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Effects of Pertussis Toxin on cAMP and cGMP Responses to Carbamylcholine in N1E-115 Neuroblastoma Cells

PAOLA BRUNI, DRUSILLA L. BURNS, ERIK L. HEWLETT, AND JOEL MOSS

Laboratory of Cellular Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205 (P.B., D.L.B., J.M.) and the Departments of Medicine and Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22908 (E.L.H.)

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SUMMARY

As noted previously, in N1E-115 neuroblastoma cells, carbamylcholine, a muscarinic cholinergic agonist, increased cGMP over 15-fold and decreased basal and prostaglandin E_1 (PGE_1)-stimulated cAMP content. In contrast to the stimulatory effects of PGE_1 on cAMP, which were immediate, the carbamylcholine-induced decrease in basal and PGE_1-stimulated cAMP exhibited a delay. The delay in carbamylcholine inhibition was independent of the extent of adenylate cyclase activation. Although basal cAMP content was suppressed within 30 sec after addition of carbamylcholine, inhibition was not maximal for at least 2 min following agonist addition; the delay was similar in cells exposed to PGE_1 for 10 min prior to carbamylcholine but could be eliminated by incubation of the cells with muscarinic cholinergic agonist for 5 min prior to addition of prostaglandin. N1E-115 neuroblastoma cells possess a 41,000-Da membrane protein believed to be a component of the inhibitory GTP-binding protein of adenylate cyclase that is ADP ribosylated by pertussis toxin. Incubation of the cells with pertussis toxin prior to the addition of carbamylcholine reduced the maximal extent of inhibition of cAMP content and prevented the [^{32}P]ADP-ribosylation of a 41,000-Da protein by toxin and [^{32}P]NAD in membrane preparations from these cells. Incubation of cells with pertussis toxin, however, did not significantly alter the dose-response curve for carbamylcholine effects on cGMP. Even high concentrations of carbamylcholine, effective in stimulating cGMP, had minimal effects on cAMP content in toxin-treated cells; thus, ADP-ribosylation of G_i converts the adenylate cyclase but not the guanylate cyclase system to an agonist-insensitive state.

INTRODUCTION

cAMP and cGMP content of animal cells is regulated in part by external agents, such as hormones, drugs, and bacterial toxins (1–3). Control over cAMP synthesis appears to be exerted through the hormone-sensitive adenylate cyclase system (4). Cyclase activity is regulated by stimulatory and inhibitory agonists operating through their respective cell surface receptors (4, 5); these receptors are linked to the catalytic unit through different GTP-binding proteins, termed G_s[1] and G_i, respectively (4). G_s and G_i are similar in structure and possess GTP-binding subunits $G_{s\alpha}$ and $G_{i\alpha}$ of 45,000 and 41,000 Da, linked to a common heterodimer, $G_{\beta\gamma}$ of 35 kDa (G_β) and ~10 kDa (G_γ) (4, 6). These proteins either directly or indirectly alter catalytic unit activity (4, 7). The 41,000-Da protein is the target of pertussis toxin (islet-activating protein) (8, 9), a protein product of *Bordetella pertussis*, an etiologic agent of whooping cough (10). The toxin catalyzes the transfer of ADP-ribose from NAD to the 41,000-Da subunit of G_i (8, 9) and thereby inactivates the protein (11); following incubation of cells with pertussis toxin, inhibitory agents such as opiates, muscarinic cholinergic agonists, and α_2-adrenergic agonists no longer inhibit adenylate cyclase (11). Binding studies are consistent with the hypothesis that ADP-ribosylation of G_i by pertussis toxin uncouples the inhibitory receptor from the GTP-binding protein, resulting in a loss of cyclase inhibition (11, 12). In addition to regulating cAMP by control over synthesis, it also appears that in some cells, muscarinic cholinergic agonists may depress cAMP content by stimulating hydrolysis through activation of a cyclic nucleotide phosphodiesterase (13, 14).

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[1] The abbreviations used are: G_s, the stimulatory guanine nucleotide-binding protein of the adenylate cyclase system; G_i, the inhibitory guanine nucleotide-binding protein of the adenylate cyclase system; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PGE_1, prostaglandin E_1; ADP-ribosylation, adenosine 5′-diphosphoribosylation; K_a, the concentration of agonist producing half-maximal effects; IBMX, 3-isobutyl-1-methylxanthine.

