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Biochemistry

Pertussis toxin differentiates between two mechanisms of attenuation of cyclic AMP accumulation by muscarinic cholinergic receptors

(adenylate cyclase/phosphodiesterase/guanine nucleotide regulatory proteins)

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ABSTRACT **It has been proposed elsewhere [Meeker, R. B. & Harden, T. K. (1982) *Mol. Pharmacol.* 22, 310–319] that muscarinic cholinergic receptor-mediated attenuation of cAMP accumulation occurs through activation of phosphodiesterase in 1321N1 human astrocytoma cells. Pertussis toxin, which ADP-ribosylates the guanine nucleotide regulatory protein involved in receptor-mediated inhibition of adenylate cyclase (N_i), has been utilized to further differentiate between the mechanism of cholinergic regulation of cAMP metabolism in 1321N1 cells and the mechanism involving inhibition of adenylate cyclase in other tissues. Muscarinic receptor-mediated regulation of cAMP accumulation in NG108-15 neuroblastoma–glioma cells occurs through inhibition of adenylate cyclase. Pretreatment of these cells with pertussis toxin completely blocked the capacity of carbachol to attenuate cAMP accumulation. In contrast, concentrations of pertussis toxin two to three orders of magnitude higher than those effective in NG108-15 cells had no effect on muscarinic receptor-mediated attentuation of cAMP accumulation in 1321N1 cells. In addition, no effect of pertussis toxin was observed either on the control rate or the carbachol-stimulated rate of cAMP degradation measured directly in intact 1321N1 cells. A 41,000 M_r protein previously proposed to be the α subunit of N_i was labeled during incubation of a plasma membrane fraction from 1321N1 cells with [^{32}P]NAD and pertussis toxin. Pertussis toxin is apparently active in 1321N1 cells, since this protein substrate was not labeled in plasma membrane preparations from cells previously incubated with toxin. Functional activity of N_i was demonstrated by the observation that guanosine 5′-[γ-thio]triphosphate- and GTP-mediated inhibition of forskolin-stimulated adenylate cyclase activity occurred in cell-free preparations from 1321N1 cells. The inhibitory activity of these guanine nucleotides was lost in membrane preparations from pertussis toxin-treated cells. The data suggest that adenylate cyclase is not involved in cholinergic action in 1321N1 cells and, furthermore, N_i is not involved in muscarinic receptor-mediated activation of phosphodiesterase in these cells. Thus, pertussis toxin can be used to differentiate between two mechanisms of cholinergic regulation of cAMP metabolism.**

Activation of muscarinic cholinergic receptors results in attenuation of cAMP accumulation in intact cells and inhibition of adenylate cyclase activity in cell-free preparations (1–6). The GTP dependence of this inhibition (4, 5), the negative heterotropic effects of guanine nucleotides on agonist binding to inhibitory receptors (6–8), and knowledge of the role of the stimulatory guanine nucleotide regulatory protein (N_s) in the mechanism of activation of adenylate cyclase (9) have led to the proposal that an inhibitory guanine nucleotide regulatory protein (N_i) is involved in the mechanism of receptor-mediated inhibition of adenylate cyclase (10). Activation and inhibition of adenylate cyclase are differentially sensitive to Mn^{2+} (10), GTP (10–12), and chemical modification by *N*-ethylmaleimide (6, 13). Platelets from pseudohypoparathyroid patients are functionally deficient in N_s but express both epinephrine-mediated inhibition of adenylate cyclase and GTP-sensitive high-affinity binding of agonists to α_2-adrenergic receptors, suggesting that N_i is fully functional in these cells (14). Furthermore, cyc$^-$ S49 lymphoma cells lack functional N_s but express active N_i (15).

Pertussis toxin, which ADP-ribosylates N_i, blocks inhibitory coupling of muscarinic and other receptors to adenylate cyclase (16, 17). N_s is not a substrate for pertussis toxin (18, 19). Cholera toxin, which ADP-ribosylates N_s (9), does not ADP-ribosylate N_i (18, 19) and has no effect on receptor-mediated inhibition of adenylate cyclase (20). Thus, muscarinic and other inhibitory receptors regulate adenylate cyclase through a guanine nucleotide regulatory protein that is distinct from the protein involved in regulation of adenylate cyclase by stimulatory receptors; this inhibitory regulatory protein can be identified by its susceptibility to modification by pertussis toxin.

