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Cholera toxin inhibits chemotaxis by a cAMP-independent mechanism

(adenylate cyclase/guanine nucleotide binding protein/ADP-ribosylation)

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ABSTRACT **Cholera toxin inhibits chemotaxis of the RAW264 mouse macrophage cell line. The degree of inhibition by cholera toxin increases upon incubation with the cells, suggesting that the entry of the toxin is required for inhibition of chemotaxis. In the absence of guanine nucleotides, cholera toxin catalyzes the [^{32}P]ADP-ribosylation of RAW264 cell membrane proteins of M_r 41,000, M_r 45,000, and a doublet of M_r 48,000–50,000. GTP increases the labeling of the M_r 45,000 protein and the M_r 48,000–50,000 doublet, and it decreases the labeling of the M_r 41,000 protein. Experiments with cholera toxin treatment of intact cells indicate that the M_r 45,000 protein is the major membrane protein ADP-ribosylated by the toxin *in vivo*. Cholera toxin increases cAMP levels in RAW264 cells, but increased cAMP levels do not correlate with inhibition of chemotaxis, because isoproterenol and forskolin, which also increase cAMP levels, have no effect on chemotaxis.**

A role for a guanine nucleotide binding protein has been implicated in the chemotactic response of leukocytes (1–4). It has been reported recently that pertussis toxin inhibits chemotaxis of mouse peritoneal macrophages (5), the RAW264 mouse macrophage cell line (5), the WBC264-9 hybrid cell line that expresses chemotaxis to fMet-Leu-Phe (1), and human neutrophils (2). From studies of the WBC264-9 cell line, we have concluded that the inhibition of chemotaxis by pertussis toxin is correlated with a toxin-catalyzed ADP-ribosylation of a M_r 41,000 membrane protein that appears to be identical with, or similar to, the α-subunit of N_i, the inhibitory protein of adenylate cyclase (1). However, binding of fMet-Leu-Phe to membranes from WBC264-9 cells did not change adenylate cyclase activity (1).

Several guanine nucleotide binding proteins have been described, including transducin, which regulates the light-dependent cGMP phosphodiesterase activity in retina (6–8), and the stimulatory and inhibitory proteins of adenylate cyclase, N_s and N_i, respectively (6, 9–15). Recently, a protein similar to N_i has been identified, designated N_o, whose function is not known (16–19). The α-subunits of both N_i and N_o are substrates for ADP-ribosylation by pertussis toxin (14–19). In addition to regulating adenylate cyclase, these proteins may also have other functions, such as regulation of Ca^{2+} transport and phosphatidylinositol metabolism (20–28).

Cholera toxin has been shown by other investigators to inhibit chemotaxis of human and rabbit neutrophils (29–31). Treatment of cells with cholera toxin is known to increase cAMP because of ADP-ribosylation of N_s (9–11). Because cholera toxin and pertussis toxin ADP-ribosylate different guanine nucleotide binding proteins that have opposing effects on cAMP levels, we decided to examine the role of cAMP levels and N_s in chemotaxis by the RAW264 cell line.

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METHODS

Cell Culture and Assays. The RAW264 cell line was grown in minimum essential medium containing 10% heat-inactivated fetal calf serum (56°C for 30 min) as described (32). For the various assays, cells were scraped from flasks, centrifuged at 250 × *g* for 10 min, and suspended in medium containing 10% fetal calf serum.

Chemotaxis was determined by the capacity of RAW264 cells to cross a 10-μm-thick Nuclepore polycarbonate membrane containing 5-μm holes when endotoxin-activated mouse serum was the attractant (32). Compounds tested as potential inhibitors were included at equal concentrations in both the top and bottom wells. For cAMP determinations, an equal volume of 1 M perchloric acid was added to cell suspensions, and the acid-soluble fraction was purified on Dowex and alumina as described by Zimmerman *et al.* (33). cAMP was determined by radioimmune assay (New England Nuclear).