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A direct coupling of cell surface receptors to particulate or soluble guanylate cyclases has not been established; muscarinic cholinergic agonists may, however, increase intracellular Ca, leading by independent mechanisms to increased phosphodiesterase activity and an increase in cGMP (13, 15, 16). Of interest is the fact that certain agents, such as the muscarinic cholinergic agonists, operate through both the cAMP and cGMP systems (17), even though physically only one receptor type has been identified (18–20). Based on the differential effects of muscarinic cholinergic agonists and antagonists, it would appear that there are multiple receptor subtypes; it is not clear whether these subtypes result from differences in the receptor itself or from differences in the coupling protein associated with the receptor (21, 22).

In N1E-115 cells, studies by Matsuzawa and Nirenberg (23) demonstrated that muscarinic receptors both suppress intracellular cAMP content and increase cellular cGMP (23). These cells were used as a model system to study the kinetics of muscarinic cholinergic agonist effects on cAMP and cGMP; pertussis toxin was used to examine the role of G_i in carbamylcholine action on both cyclic nucleotides.

EXPERIMENTAL PROCEDURES

Materials

PGE_1 was purchased from Seragen; carbamylcholine, HEPES, tri-*n*-octylamine, acetyl-β-methylcholine, and oxotremorine sesquifumarate from Sigma, Dulbecco's modified Eagle medium from HEM Research, and fetal bovine serum from KC Biologicals; Ro-20-1724 was a gift from Hoffmann-LaRoche. C-1300 mouse neuroblastoma, clone N1E-115 cells, were kindly provided by Dr. Steven L. Sabol (National Heart, Lung, and Blood Institute); cAMP and cGMP radioimmunoassay kits were purchased from New England Nuclear; 3-isobutyl-1-methylxanthine from Aldrich, and [^{32}P]NAD (10–50 Ci/mmol) from New England Nuclear.

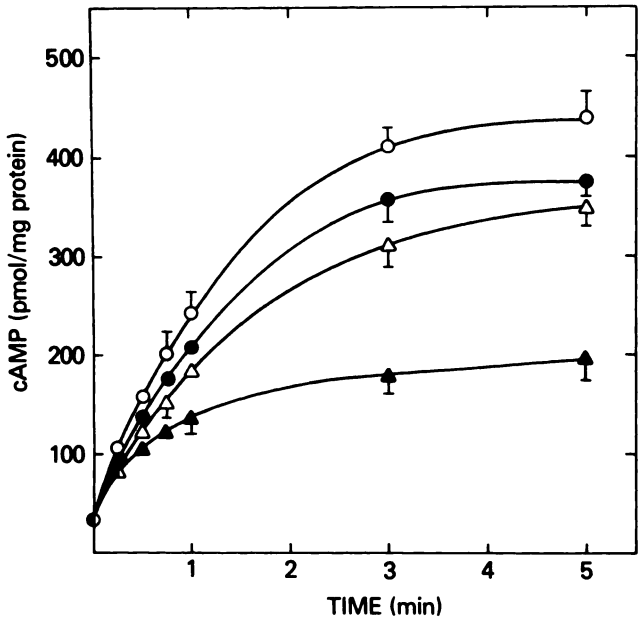

FIG. 1. *Effects of PGE_1 and carbamylcholine on cAMP content of N1E-115 cells*

Control (●, ▲) and pertussis toxin-treated cells (○, △) were incubated for the indicated time with PGE_1, 10 μg ml (●, ○), or with PGE_1 plus 1 mM carbamylcholine (▲, △). The experiment was repeated on three occasions; a representative experiment is presented.

TABLE 1

Effect of carbamylcholine on cAMP content of N1E-115 cells in the presence or absence of phosphodiesterase inhibitors

Control or toxin-treated cells were incubated for 20 min at 37° with 0.1 mM Ro-20-1724 or 0.1 mM IBMX, or both, and then further incubated with PGE_1, 10 μg/ml for 5 min with or without added 1 mM carbamylcholine. The cAMP content of cells incubated without PGE_1 or carbamylcholine was 27 and 28 pmol/mg protein for control and toxin-treated cells, respectively, in the presence of Ro-20-1724; 25 and 29, respectively, in the presence of IBMX; and 32 and 34 in the presence of both Ro-20-1724 and IBMX. Experiment was repeated on 4 separate occasions; a representative experiment is presented.