Although muscarinic receptor activation clearly inhibits adenylate cyclase in cell-free preparations from a variety of tissues, analyses of cAMP metabolism in 1321N1 astrocytoma cells (21, 22), WI-38 fibroblasts (23, 24), and thyroid slices (25) have led to the conclusion that muscarinic receptor stimulation also results in activation of phosphodiesterase. We have proposed that this is the sole mechanism by which cAMP levels are reduced in 1321N1 astrocytoma cells, since, in the presence of phosphodiesterase inhibitors, cholinergic agonist-mediated decreases in cAMP levels do not occur, and muscarinic receptor-mediated inhibition of adenylate cyclase is not detected in membrane preparations from these cells (21).

One explanation for the mechanism of regulation of cAMP metabolism in 1321N1 cells is that muscarinic receptors are coupled through N_i to activation of phosphodiesterase rather than inhibition of adenylate cyclase. The occurrence of guanine nucleotide-mediated activation of phosphodiesterase in several tissues supports this possibility. For example, a well-characterized GTP regulatory protein, transducin, couples the light-activated receptor (rhodopsin) of the retinal rod outer segment to activation of a cGMP phosphodiesterase (26). On the basis of amino acid composition and electrophoretic patterns of the proteolyzed polypeptides, transducin, N_s, and N_i appear to be structurally similar guanine nucleotide regulatory proteins (27). A role for a guanine nucleotide

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Abbreviations: N_s, stimulatory guanine nucleotide regulatory protein; N_i, inhibitory guanine nucleotide regulatory protein; ISO, (−)-isoproterenol; CARB, carbamoylcholine; PGE_1, prostaglandin E_1; GTP[γS], guanosine 5′-[γ-thio]triphosphate.

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regulatory protein in the regulation of phosphodiesterase also is suggested by the reports that guanine nucleotides activate an insulin-stimulated cAMP phosphodiesterase (28) and a soluble phosphodiesterase (29) from rat liver. In addition, de Mazancourt and Guidicelli (30) have reported that a membrane phosphodiesterase from rat brain is stimulated in a guanine nucleotide-dependent fashion by N^6-phenylisopropyladenosine. Interestingly, pertussis toxin ADP-ribosylates transducin (31, 32) and blocks the light-stimulated hydrolysis of GTP and cGMP in retinal photoreceptors (31). Elks *et al.* (33) also have reported that the insulin-induced activation of particulate cAMP phosphodiesterase activity in 3T3-L1 adipocytes is blocked by pretreatment of cells with pertussis toxin.

In this study we have utilized pertussis toxin to further examine the role of N_i in the muscarinic receptor-mediated attenuation of cAMP accumulation and activation of phosphodiesterase in 1321N1 astrocytoma cells. The results obtained with this cell line are compared to data obtained with a cell line in which regulation of cAMP levels by muscarinic receptors apparently occurs through inhibition of adenylate cyclase. Preliminary reports of this work have been presented (34, 35).

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium, trypsin, fetal calf serum, and defibrinated sheep blood were purchased from GIBCO. (−)-Isoproterenol [ISO; as the (+)-bitartrate], carbamoylcholine (CARB), (±)-propranolol·HCl, cAMP, creatine phosphate, creatine kinase, ATP, GTP, and isobutylmethylxanthine were obtained from Sigma. Guanosine 5′-[γ-thio]triphosphate (GTP[γS]) was purchased from Boehringer Mannheim and forskolin was from Calbiochem. Bordet–Gengou agar base was purchased from Oxoid (Columbia, MD) and [2,8-^{3}H]adenine (25–50 Ci/mmol; 1 Ci = 37 GBq) was from ICN. Affi-Gel blue (100–200 mesh) was obtained from Bio-Rad and hydroxylapatite was from Calbiochem.

Cell Culture. 1321N1 human astrocytoma cells (21) and NG108-15 neuroblastoma–glioma cells (36) were grown as previously described. For pertussis toxin treatment, fresh medium containing toxin, penicillin (50 units/ml), and streptomycin (50 μg/ml) was transferred to the dishes.