ADP-Ribosylation Assays. The ADP-ribosyltransferase activity of cholera toxin was determined by the incorporation of radioactivity from [^{32}P]NAD into membrane proteins (34). The standard reaction mixture (0.05 ml) contained 0.25 M potassium phosphate (pH 7.0), 1 mM ATP, 0.1 mM GTP, 0.1 M arginine, 0.1 M thymidine, 25 μM [^{32}P]NAD (1.6 Ci/mmol; 1 Ci = 37 GBq), and 10 μg of cholera toxin per ml, which had been activated with 25 mM dithiothreitol. The reaction was started by the addition of cell membranes (25–75 μg of protein), and the mixture was incubated 45 min at 30°C. The reaction was stopped by addition of 0.5 ml of ice-cold 10 mM Tris·HCl (pH 7.5), and the membranes were collected by centrifugation. [^{32}P]-labeled proteins were solubilized and separated by NaDodSO$_4$/10% PAGE (35). The determination of the ADP-ribosyltransferase activity of pertussis toxin and the preparation of cell membranes from RAW264 cells has been described (1).

Cholera Toxin. Cholera toxin and its A and B subunits were purchased from Calbiochem, Sigma, or Schwarz/Mann. The B subunit of cholera toxin was free from contamination with holotoxin as judged by the absence of the M_r 22,000 A_1 subunit on NaDodSO$_4$/PAGE in the presence of 2-mercaptoethanol or by the absence of ADP-ribosyltransferase activity using human erythrocyte ghosts as a substrate for the toxin-dependent [^{32}P]ADP-ribosylation of the α-subunit of N_s. Either assay for the A subunit could have detected a contaminate in the B subunit of <1%. Human erythrocyte ghosts, prepared by the method of Cassel and Selinger, omitting the DNase treatment (36), were a gift from T. Hudson.

Abbreviation: Guo(5')*PP*[NH]*P*, guanosine 5'-[β,γ-imido]triphosphate.

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RESULTS

Inhibition of Chemotaxis by Cholera Toxin. When RAW264 cells and cholera toxin were mixed and immediately placed in chemotaxis chambers, chemotaxis was inhibited in a dose-dependent manner with 50% inhibition at 2.5 μg of toxin per ml (Fig. 1). The sensitivity to inhibition by cholera toxin was increased 25-fold by preincubation of the toxin with cells for 3 hr at 37°C; 50% inhibition now occurred at 0.1 μg of toxin per ml.

Because the entry of cholera toxin into various cells is known to require several minutes, the temporal dependence for the inhibition of chemotaxis suggested that entry of the toxin into RAW264 cells was required. Cholera toxin consists of two components, designated the A and B subunits, to which different roles have been assigned (37). The B subunit binds to gangliosides on the cell surface and is required for entry of the A subunit into the cell, while the A subunit utilizes intracellular NAD to catalyze an ADP-ribosylation reaction. In an effort to demonstrate that entry of the toxin was required for inhibition of chemotaxis, the effects of the purified A and B subunits were determined. In the absence of the B subunit, the A subunit should not enter the cell, and incubation of RAW264 cells with concentrations of the A subunit from 0.3 to 3 μg/ml had no effect on chemotaxis (data not shown). At the highest concentration of the A subunit tested (8 μg/ml), a small amount of inhibition did occur, which was possibly due to the entry of a small amount of the A subunit by a nonspecific mechanism. In contrast to the holotoxin, the effect of the A subunit on chemotaxis did not change when the cells and toxin were preincubated for 3 hr.