IBMX	Ro-20-1724	Carbamylcholine	cAMP content	
			No toxin	Plus toxin
			pmol·mg protein^{-1}	
0	+	0	300 ± 4.4	312 ± 13.2
0	+	+	189 ± 4.6 (37)[a]	273 ± 8.7 (13)
+	0	0	229 ± 11.5	237 ± 27.6
+	0	+	174 ± 5.9 (24)	204 ± 25 (14)
+	+	0	657 ± 2.1	676 ± 36.7
+	+	+	318 ± 26.2 (52)	632 ± 30.3 (7)

[a] In parenthesis, percentage decrease in cAMP content due to carbamylcholine.

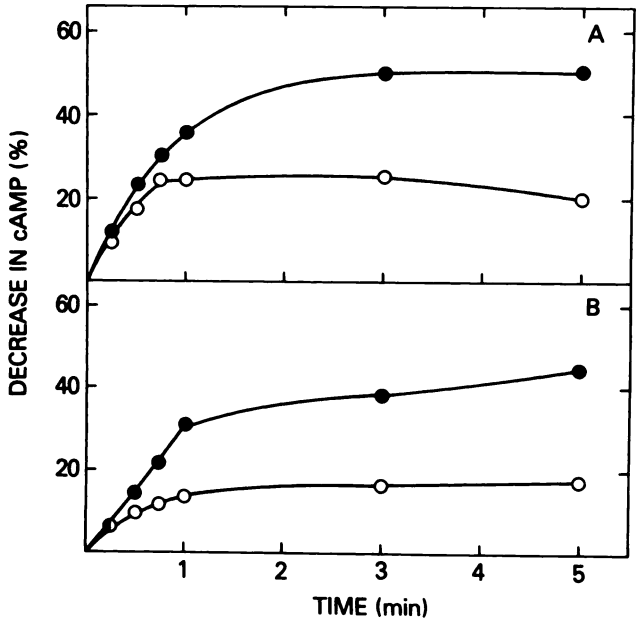

FIG. 2. *Effect of carbamylcholine on cAMP content of cells with and without PGE_1*

Control (●) and toxin-treated (○) cells were incubated with and without 1 mM carbamylcholine. A. with PGE_1, 10 μg/ml; or B. without PGE_1 for the indicated time. The percentage decrease in cAMP content caused by carbamylcholine is shown. Data in A are from the experiment shown in Fig. 1. The basal cAMP content in the absence or presence of pertussis toxin was 45 and 47 pmol/mg, respectively.

Methods

Tissue culture. Cells released from Falcon flasks (75 cm^2) by shaking were grown in 60-mm Petri dishes in 3 ml of 90% Dulbecco's modified Eagle medium, 10% fetal bovine serum in a humidified atmosphere of 5% CO_2-95% air at 37°. Medium was replaced on days 3, 5, 6, and 7 after plating; experiments were carried out on the 8th day.

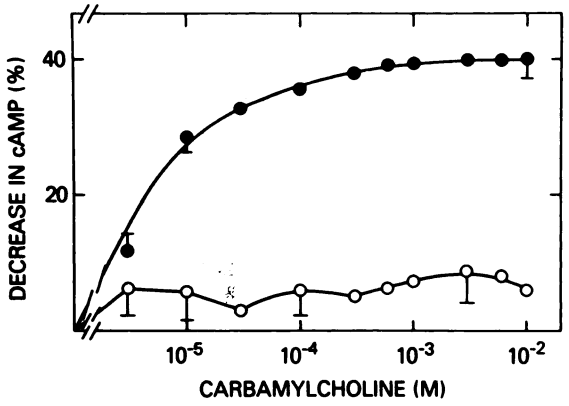

FIG. 3. *Effect of carbamylcholine on cAMP content of N1E-115 cells*
Control (●) and pertussis toxin-treated (○) cells were incubated for 30 sec with PGE_1, 10 μg/ml, and the indicated concentration of carbamylcholine. The decrease in cAMP content caused by carbamylcholine is expressed as a percentage of the cAMP content of cells incubated with PGE_1 alone for 30 sec which was 126 and 132 pmol/mg protein for control and treated cells, respectively. Basal cAMP content was 41.5 for control and 51.9 pmol/mg protein for treated cells. Experiment was repeated in triplicate.