Preparation of Pertussis Toxin. *Bordetella pertussis* (strains 165 and Tohama, phase I) was grown for 4 days at 37°C on Bordet–Gengou blood agar plates, harvested, and inoculated into a small volume of modified Stainer–Scholte liquid culture medium supplemented with 3% fetal calf serum (37). The cell suspension was maintained in roller bottles at 37°C for 48 hr, diluted 1:100 with serum-free Stainer–Scholte medium, and shaken for an additional 48 hr. The cells were removed from the suspension by centrifugation for 30 min and the supernatant was stored at 4°C. The pertussis supernatant was partially purified by chromatography on hydroxylapatite according to the method of Yajima *et al.* (38) or by chromatography on Affi-Gel blue according to the method of Sekura *et al.* (39).

Measurement of cAMP Accumulation. cAMP accumulation was monitored as previously described (21). Briefly, 1321N1 cells grown for 7–10 days in 12-well culture dishes (at an initial seeding density of 40,000 cells per well) or confluent NG108-15 cells in 12-well dishes (subcultured 2–3 days previously from a confluent stock plate) were incubated for 1 hr with 1 ml of Hepes-buffered (20 mM, pH 7.5) Eagle's medium containing [^{3}H]adenine (1–2 μCi/ml for 1321N1 cells and 0.5 μCi/ml for NG108-15 cells). The medium was aspirated, the cells were washed twice with 1 ml of Hepes/Eagle's medium, and a fresh 0.95-ml aliquot of Hepes/Eagle's medium was added. [^{3}H]cAMP synthesis was induced by the addition of 50 μl of 10 μM ISO or 1 μM PGE_1, and inhibition of [^{3}H]cAMP accumulation was measured by the simultaneous addition of CARB. The reactions proceeded for 10 min and were terminated by aspiration and the immediate addition of 1 ml of 5% trichloroacetic acid containing 0.5 mM cAMP. The [^{3}H]cAMP formed during the hormonal challenge was separated from [^{3}H]ATP by sequential passage over Dowex and alumina columns as previously described (6). Aliquots of the cAMP fractions were assayed spectrophotometrically at 259 nm to correct for recovery of cAMP (usually 50–80%).

Preparation of [α-^{32}P]NAD. [α-^{32}P]ATP synthesized by the method of Johnson and Walseth (40) was used to synthesize [α-^{32}P]NAD according to the method of Cassel and Pfeuffer (41). Purity of nucleotides was assessed by chromatography on polyethyleneimine (PEI)-cellulose (Brinkmann).

[^{32}P]ADP-ribosylation. Preparation of plasma membrane fractions was as previously described (42). ADP-ribosylation was carried out at 30°C for 90 min in a final volume of 0.5 ml containing pertussis toxin (10 μg of protein), gradient-purified membranes (120 μg of protein), 10 μM [α-^{32}P]NAD (1–2 Ci/ mmol), 1 mM thymidine, 0.5 mM ATP, 5 mM dithiothreitol, 2.5 mM $MgCl_2$, 6 mM potassium phospho*enol*pyruvate, pyruvate kinase at 10 μg/ml, and 100 mM Tris, pH 8.0. The reaction was stopped by the addition of 1 ml of ice-cold 50 mM Tris, pH 7.4, and centrifugation at 10,000 × *g* for 5 min. The labeled membranes were washed three times by resuspension in ice-cold 50 mM Tris, pH 7.4, and centrifugation, incubated overnight in 100 μl of Laemmli sample buffer (43), and then applied to a 12.5% sodium dodecyl sulfate discontinuous polyacrylamide gel with a 4% stacking gel. Gels were fixed, stained with Coomassie brilliant blue, and dried, and autoradiograms were developed, using Kodak XAR-15 film.

Adenylate Cyclase Assay. Adenylate cyclase activity was quantitated as previously described (6). Each assay tube contained (final concentrations) 0.1 mM [α-^{32}P]ATP (1.5 μCi per assay), 1 mM [^{3}H]cAMP (40,000 cpm/assay), 8 mM phosphocreatine, creatine kinase (6 units per assay), 1 mM isobutylmethylxanthine, 0.1 mM EGTA, 1.2 mM $MgCl_2$, 30 mM Tris at pH 8.25, 100 μM forskolin, and various concentrations of guanine nucleotides in a final volume of 0.1 ml. Reactions were started by adding 10–50 μg of membrane protein. The tubes were incubated for 3 min at 30°C and the reactions were terminated by the addition of 0.9 ml of 5% trichloroacetic acid. [α-^{32}P]ATP was separated from [^{32}P]-cAMP by sequential column chromatography over Dowex and neutral alumina (6). Membrane protein was determined by the method of Lowry *et al.* (44).