The B subunit of cholera toxin, which lacks the ADP-ribosyltransferase activity of the A subunit, did not inhibit chemotaxis as effectively as the holotoxin. Partial inhibition of chemotaxis occurred at relatively high concentrations (3–30 μg/ml) (Fig. 1). However, the properties of inhibition by the B subunit were clearly different from those of the holotoxin. There was essentially no inhibition by the B subunit at concentrations <1 μg/ml, whereas the holotoxin at 1 μg/ml inhibited chemotaxis by 40% without preincubation and by 85% with preincubation. In addition, the inhibition of chemotaxis by the B subunit was not complete; only 40% inhibition occurred at concentrations from 3 to 30 μg/ml (Fig. 1). Preincubation of the cells with the B subunit did not significantly increase the inhibition of chemotaxis, with the possible exception of a small increase at the two highest concentrations. These differences in the properties of the inhibition of chemotaxis between the B subunit and the holotoxin suggest that two separate mechanisms may be involved. Although we have not further investigated the inhibition of chemotaxis by the B subunit, this inhibition may involve cell-surface components, based on the relatively high concentration at which it occurs and the fact that the B subunit is known to bind to gangliosides on the cell surface. In this regard, it is believed that the B subunit can act as a lectin as well as change the permeability of the cell membrane (37, 38). Since the holotoxin was a much better inhibitor of chemotaxis after preincubation with the cells than either the A or B subunit alone, the data suggest that both entry of the toxin and the ADP-ribosyltransferase activity are involved in the inhibition of chemotaxis.

ADP-Ribosylation of Membrane Proteins by Cholera Toxin. Cholera toxin is known to ADP-ribosylate the α-subunit of N_s, which has a M_r of 45,000 (9–11). In addition to the α-subunit, N_s also contains β- and γ-subunits of M_r 35,000 and 10,000, respectively. When membranes from RAW264 cells were incubated with [^{32}P]NAD and cholera toxin, radioactive bands were observed for proteins of M_r 41,000, M_r 45,000 and a doublet of M_r 48,000–50,000 (Fig. 2). The M_r 41,000 protein ADP-ribosylated by cholera toxin migrated with the same molecular weight as the pertussis toxin substrate in these membranes (Fig. 2). The ADP-ribosylation by cholera toxin was greatly altered by guanine nucleotides. The addition to the reaction of GTP or the nonhydrolyzable analog of GTP, guanosine 5′-[β,γ-imido]triphosphate {Guo(5′)*PP*[NH]*P*}, greatly increased ADP-ribosylation of the M_r 45,000 and the M_r 48,000–50,000 doublet. However, GTP inhibited the incorporation of radioactivity into the M_r 41,000 protein and labeling of the M_r 41,000 band still occurred with Guo(5′)*PP*[NH]*P*. A similar guanine nucleotide dependence for the labeling of a M_r 41,000 protein by cholera toxin has been reported in adipocyte membranes (39). The guanine nucleotide dependence for ADP-ribosylation of the M_r 41,000 protein by cholera toxin is opposite that