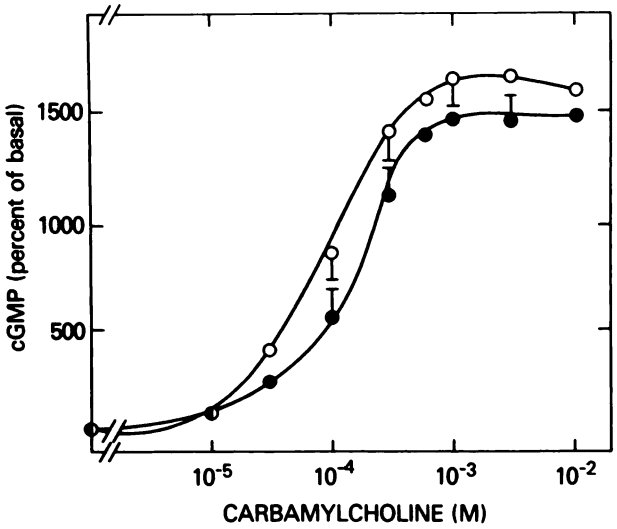

FIG. 4. *Effect of carbamylcholine on cGMP content of N1E-115 cells*
Control (●) and pertussis toxin-treated (○) cells were incubated for 30 sec with PGE_1, 10 μg/ml, and the indicated concentration of carbamylcholine. cGMP content is recorded as a percentage of basal which was 2.0 and 1.9 pmol/mg protein for control and toxin-treated cells, respectively. Values for cells incubated with PGE_1 alone were not significantly different. The experiment was repeated in triplicate.

Cells were washed twice with 3 ml of Hanks' balanced salt solution containing 10 mM HEPES, pH 7.6, and incubated for 20 min at 37° with 2 ml of the same solution containing 0.1 mM Ro-20-1724. Additions (40 μl) of carbamylcholine (in Hanks' medium with 10 mM HEPES, pH 7.6) and/or PGE_1 (in ethanol) or both were then made; variations were as indicated in figure legends. When PGE_1 was used, controls contained 0.2% ethanol. After incubation for the indicated time at 37°, 0.2 ml of 100% trichloracetic acid was added, and cells and medium were rapidly frozen on dry ice. After thawing, medium was collected and centrifuged; 1 ml of supernatant was treated with 1 ml of 0.5 M tri-*n*-octylamine solution in Freon (24). The upper phase was used for radioimmunoassay of cGMP and cAMP. Cells attached to dishes were solubilized with 1 N NaOH for determination of protein content (25). Data reported are means ± S.E. of values from triplicate dishes; each dish was assayed in duplicate. In figures, absence of a standard error indicates that it is within the symbol. Cells treated with pertussis toxin were incubated with toxin, about 300 ng/ml, for 16–20 hr at 37° before experiments were begun. Pertussis toxin, purified as described (26), was stored at 4° in 0.5 M NaCl, 0.1 M sodium phosphate, pH 7.5.

ADP-ribosylation of membranes. Membranes (about 40 μg of protein) were incubated in 25 mM glycine, 40 mM potassium phosphate, pH 7.5, containing 20 μM [^{32}P]NAD (about 20 μCi/ml), 0.5 mM ATP, 20 mM thymidine, 10 mM dithiothreitol and ovalbumin (0.1 mg/ml) with or without pertussis toxin for 2 hr at 30° (total volume, 0.120 ml). After addition of 2 ml of 10% trichloracetic acid and centrifugation (3000 ×

TABLE 2

Effect of different muscarinic agonists on cAMP and cGMP of N1E-115 cells

Cells were incubated for the indicated times with PGE_1, 10 μg/ml, with or without added carbamylcholine, acetyl-*p*-methylcholine, or oxotremorine (all 1 mM). The cAMP content of cells incubated without PGE_1 or muscarinic agents was 36 pmol/mg protein. The cGMP content of cells incubated without PGE_1 or muscarinic agents was 4.8 pmol/mg of protein. The lack of an effect of oxotremorine was observed on three separate occasions; the effectiveness of the ligand was confirmed in human fibroblasts.