RESULTS

The activity of pertussis toxin was examined in a cell line (NG108-15 neuroblastoma–glioma cells) that expresses a well-characterized muscarinic receptor-linked adenylate cyclase system. Concentration-effect curves for CARB-mediated inhibition of cAMP accumulation were generated in NG108-15 cells pretreated overnight with pertussis toxin or vehicle (Fig. 1). CARB (100 μM) inhibited PGE_1-stimulated cAMP accumulation by 65% in control cells. Pretreatment with pertussis toxin enhanced PGE_1-stimulated cAMP accumulation. At the high concentration of pertussis toxin, CARB failed to attenuate PGE_1-stimulated cAMP accumulation. In addition, the capacity of epinephrine and morphine to inhibit cAMP accumulation through α_2-adrenergic and opiate receptors, respectively, was blocked by pretreatment with pertussis toxin (data not shown). These results are consistent with those previously reported by Kurose *et al.* (17). Pretreatment of NG108-15 cells with pertussis toxin also resulted in a loss of guanine nucleotide-sensitive binding of the agonist [^{3}H]oxotremorine-M (45) to muscarinic receptors without affecting the binding of the antagonist [^{3}H]quinuclidinyl benzilate (data not shown). In contrast to the results

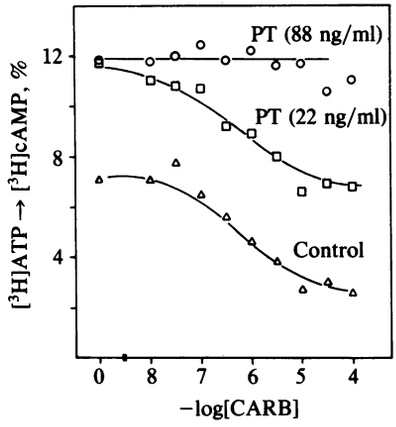

FIG. 1. Pertussis toxin-induced blockade of muscarinic receptor-mediated inhibition of cAMP accumulation in NG108-15 cells. NG108-15 cells were treated overnight with pertussis toxin at 88 ng/ml (○) or 22 ng/ml (□) or with vehicle (△). cAMP accumulation was measured in the presence of 1 μM PGE_1 and the indicated concentrations of CARB. The data are plotted as the percent conversion of [^{3}H]ATP to [^{3}H]cAMP and are the mean of quadruplicate determinations. The data are representative of results from two experiments.

obtained with NG108-15 cells, concentrations of toxin greater than two orders of magnitude higher than those maximally effective in NG108-15 cells had no effect on the capacity of muscarinic receptors to attenuate ISO-stimulated cAMP accumulation in 1321N1 cells (Fig. 2).

Since muscarinic receptor activation enhances cAMP degradation in 1321N1 cells (21, 22), the effect of pertussis toxin on the kinetics of cAMP degradation was examined directly. cAMP synthesis was stimulated with 10 μM ISO for 10 min. Propranolol then was added to the cells to block further cAMP synthesis and the decrease in cAMP levels, which is a function of phosphodiesterase activity, was measured in the absence or presence of CARB over the following 3 min. As we have previously shown (21, 22), the disappearance of cAMP was a first-order process (Fig. 3). The rate constant for [^{3}H]cAMP degradation in control cells was 0.46 min^{-1}, and CARB enhanced degradation approximately 2-fold (k_{deg} = 0.90 min^{-1}). cAMP degradation either in the absence (k_{deg} = 0.44 min^{-1}) or presence (0.82 min^{-1}) of CARB was not affected by pretreatment of the cells with pertussis toxin.

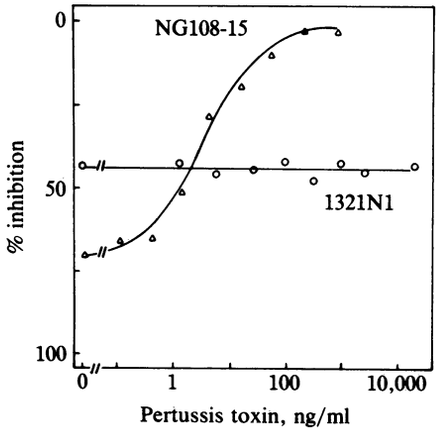

FIG. 2. Differential effects of pertussis toxin on muscarinic receptor-mediated attenuation of cAMP accumulation in NG108-15 and 1321N1 cells. NG108-15 cells (△) and 1321N1 cells (○) were preincubated overnight with the indicated concentrations of pertussis toxin. The cells were challenged with 1 μM PGE_1 (NG108-15) or 10 μM ISO (1321N1) alone or in the presence of 100 μM CARB. The results are plotted as the percent inhibition of cAMP accumulation observed in the presence of CARB. The data are from quadruplicate determinations and are representative of experiments carried out with three different preparations of toxin.