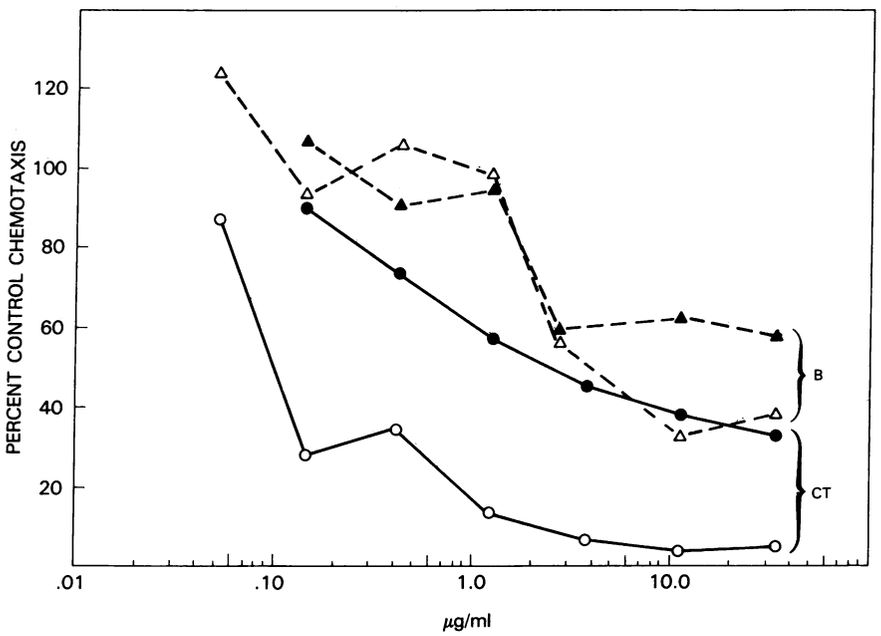

FIG. 1. Inhibition of RAW264 chemotaxis by cholera toxin. Cholera toxin (CT) or the B subunit of cholera toxin (B) was added to RAW264 cells, and the cells were assayed immediately (●, cholera toxin; ▲, B subunit) or after a 3-hr incubation (○, cholera toxin; △, B subunit).

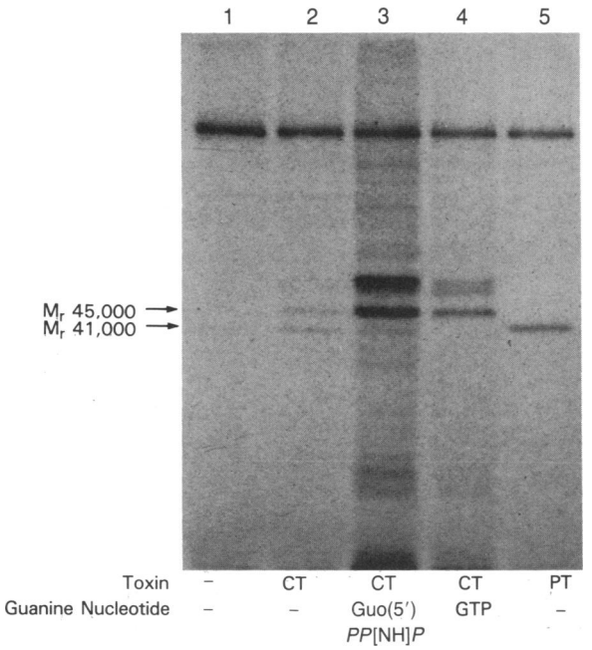

FIG. 2. Effect of guanine nucleotides on ADP-ribosylation by cholera toxin. Membranes from RAW264 cells were incubated with [^{32}P]NAD and the indicated additions of toxins and guanine nucleotides for 45 min. Membrane proteins were separated by NaDodSO$_4$/PAGE, and the incorporation of radioactivity was determined by autoradiography. Lanes: 1, no additions; 2, cholera toxin (CT); 3, cholera toxin, 1 mM Guo(5')*PP*[NH]*P*; 4, cholera toxin, 1 mM GTP; 5, pertussis toxin (PT). Molecular weights for indicated radioactive bands were calculated from the relative migration of standard protein molecular weight markers.

observed with pertussis toxin in both RAW264 and WBC264-9 cell membranes, where labeling of the M_r 41,000 protein was stimulated by GTP, and very little labeling occurred in the presence of Guo(5')*PP*[NH]*P* or in the absence of guanine nucleotides (1). The effect of GTP and Guo(5')*PP*[NH]*P* on ADP-ribosylation by cholera toxin and pertussis toxin for the different protein substrates is summarized in Table 1.

To demonstrate that cholera toxin enters RAW264 cells and ADP-ribosylates the α subunit of N_s under conditions that inhibit chemotaxis, cells were incubated with the toxin, and membranes were isolated and treated with [^{32}P]NAD and cholera toxin. With this assay, a decrease in the labeling of membranes indicates that ADP-ribosylation by the toxin occurred in the intact cells. Indirect measurement of ADP-ribosylation was used because NAD does not enter cells. Significant ADP-ribosylation of the α subunit of N_s occurred when cells were incubated with cholera toxin, as shown by the decreased radioactivity in the M_r 45,000 protein (Fig. 3). However, there was little effect on the labeling of the M_r 48,000–50,000 doublet, indicating that these proteins were not ADP-ribosylated by the toxin in intact cells. Treatment of the cells with the purified B subunit of cholera toxin did not change the amount of N_s α-subunit available for ADP-ribosylation (data not shown), and it showed that the A chain of the toxin was required.

