blockade was shown to occur at a step downstream from the stimulus-dependent intracellular increase in free Ca^{2+} (26). Subsequent studies, however, have raised questions about both the functional involvement of a BT-D-sensitive G_{22k} protein in stimulated exocytosis and the intrinsic ADP-ribosyltransferase activity of BT-D (47). Nonetheless, a review of the existing literature provides substantial experimental support for a functional role of cellular G proteins (both PT-sensitive and PT-insensitive) in regulated secretion, at a site or sites distal to the generation of immediate, receptor-linked second messengers (21, 27, 28, 42). For example, studies conducted with permeabilized rabbit neutrophils have shown that GTPγS can activate secretory processes by acting both at the level of second messenger generation and at more downstream steps that directly activate exocytotic mechanisms (21,

30). In analogous studies, synergistic effects of Ca^{2+} and GTPγS have been demonstrated in permeabilized mast cell degranulation (27, 28, 42).

Our present studies confirm prior reports (23, 24) that human PMNs contain a G_{22k} protein that can be specifically ADP-ribosylated by BT-D (Fig. 1). Based on the one-dimensional gel electrophoresis data, we cannot exclude the possibility of more than one G protein of similar molecular mass being ADP-ribosylated by BT-D; however, prior studies have purified and characterized a 22-kDa PMN G protein, which was shown to be the substrate for ADP-ribosylation by BT-D (24). Our studies also provide evidence for a functional involvement of the BT-D-sensitive G_{22k} in the regulation of exocytosis in FMLP-stimulated human PMNs (Figs. 2–4). Furthermore, the additive inhibition by PT and BT-D (Fig. 3) suggests the involvement of multiple G proteins, at different sites, in the regulation of exocytosis in PMNs. Moreover, the data presented in Figure 4 demonstrate that at least 85% of G_{22k} needs to be irreversibly modified by BT-D, before an inhibitory effect of the toxin on PMN secretory responses is evident. This finding provides an explanation for the reported failure to observe inhibition of β-glucuronidase release from BT-D-treated PMNs under experimental conditions which we found to modify only 50% to 60% of the PMN G_{22k} (23).

In secretion studies with digitonin-permeabilized PMNs, BT-D was found to significantly inhibit Ca^{2+} minus and Ca^{2+} + GTP-induced exocytosis of both primary and secondary granules, whereas PT only inhibited the release of secondary granule contents without affecting β-glucuronidase release, a marker for primary granules (Fig. 5). Furthermore, while BT-D inhibited Ca^{2+}-independent, GTPγS-induced exocytosis substantially in digitonin-permeabilized PMNs, the extracellular release of primary granule contents was found to be more sensitive to toxin-induced inhibition than was release of secondary granules (>80% vs 40%). In contrast, PT treatment of PMNs did not affect the Ca^{2+}-independent, GTP- duced secretion (data not shown). In a recent report (48), evidence was presented for the association of distinct subsets of small G proteins with the primary and secondary granules of human PMNs, and it is tempting to speculate that a differential sensitivity of these PMN G proteins to BT-D may distinguish the G proteins that are involved in mediating exocytosis of one or the other granular type.

Although the nature of the accumulated membrane-bound vesicles is not known at this time, ultrastructural changes of BT-D-treated PMNs after activation with FMLP, as shown in Figure 6, mimic those of secretion-defective mutant yeast strains, deficient in the ras-related small G protein, sec4p, in which sub-plasmalemmal accumulations of secretory vesicles are observed (15–18). Recently, the rab family of small G proteins have been identified as mammalian homologues of sec4p and ypt1p (49), and distinct rab proteins of 22- to 25-kDa molecular mass have been shown to be associated with endocytic (11), exocytotic (12), and synaptic (50) vesicles, suggesting that the rab proteins may regulate vesicular trafficking in an organelle-specific manner. Furthermore, experimental evidence for an association-dissociation cycle of a small G protein, rab3A, during Ca^{2+}-dependent exocytosis of synaptic vesicles (the secretory organelles of nerve terminals) has been presented (19). In addition, the dissociation of rab3A was shown to be dependent on the exocytotic fusion of the vesicles and not on the stimulus-induced increase in intracellular Ca^{2+} (19). The results of our present study do not provide information regarding the mechanism or site of action of the BT-D-sensitive G_{22k} in PMN exocytosis; however, they clearly indicate a functional involvement of BT-D-sensitive 22-kDa small G protein(s) in PMN exocytosis.

Although the intrinsic ADP-ribosyltransferase activity of BT-D has been called into question (47), there is convincing biochemical evidence in support of an ADP-ribosyltransferase activity of the toxic component of the neurotoxin (37, 45, 46). The results of our present studies are consistent with the reported ADP-ribosylation of G_{22k} by BT-D in bovine adrenal tissue (37) and in other cells including PMNs (23, 24), and also with the reported inhibition of neurotransmitter release by BT-D in bovine adrenal medullary cells (26). The observed correlation of inhibition of PMN exocytosis with the degree of ADP-ribosylation of PMN G_{22k} by BT-D (Fig. 4) and the lack of an inhibitory effect of exoenzyme BT-C3 on PMN secretion in permeabilized cells (data not shown), suggest an involvement of the BT-D sensitive G_{22k} in the regulation of stimulated exocytosis of human PMNs. In this context, it is worthwhile to note that the [rho]-related small G proteins rac1 and rac2, which are substrates for ADP-ribosylation by BT-C3 (31), have been studied extensively in the past few years, and are believed to be involved in cytoskeletal reorganization (51) and in the activation and/or assembly of NADPH oxidase complex of phagocytic cells (52, 53).

Recent evidence from several laboratories indicate that the rab proteins, the mammalian homologues of sec4 and ypt1 gene products, which regulate yeast secretory vesicle fusion and endoplasmic reticulum-to-Golgi transport, respectively, represent a large family of ras-like small G proteins (or GTPases) that regulate distinct vesicular transport events at the level of membrane targeting and/or fusion (12, 44, 49). Based on evidence accumulated from recent studies in cell-free systems, it is currently believed that most organelles of the endocytic and secretory pathways bear distinct rab proteins on their surfaces, and that the rab proteins may function to ensure the accuracy of vesicle targeting events (12, 44, 49). In view of the differential regulation of distinct PMN granule types (7, 8), it is conceivable that such a regulatory mechanism also exists in the targeting and/or fusion of PMN granules during

stimulated exocytosis. Future studies directed at characterization and subcellular localization of the BT-D-sensitive G_{22k} in stimulated and unstimulated PMNs should clarify this possibility.

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