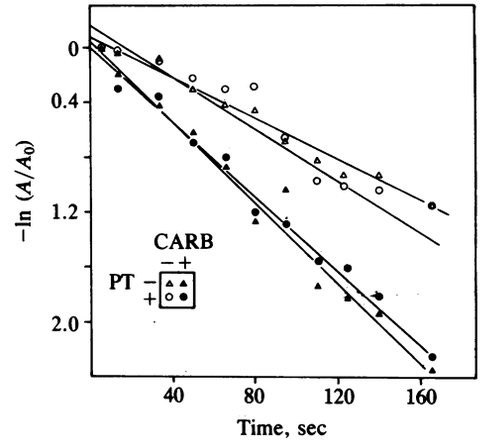

FIG. 3. Effect of pertussis toxin on cAMP degradation. 1321N1 cells were preincubated overnight in the presence (○, ●) or absence (△, ▲) of pertussis toxin (PT) at 100 ng/ml. The cells were then labeled with [^{3}H]adenine and [^{3}H]cAMP synthesis was induced with 10 μM ISO. After 10 min, the synthetic reaction was stopped by the addition of 50 μl of propranolol (△, ○; 10 μM final concentration) or propranolol (10 μM, final) + CARB (▲, ●; 100 μM final concentration). The reaction was stopped at the indicated times by aspiration and addition of 1 ml of 5% trichloroacetic acid containing 0.5 mM cAMP. The results are plotted as the natural logarithm of the cAMP concentration at the indicated times (A) divided by the initial cAMP concentration (A_0). The data are representative of results from two experiments.

Any conclusions from the results presented thus far would have to be based on the supposition that pertussis toxin was fully active in intact 1321N1 cells. Experiments were carried out to examine this possibility. As we have previously reported (42), the presence of N_i in 1321N1 cells is suggested by the occurrence of a 41,000 M_r substrate for pertussis toxin. To confirm that pertussis toxin indeed modified this protein in intact 1321N1 cells, cells were pretreated with toxin and lysed, and a membrane fraction was prepared. This fraction then was incubated in the presence of pertussis toxin and [^{32}P]NAD. As is illustrated in Fig. 4, a 41,000 M_r polypeptide was labeled in the presence of pertussis toxin and [^{32}P]NAD in control membranes, but no ^{32}P was incorporat-

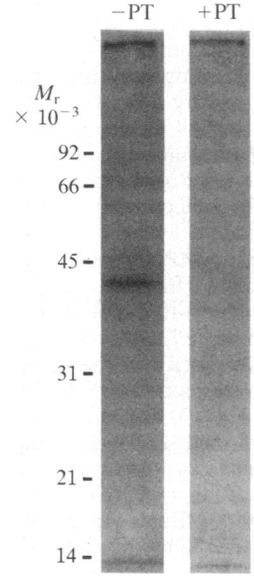

FIG. 4. Inhibition of ADP-ribosylation of the M_r 41,000 protein by prior treatment of 1321N1 cells with pertussis toxin. 1321N1 cells were treated with either vehicle (−PT) or with partially purified pertussis toxin at 250 ng/ml (+PT). Plasma membranes were prepared, incubated with pertussis toxin and [α-^{32}P]NAD, and electrophoresed on 12.5% polyacrylamide gels. The autoradiogram of the dried gel was exposed for 72 hr. The positions of standard proteins of known molecular weight are shown on the left. Similar results were obtained in four additional experiments.