Table 1. Guanine nucleotide specificity of ADP-ribosylation of cholera toxin and pertussis toxin substrates in RAW264 membranes

M_r of membrane polypeptide	Toxin	Guanine nucleotide	
		GTP	Guo(5')*PP*[NH]*P*
45,000	Cholera	↑	↑
41,000	Cholera	↓	(−)
41,000*	Pertussis	↑	−

↑, Increased labeling; ↓, decreased labeling; −, no change; (−), little or no change.

*The same guanine nucleotide dependence is observed in WBC264-9 cells (1).

In addition to ADP-ribosylation of N_s, treatment of cells with 1 μg of cholera toxin per ml increased the labeling of the M_r 41,000 protein (Fig. 3). This was a surprising result because no labeling of the M_r 41,000 protein by cholera toxin was observed with untreated cell membranes unless the reaction was carried out in the absence of GTP. A possible explanation for this finding is that treatment of intact cells with cholera toxin resulted in a conformational change in the M_r 41,000 protein that made it a substrate for ADP-ribosylation by cholera toxin in membranes. Treatment of the cells with cholera toxin under conditions that ADP-ribosylate N_s, did not significantly change the ADP-ribosylation of the M_r 41,000 substrate by pertussis toxin in isolated membranes (Fig. 3).

Inhibition of Chemotaxis Does Not Correlate with Increased Levels of cAMP. The best documented effect of the ADP-ribosylation of N_s by cholera toxin is an increase in adenylate cyclase activity, which results in the accumulation of cAMP (9–11). As expected from studies of the effects of cholera toxin on other cells, incubation of RAW264 cells with the toxin increased the level of cAMP (Table 2). cAMP increased ≈6-fold during a 2-hr incubation at 10 μg of cholera toxin per ml, a concentration of toxin that causes nearly complete inhibition of chemotaxis. Higher cAMP levels were obtained in the presence of cholera toxin and isobutylmethylxanthine (IBMX), a cAMP phosphodiesterase inhibitor; however, IBMX did not potentiate the inhibition of chemotaxis by cholera toxin.

Incubation of the cells with other effectors of the adenylate cyclase system, such as 10 μM isoproterenol or 100 μM forskolin, also increased cAMP both in the presence and absence of IBMX. However, neither isoproterenol nor forskolin inhibited chemotaxis when added to the cells and assayed immediately, or after a 3-hr preincubation with the compounds (data not shown). Forskolin or isoproterenol in the presence of IBMX increased cAMP levels to a greater extent than did cholera toxin in the absence of IBMX (Table 2), while cholera toxin treatment alone inhibited chemotaxis. Therefore, these data indicate that increases in total cAMP were not correlated with the inhibition of chemotaxis.

DISCUSSION

We have demonstrated that RAW264 chemotaxis to endotoxin-activated mouse serum is inhibited by cholera toxin. Other investigators have also shown that cholera toxin inhibits chemotaxis to fMet-Leu-Phe in human (29, 31) and rabbit neutrophils (30); however, previous studies did not show a correlation between increases in cAMP levels and the inhibition of chemotaxis. Our studies also indicate that inhibition of RAW264 chemotaxis by cholera toxin is not the result of increased cAMP levels. Thus, cAMP levels can be increased with isoproterenol or forskolin to levels that are comparable to those obtained with cholera toxin, and yet chemotaxis is inhibited only with cholera toxin.