Muscarinic agonist (1 mM)	cGMP content (30 sec)	cAMP content	
		30 sec	5 min
	pmol·mg protein^{-1}	*pmol·mg protein*$^{-1}$	
None	6.5 ± 0.4	159 ± 23.7	677 ± 40.0
Carbamylcholine	47.6 ± 2.0	131 ± 11.7	404 ± 21.3
None	4.8 ± 0.53	153 ± 2.5	639 ± 32.5
Acetyl-*p*-methylcholine	30.2 ± 1.1	139 ± 4.8	605 ± 3.4
None	5.1 ± 0.6	151 ± 3.2	642 ± 35.0
Oxotremorine	7.1 ± 0.7	148 ± 20.7	613 ± 16.6

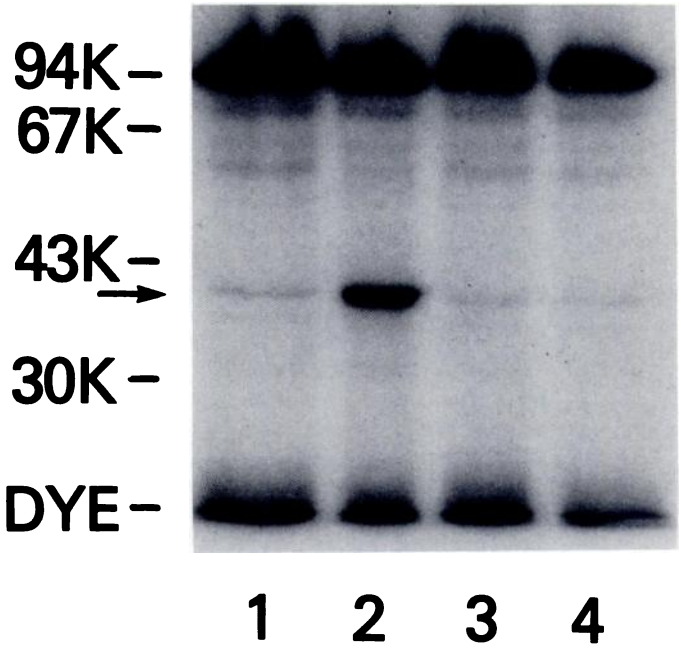

FIG. 5. *ADP-ribosylation of the 41,000-Da pertussis toxin substrate in isolated membranes*
Control cells (lanes *1* and *2*) and toxin-treated cells (lanes *3* and *4*) were used. Membranes were isolated and incubated with [^{32}P]NAD and without (lanes *1* and *3*) or with (lanes *2* and *4*) pertussis toxin (10 μg/ml) before samples (40 μg of membrane protein) were subjected to electrophoresis and autoradiography. *Arrow* points to pertussis toxin substrate, M_r = 41,000 Da. The experiment was repeated in duplicate.

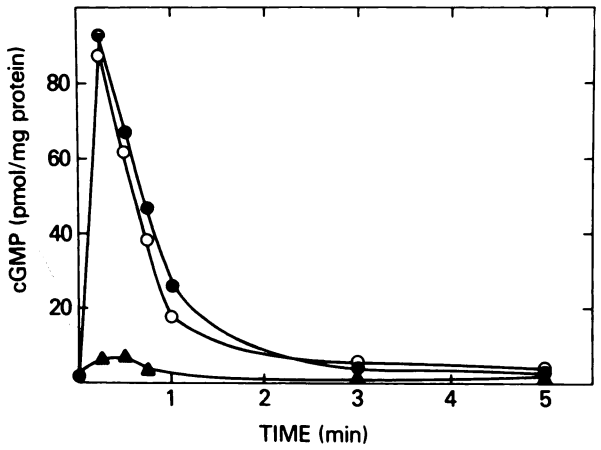

FIG. 6. *Effects of PGE_1 and carbamylcholine on cGMP content of N1E-115 cells*

Control (●, ▲) and pertussis toxin-treated (○, △) cells were incubated for the indicated time with PGE_1, 10 μg/ml (▲, △) or with PGE_1 plus 1 mM carbamylcholine (●, ○). The experiment was repeated in triplicate; the cGMP peak was confirmed on other occasions.

g, 30 min), the precipitated material was solubilized in 1% sodium dodecyl sulfate. Electrophoresis (in 12% polyacylamide gels, containing sodium dodecyl sulfate) and autoradiography were performed as previously described (27).

