MOLECULAR PHARMACOLOGY, 27:32–37

Guanine Nucleotide-Sensitive, High Affinity Binding of Carbachol to Muscarinic Cholinergic Receptors of 1321N1 Astrocytoma Cells Is Insensitive to Pertussis Toxin

TONY EVANS,[1] MICHAEL W. MARTIN, ARLENE R. HUGHES, AND T. KENDALL HARDEN[2]

Department of Pharmacology, University of North Carolina, School of Medicine, Chapel Hill, North Carolina 27514

Received July 2, 1984; Accepted September 21, 1984

SUMMARY

Activation of muscarinic cholinergic receptors of 1321N1 human astrocytoma cells attenuates cyclic AMP accumulation. This effect results from an activation of phosphodiesterase with no direct inhibition of adenylate cyclase activity. In spite of this lack of coupling of muscarinic receptors to adenylate cyclase, guanine nucleotides reduce the apparent binding affinity of the agonist carbachol in a washed membrane preparation of 1321N1 cells. The order of potency for this effect is guanosine 5′-*O*-(3-thiotriphosphate) > 5′-guanylyl-imidodiphosphate = GTP = GDP; ATP has no effect. The occurrence of a M_r = 41,000 protein labeled in the presence of [^{32}P]NAD and pertussis toxin as well as the occurrence of guanine nucleotide-mediated inhibition of forskolin-stimulated adenylate cyclase activity indicate that the functional inhibitory guanine nucleotide regulatory component of adenylate cyclase (N_i) is present in 1321N1 cells. Pertussis toxin pretreatment of NG108-15 neuroblastoma × glioma cells, which express muscarinic receptors that link through N_i to inhibit adenylate cyclase, blocked the GTP-sensitive, high affinity binding of carbachol. In contrast, pretreatment of 1321N1 cells with a concentration of pertussis toxin that blocked [^{32}P]ADP ribosylation of the M_r = 41,000 substrate and GTP-mediated inhibition of forskolin-stimulated adenylate cyclase activity had no effect on GTP-sensitive high affinity binding of carbachol. These results suggest that muscarinic cholinergic receptors of 1321N1 cells couple to a guanine nucleotide regulatory protein that is distinct from N_i.

INTRODUCTION

The biochemical sequelae of the interaction of agonists with muscarinic cholinergic receptors have not been completely defined. However, in several target tissues, stimulation of muscarinic receptors results in an attenuation of adenylate cyclase activity (1–3). Advances in knowledge of the mechanisms of hormonal regulation of adenylate cyclase are thus applicable to the quest for understanding of cholinergic action. A key step in defining the mechanism of hormonal stimulation of adenylate cyclase was the realization that a guanine nucleotide regulatory protein, N_s,[3] subserved a central role in information transfer from agonist-occupied receptors to the catalytic component of adenylate cyclase (4). It has recently become clear that a second regulatory protein, N_i, which shares considerable structural homology with N_s (5, 6), serves to couple muscarinic cholinergic and other inhibitory receptors to the attenuation of adenylate cyclase (7). A number of functional properties of N_i also closely resemble those of N_s (7). Gilman and co-workers (7, 8) recently have proposed that agonist-occupied inhibitory receptors catalyze the dissociation of N_i into its M_r = 41,000 and 35,000 subunits with the latter in turn interacting with the active M_r = 45,000 subunit of N_s to inhibit enzyme activity.

The elegant work of Ui and co-workers (9–12) has provided a basis for both physically and biochemically distinguishing N_i from N_s. That is, the M_r = 41,000 α-subunit of N_i is selectively ADP-ribosylated and inactivated by pertussis toxin (5, 9, 12). Thus, the capacity of muscarinic and other inhibitory receptors for inhibition of adenylate cyclase is lost in pertussis toxin-treated cells; stimulatory receptor-mediated regulation of ade-

This work was supported by United States Public Health Service Grant GM29536.

[1] Present address: Department of Pharmacology, The University of Calgary, Calgary, Alberta, Canada T2N 4NI.

[2] Established Investigator of the American Heart Association.

[3] The abbreviations used are: N_s, stimulatory guanine nucleotide regulatory component of adenylate cyclase; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Gpp(NH)p, 5′-guanylylimidodiphosphate; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; N_i, inhibitory guanine nucleotide regulatory component of adenylate cyclase; QNB, quinuclidinyl benzilate.

0026-895X/85/01032-06$02.00/0

nylate cyclase remains intact. Not only does pertussis toxin block receptor-mediated inhibition of adenylate cyclase, but the capacity of agonists to form a GTP-sensitive high affinity binding complex with muscarinic and other inhibitory receptors also is lost in pertussis toxin-treated membranes (11, 13, 14). In analogy with the β-adrenergic receptor system (4), it is likely that the negative heterotropic effect of guanine nucleotides on agonist binding to muscarinic receptors is a reflection of the dissociation of a high affinity complex of agonist·receptor·N_i.