In human neutrophils a rapid transient increase in cAMP has been observed when cells are incubated with the attractant fMet-Leu-Phe (40–42). However, no change in the activity of adenylate cyclase has been observed when membranes from human neutrophils (43) or WBC264-9 cells (1) are incubated with fMet-Leu-Phe, indicating that adenylate cyclase is not coupled to *N*-formyl peptide receptors. Verghese *et al.* (43) have recently suggested that the attractant-induced increase in cAMP requires Ca^{2+} mobilization and could be

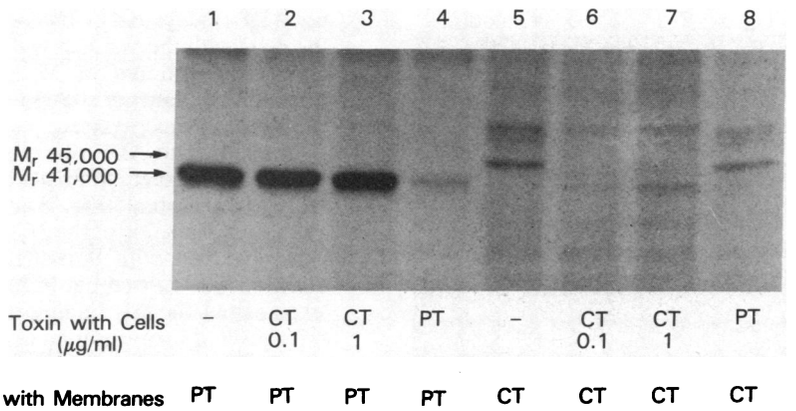

FIG. 3. Effect of cholera toxin on ADP-ribosylation of intact cells. RAW264 cells were suspended in minimal essential medium and incubated for 3 hr at 37°C with the indicated toxins. Lanes: 1 and 5, no toxin; 2 and 6, 0.1 μg of cholera toxin (CT) per ml; 3 and 7, 1 μg of cholera toxin per ml; 4 and 8, 0.01 μg of pertussis toxin (PT) per ml. Membranes were prepared and incubated with [^{32}P]NAD, 0.1 mM GTP, and either 8 μg of pertussis toxin per ml (lanes 1–4) or 10 μg of cholera toxin per ml (lanes 5–8). The other reaction components are described in *Methods* for cholera toxin and in ref. 1 for pertussis toxin. Membrane proteins (45 μg) were solubilized, separated by NaDodSO$_4$/PAGE, and the incorporation of radioactivity was determined by autoradiography.

mediated in part through a transient inhibition of phosphodiesterases.

Although a role for adenylate cyclase has not been demonstrated in chemotaxis, evidence from several laboratories has suggested a role for a guanine nucleotide binding protein (1–5). The inhibition of chemotaxis by pertussis toxin is correlated with the toxin-catalyzed ADP-ribosylation of a M_r 41,000 protein. The molecular weight of the ADP-ribosylated protein is consistent with either N_i or N_o, but because of the similar mobilities of these proteins on NaDodSO$_4$/PAGE, it has not yet been possible to determine which, if either, of these proteins is involved in chemotaxis. However, the coupling of N_i to fMet-Leu-Phe receptors would be expected to affect adenylate cyclase activity, and a change in cyclase activity in the presence of fMet-Leu-Phe has not been observed in human neutrophils (43) or the WBC264-9 cell line (1). In some systems, it has also been possible to show that binding of the attractant fMet-Leu-Phe is affected when cells are treated with pertussis toxin (2, 44) or guanine nucleotides (2–4), and that pertussis toxin inhibits high affinity GTPase activity believed to be catalyzed by N proteins (1, 44). Because arachidonic acid release and Ca^{2+} mobilization induced by fMet-Leu-Phe are inhibited by pertussis toxin (20, 22–25), it has been suggested that a guanine nucleotide binding protein is coupled to phospholipases, and recent studies with human neutrophils (27) and the human HL-60 cell line (28) indicate that phospholipase C is regulated by a guanine nucleotide binding protein.