RESULTS

PGE_1 caused a marked increase in cAMP content of N1E-115 cells, which reached a maximum in about 3 min (Fig. 1); maximal effects of PGE_1 were observed at about 0.1 μg/ml (data not shown). Addition of carbamylcholine with PGE_1 decreased cAMP accumulation (23) (Fig. 1). Reduction by carbamylcholine of PGE_1-stimulated cAMP levels was observed in the presence of the phosphodiesterase inhibitors 3-isobutyl-1-methylxanthine, Ro-20-1724, or both (Table 1). Decreases in cAMP content caused by carbamylcholine were abolished or greatly reduced by prior treatment of cells with pertussis toxin (Figs. 1 and 2). As noted below, in the presence of [^{32}P]NAD, the toxin catalyzed the [^{32}P]ADP-ribosylation of a 41,000-Da protein in membranes prepared from these cells. Percentage inhibition by carbamylcholine in control and toxin-treated cells was greater after 3–5 min than it was at earlier times (Figs. 1, 2, A and B). The reduction in cAMP content was maximal (about 40%) at all times from 0 to 5 min after addition of PGE_1 when carbamylcholine was added 5 min before PGE_1 (data not shown). A similar delay in carbamylcholine action was observed in cells incubated with PGE_1 for 10 min prior to addition of muscarinic cholinergic agonist (data not shown). In the absence of PGE_1, carbamylcholine also decreased basal cAMP content; maximal effects (about 40%) were seen after 1–3 min (Fig. 2B). Thus, carbamylcholine suppression of cAMP content appeared to be independent of adenylate cyclase activation.

Maximal inhibition of PGE_1-stimulated cAMP accumulation was observed with 0.1 mM carbamylcholine and half-maximal inhibition with <0.01 mM (Fig. 3). The effect of carbamylcholine on PGE_1-stimulated cAMP content was dependent on the passage of cells; it was reduced in older cells (data not shown). Concentrations of carbamylcholine required to increase cGMP in these cells were an order of magnitude higher; maximal effects on cGMP content were observed with about 1 mM and half-maximal effects with 0.1 mM (Fig. 4). At 1 mM, carbamylcholine was more effective than acetylmethylcholine or oxotremorine on either stimulation of cGMP or inhibition of cAMP (Table 2). Prior treatment of cells with pertussis toxin had no effect on the cGMP response to carbamylcholine in a 30-sec incubation (Fig. 4), whereas it greatly decreased inhibition of cAMP accumulation at all concentrations of carbamylcholine (Fig. 3). The concentration of pertussis toxin used in these experiments appeared to be maximally effective; membranes prepared from toxin-treated N1E-115 cells, incubated with [^{32}P]NAD and toxin, no longer showed modification of the 41,000 Da, suggesting that the protein had been modified completely in the intact cells (Fig. 5). In experiments performed with N1E-115 cells incubated with pertussis toxin under conditions identical to those used for Fig. 5, there was no significant effect of carbachol on PGE_1-stimulated cAMP content (data not shown); pertussis toxin treatment still did not affect cGMP (data not shown). To examine in more detail whether pertussis toxin altered carbachol responsiveness, the effect on cGMP content of time of incubation with carbachol was determined (Fig. 6). After addition of carbamylcholine, the cGMP content increased rapidly, reached a maximum in about 30 sec, and then declined to almost basal levels by 3 min. The time course and magnitude of changes were unaltered by pertussis toxin (Fig. 6). Most of the guanylate cyclase activity in these cells was present in a 30,000 × *g* supernatant. The activity of the particulate and soluble enzyme was unaffected by treatment of cells with carbachol or pertussis toxin (data not shown).