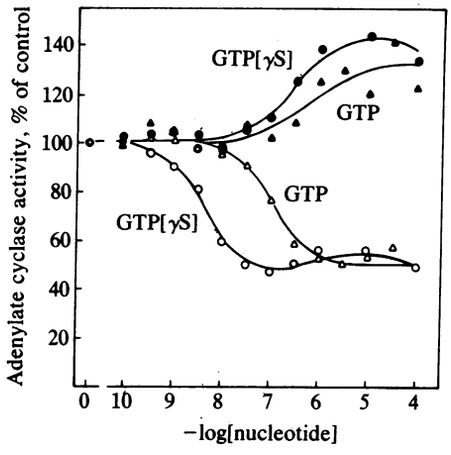

FIG. 5. Guanine nucleotide-mediated inhibition of forskolin-stimulated adenylate cyclase activity in 1321N1 cells. 1321N1 cells were incubated overnight in the absence (○, △) or presence (●, ▲) of pertussis toxin (100 ng/ml) and lysed, and adenylate cyclase was assayed for 3 min in the presence of 100 μM forskolin and the indicated concentrations of GTP or GTP[γS]. The results are plotted as the percent of adenylate cyclase activity in the absence of guanine nucleotides and are the mean of quadruplicate determinations. Forskolin-stimulated adenylate cyclase activity in the absence of guanine nucleotides was 1000 pmol/min per mg and 420 pmol/min per mg for membranes from control and pertussis toxin-treated cells, respectively. The data are representative of results obtained in two experiments.

ed in membranes prepared from cells previously treated with toxin. This result is consistent with the idea that pertussis toxin ADP-ribosylates N_i in intact 1321N1 cells.

The presence of a 41,000 M_r substrate for pertussis toxin in 1321N1 cells does not necessarily prove the presence of functional N_i. Indeed, the lack of muscarinic receptor-mediated inhibition of adenylate cyclase in spite of the presence of muscarinic receptors and adenylate cyclase could be explained by the absence of N_i or its presence in a functionally altered form. Seamon and Daly (46) have presented data supporting the idea that inhibition of forskolin-stimulated adenylate cyclase activity by guanine nucleotides provides a measure of the functional activity of N_i. That is, since forskolin "maximally" activates adenylate cyclase, the stimulatory effects of guanine nucleotides are not observed in the presence of forskolin, and, thus, the inhibitory effects of these nucleotides mediated through N_i become apparent. As illustrated in Fig. 5, both GTP and GTP[γS] inhibited forskolin-stimulated adenylate cyclase activity in 1321N1 membranes. The values for the half-maximal effect for each of the nucleotides were similar to those observed in membranes from other tissues (15, 36). In contrast to control membranes, neither guanine nucleotide inhibited (in fact, a stimulation was observed) adenylate cyclase in membranes from pertussis toxin-pretreated cells. In addition to blockade of the inhibitory effects of guanine nucleotides, pertussis toxin pretreatment resulted in a decrease in forskolin-stimulated activity measured in the absence of a guanine nucleotide.

DISCUSSION

Earlier work by this laboratory (21, 22) led to the proposal that activation of muscarinic cholinergic receptors in 1321N1 cells reduces cAMP levels by activating phosphodiesterase: (*i*) the time necessary to attain maximal cAMP levels is reduced in the presence of a muscarinic receptor agonist; (*ii*) inhibitors of phosphodiesterase noncompetitively block the inhibitory effect of muscarinic receptors on cAMP accumulation; (*iii*) muscarinic receptor agonists increase the rate of cAMP degradation assessed directly in intact cells; (*iv*) the Ca^{2+} ionophore A23187 mimics the effects of muscarinic receptor agonists, and in the absence of extracellular Ca^{2+} the effects of muscarinic receptor stimulation are lost; (*v*) although muscarinic receptor-mediated inhibition of adenylate cyclase occurs in broken cell preparations from other cell types, no evidence for inhibition has been obtained in homogenates of 1321N1 cells.

The current work with pertussis toxin adds further support to the idea that a mechanism not involving adenylate cyclase is responsible for muscarinic receptor-mediated reduction in cAMP levels in 1321N1 cells. The functional presence of N_i is apparently necessary for the occurrence of receptor or guanine nucleotide-mediated inhibition of adenylate cyclase; pertussis toxin ADP-ribosylates and functionally inactivates N_i. Thus, the lack of an effect of pertussis toxin on muscarinic receptor-mediated attenuation of cAMP accumulation is further proof of the hypothesis that cholinergic regulation of cAMP metabolism in 1321N1 cells occurs through phosphodiesterase rather than adenylate cyclase.