Table 2. cAMP levels in RAW264 cell stimulated with cholera toxin forskolin or isoproterenol

Stimulus	cAMP, pmol per 10^6 cells	
	No IBMX	0.5 mM IBMX
45-min incubation		
None	7.9 ± 1.0	32.8 ± 1.1
Forskolin (100 μM)	17.6 ± 1.3	153 ± 7
Isoproterenol (10 μM)	19.0 ± 1.6	209 ± 16
2-hr incubation		
None	6.4 ± 1.6	37.7 ± 7.9
Cholera toxin (10 μg/ml)	35.9 ± 0.1	>230
Medium	8.7 ± 0.0	27.1*

Cell (3×10^6 per ml) were incubated with stimuli for either 45 min or 2 hr, an equal volume of 1 M perchloric acid was added, and the amount of cAMP in the supernatant was determined. Data are expressed as the mean of triplicates ± SEM.
*A single determination.

Experiments similar to those that indicate a role for N_i or N_o in chemotaxis can also indicate an involvement of N_s. In RAW264 cells, evidence was obtained to suggest that inhibition of chemotaxis occurs when cholera toxin enters the cells and the α-subunit of N_s is ADP-ribosylated. This hypothesis is supported by the following observations. First, the intact cholera holotoxin was a better inhibitor of chemotaxis than either the purified A or B subunits. Although some inhibition of chemotaxis occurred with the isolated B subunit of the toxin, the mechanism appeared to be different from that of the holotoxin. The B subunit inhibited chemotaxis only at concentrations higher than that of the holotoxin, and the inhibition by the B subunit was not increased by preincubation with the cells. Second, because entry of cholera toxin is known to require several minutes, our observation that preincubation of the cells with toxin increased the inhibition of chemotaxis is consistent with a requirement for the entry of toxin. Third, the only known activity of the toxin once it enters the cell is to ADP-ribosylate proteins, and the α-subunit of N_s is believed to be the primary substrate in intact cells. We demonstrated that N_s was ADP-ribosylated in cells under conditions where cholera toxin inhibits chemotaxis, both by the decrease in the incorporation of [^{32}P]NAD into the M_r 45,000 membrane protein from cells treated with toxin and by the increase in cAMP produced in treated cells.

Several observations have been made that suggest some type of interaction between different N proteins. For example, it has been shown that cholera toxin can partially attenuate the opiate- and N_i-dependent inhibition of adenylate cyclase, although the attenuation is not as effective as with pertussis toxin (45). Similarly, fMet-Leu-Phe-stimulated arachidonic acid release in guinea pig neutrophils is also partially inhibited by cholera toxin, while pertussis toxin completely inhibited the release (20, 22). For the chemotaxis of RAW264 cells, evidence has been presented for an involvement of both the N_s and N_i (N_o) guanine binding proteins.

These findings could be explained if, like transducin, a single N protein was ADP-ribosylated by both cholera and pertussis toxins. However, transducin is found only in retina, and in other systems the pertussis toxin and cholera toxin membrane substrates are different, because the α-subunits differ in their molecular weights. The radiolabeled M_r 41,000 band in RAW264 cell membranes may be an exception, in

that conditions were found where ADP-ribosylation occurred with both cholera and pertussis toxins. However, we have not determined whether the M_r 41,000 band contains two separate proteins that are ADP-ribosylated by different toxins, or whether the two toxins ADP-ribosylate different sites on the same protein. It is important to note that the M_r 41,000 band did not appear to be ADP-ribosylated in intact cells treated with cholera toxin (Fig. 3).

A model that would allow for interaction between guanine nucleotide binding proteins has been suggested by Gilman (6). Although N_s and N_i have different α-subunits, which are ADP-ribosylated by cholera toxin and pertussis toxin, respectively, these guanine nucleotide binding proteins probably share common $\beta\gamma$-subunits. It has been established that the activity of the α-subunits are altered by the association with the $\beta\gamma$-subunits, and that the association between these subunits is also altered by ADP-ribosylation. Therefore, the $\beta\gamma$-subunits can provide a link between different N proteins, and it is possible that inhibition of chemotaxis by both toxins may be mediated by alteration of the equilibrium between the fully associated form of the N proteins and the α- and $\beta\gamma$-subunits.

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