DISCUSSION

Increases in cGMP have been observed following sensitization of rodents to *B. pertussis* and pertussis vaccine (28, 29). In the present studies, with N1E-115 neuroblastoma cells, the toxin had little effect on basal cGMP. In contrast to the loss of agonist effects on cAMP content following toxin treatment, the K_a for cGMP elevation by carbamylcholine was not significantly altered by prior exposure of cells to pertussis toxin. This means that in toxin-treated cells, it may be possible to study the mechanism and consequences of cGMP accumulation (guanylate cyclase activation) induced by muscarinic or similar agonists in the absence of effects secondary to adenylate cyclase inhibition. Snider *et al.* (30) proposed that the increase in cGMP caused by muscarinic agonists in N1E-115 cells results from receptor-stimulated arachidonate release. It has been suggested that in mast cells and neutrophils, pertussis toxin-catalyzed ADP-ribosylation inhibits arachidonate release and other related events (31–37). If receptor-stimulated arachidonate release is responsible for the elevation of cGMP content in N1E-115 cells, the lack of a toxin effect on cGMP content is consistent with the hypothesis that inhibition of arachidonate release by toxin-catalyzed ADP-ribosylation may be cell-type specific.

The inhibitory effect of carbamylcholine on cAMP content appeared to be greater than that obtained with oxotremorine; in other systems, carbamylcholine and oxotremorine have similar effects on cAMP accumulation (22). Effects on cAMP content were observed with much lower concentrations of carbamylcholine than those necessary to increase cGMP content. It is not clear whether the difference in carbamylcholine concentration required for effects on cAMP and cGMP levels reflects the presence of multiple receptor subtypes in these cells or the fact that the receptors are linked to the cAMP- and cGMP-generating systems through different coupling proteins. To date, studies on antagonists cross-linked to surface receptors or on purified receptors have revealed only one protein of about 80,000 Da; in some systems, lower molecular weight species have been identified, but these forms are believed to result from proteolysis (18–20).

Effects of muscarinic cholinergic agents on cAMP content are believed to proceed by two mechanisms. The first pathway involves activation of the ligand-receptor complex and G_i, leading to decreased turnover by the adenylate cyclase catalytic unit and thus, reduced cAMP synthesis (4). The second mechanism for decreasing cAMP content by muscarinic cholinergic agonists involves activation of cyclic nucleotide phosphodiesterase(s), leading to increased cAMP degradation (13, 14). Effects of pertussis toxin-catalyzed ADP-ribosylation are believed to proceed via the first pathway (4). Based on binding studies with inhibitory agonists (*e.g.* muscarinic, opiate, α_2-adrenergic) it appears that the effect of the pertussis toxin-catalyzed ADP-ribosylation is to convert receptors from a high affinity coupled state to a low affinity uncoupled state (11, 12). Presumably, the coupled receptor is that which is linked to the inhibitory GTP-binding subunit of adenylate cyclase (11, 12). Uncoupling of G_i from the inhibitory muscarinic receptor by toxin-catalyzed ADP-ribosylation in fact did not convert the receptor to a low affinity but active state since in toxin-treated cells even high carbamylcholine concentrations were not effective in depressing cAMP content.

Pertussis toxin sensitivity of the carbamylcholine-inhibited cAMP accumulation may be used to distinguish events dependent on adenylate cyclase from those acting through phosphodiesterase. Muscarinic cholinergic receptor-mediated inhibition of cellular cAMP levels through adenylate cyclase is sensitive to pertussis toxin (11, 38), whereas recent studies by Hughes *et al.* (39) indicate that attenuation of cAMP accumulation through phosphodiesterase activation is not subject to toxin inhibition. The effects of carbamylcholine appear to be mediated in N1E-115 cells predominantly through adenylate cyclase inhibition rather than by phosphodiesterase activation. The presence of phosphodiesterase inhibitors such as IBMX or Ro-20-1724 did not reduce carbamylcholine effectiveness; in studies by Miot *et al.* (14) on thyroid slices, agents that decreased cAMP through phosphodiesterase activation exhibited diminished potency in the presence of some phosphodiesterase inhibitors, the effectiveness of the inhibitor being related to the selectivity of phosphodiesterases present in the system. Since toxin blocked the effects of carbamylcholine, it would appear that in this system, the inhibitory agonist works primarily through the adenylate cyclase system.

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Send reprint requests to: Dr. Joel Moss, National Institutes of Health, Building 10, Room 5N307, Bethesda, MD 20205.