The work has additional ramifications. There is ample precedent regarding a role for a guanine nucleotide-binding protein in the regulation of phosphodiesterase. Studies suggesting such an involvement have ranged from the well-characterized retinal rhodopsin–phosphodiesterase system, in which transducin serves an obligatory coupling function (26, 31), to the less well-understood recent findings suggesting regulation of liver phosphodiesterase by GTP (28, 29). It recently has been reported that pertussis toxin ADP-ribosylates and modifies the function of transducin (31, 32) and that pertussis toxin blocks the insulin-induced activation of particulate phosphodiesterase activity in 3T3 adipocytes (33). Thus, not only is there precedent for a role of a guanine nucleotide regulatory protein in the regulation of phosphodiesterase activity, but pertussis toxin has been utilized to modify these activities. In spite of these levels at which N_i might subserve a role in 1321N1 cells, the complete lack of an effect of very high concentrations of pertussis toxin relative to those effective in NG108-15 cells suggests that N_i is not involved in muscarinic receptor-mediated activation of phosphodiesterase.

Although there is no reason to believe that the 41,000 M_r substrate for pertussis toxin in 1321N1 membranes is not the pertussis toxin substrate observed by others in a variety of tissues, we cannot with absolute confidence conclude that this protein is N_i that is fully functional in all regards. Nonetheless, the fact that guanine nucleotides inhibit forskolin-stimulated adenylate cyclase activity is consistent with the idea that the interface between N_i and the other components of the adenylate cyclase system is normal in 1321N1 cells. A functionally relevant interaction between N_i and muscarinic receptors in 1321N1 cells has not been identified. Perhaps our failure to observe muscarinic receptor-mediated inhibition of adenylate cyclase is due to failure to identify optimal assay conditions; alternatively, a muscarinic receptor-linked adenylate cyclase activity could become "uncoupled" upon cell lysis and membrane preparation. Our prejudice, however, is that neither of these possibilities is responsible for failure to observe cholinergic regulation of adenylate cyclase. The complete reversal of muscarinic receptor-mediated attenuation of cAMP accumulation by phosphodiesterase inhibitors, as well as the extent of the enhancement of cAMP degradation in the presence of cholinergic agonists, indicates that muscarinic receptor-mediated activation of phosphodiesterase can fully account for the attenuation of cAMP accumulation.

A possible explanation for the data obtained with 1321N1 cells is that a muscarinic receptor subtype exists on these cells that is incapable of coupling through N_i to regulate adenylate cyclase. In this regard, muscarinic receptor subtypes

have been proposed to exist (47). It is logical to propose that these receptor subtypes would be coupled to different biochemical mechanisms; the two most likely mechanisms would involve adenylate cyclase and Ca^{2+} mobilization. We have recently shown that muscarinic receptor activation in 1321N1 cells causes a marked increase in phosphoinositide breakdown and unidirectional efflux of $^{45}Ca^{2+}$ from $^{45}Ca^{2+}$-prelabeled cells (48). Thus, 1321N1 cells may express a muscarinic receptor subtype that is different from that on NG108-15 cells and is incapable of coupling to N_i. In this regard, radioligand binding studies examining the affinities of the purportedly muscarinic receptor-selective antagonist pirenzepine and a series of agonists have revealed differences in the relative drug selectivity of the muscarinic receptor populations of 1321N1 versus NG108-15 cells (49).

In summary, although examples of a role for a guanine nucleotide regulatory protein in the regulation of phosphodiesterase are available, we have been unable to confirm the involvement of such a protein in the mechanism of muscarinic receptor regulation of phosphodiesterase in 1321N1 cells. Obviously, this conclusion relies on the activity of pertussis toxin in blocking any involvement of N_i in the cholinergic regulation of phosphodiesterase. Thus, N_i could be involved in some unknown pertussis toxin-insensitive mechanism. However, in light of other findings with pertussis toxin regarding transducin and N_i, the most parsimonious interpretation of the results is that N_i is not involved in muscarinic receptor-mediated activation of phosphodiesterase and that two separate mechanisms whereby muscarinic receptors attenuate cAMP accumulation can be differentiated on the basis of their susceptibility to inactivation by pertussis toxin. In light of the recent report by Gomperts (50), the possibility that an unknown guanine nucleotide regulatory protein plays a role in coupling muscarinic receptors to mobilization of Ca^{2+} and activation of phosphodiesterase cannot be discounted on the basis of this work.

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