Although the most prominent means of regulation of cyclic AMP levels by cholinergic agonists apparently involves N_i and inhibition of adenylate cyclase, recent work from this laboratory using clonal cell lines (15–17), in conjunction with that from other laboratories (18, 19), suggests that muscarinic cholinergic receptors also regulate cyclic AMP metabolism through a second mechanism involving a cholinergic agonist-stimulated activation of phosphodiesterase. In 1321N1 astrocytoma cells, this activation of phosphodiesterase is Ca^{2+} dependent (15) and occurs concomitantly with a cholinergic agonist-stimulated increase in phosphoinositide breakdown and $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-prelabeled cells (20). No inhibition of adenylate cyclase activity by cholinergic agonists occurs in cell-free preparations from 1321N1 cells (15). The mechanisms of muscarinic receptor-mediated regulation of cyclic AMP metabolism can be further distinguished by the action of pertussis toxin. Whereas pertussis toxin has been shown to block the inhibitory effects of muscarinic receptors and other receptors on cyclic AMP accumulation or adenylate cyclase activity in many other tissues (8, 10–12, 21), it has no effect on muscarinic receptor-mediated attenuation of cyclic AMP accumulation and activation of phosphodiesterase in 1321N1 cells (21). Thus, on the basis of several lines of evidence, muscarinic receptors of 1321N1 cells apparently do not couple through N_i to attenuate cyclic AMP accumulation.

Our earlier studies indicated little or no regulation by guanine nucleotides of oxotremorine binding to membranes of 1321N1 cells. However, as observed in other systems (22, 23) our subsequent examination of the cholinergic regulation of phosphoinositide breakdown and Ca^{2+} mobilization in 1321N1 cells indicated that oxotremorine is a partial agonist at muscarinic receptors of these cells (20). We now report high affinity, guanine nucleotide-sensitive binding of the "full" agonist carbachol to the muscarinic receptors of 1321N1 cells. Since this binding is not sensitive to either pertussis toxin or cholera toxin pretreatment, it is suggested that muscarinic receptors in 1321N1 cells couple with a guanine nucleotide regulatory protein distinct from N_i and N_s.

MATERIALS AND METHODS

Materials. DMEM, trypsin, fetal calf serum, and defibrinated sheep blood were purchased from Grand Island Biological Company, Grand Island, NY. Carbachol, atropine, cyclic AMP, creatine phosphate, creatine phosphokinase, ATP, GTP, Gpp(NH)p, GDP, and IBMX were obtained from Sigma Chemical Co. (St. Louis, MO.). GTPγS was purchased from Boehringer Mannheim and forskolin and cholera toxin were from Calbiochem. Bordet-Gengou agar base was purchased from Oxoid USA (Columbia, MD) and [^{3}H]QNB (33.1 Ci/mmol) was from New England Nuclear Corporation (Boston, MA). Affi-Gel Blue (100–200 mesh) was obtained from Bio-Rad (New York).

Cell culture. 1321N1 human astrocytoma cells (15) and NG108-15 neuroblastoma × glioma cells (24) 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 (strain 165) was grown for four days as previously described (21). Pertussis toxin was partially purified by chromatography on Affi-Gel Blue according to the method of Sekura *et al.* (25).

ADP-ribosylation of N_i by pertussis toxin. [α-^{32}P]ATP synthesized by the method of Johnson and Walseth (26) was used to synthesize [^{32}P]NAD according to the method of Cassel and Pfeuffer (27). Preparation of plasma membrane fractions (21, 28) and pertussis toxin-catalyzed ADP-ribosylation (21) were carried out as previously described. The extent of incorporation of [^{32}P]ADP ribose into the M_r = 41,000 subunit of N_i was assessed by densitometric analysis of autoradiograms using a Kontes Model 800 fiber optics densitometric scanner and Hewlett Packard Model 3390A integrator.

Adenylate cyclase assay. 1321N1 membranes were prepared by aspirating the medium and hypotonically lysing the cells on ice for 15 min in 10 mM Hepes, 10 mM EDTA. The cells were scraped from the plate with a rubber spatula and centrifuged at 500 × *g* for 5 min to remove whole cells and nuclei. The resulting supernatant was prepared in 20 mM Tris (pH 8.25), 1 mM IBMX, 0.1 mM EGTA, 1.2 mM $MgCl_2$. Adenylate cyclase activity was quantitated as previously described (24).

Muscarinic cholinergic receptor assay. Competition binding experiments, using [^{3}H]QNB (20–25 pM) as the radioligand, were performed essentially as we have previously described (15). Assays were in a 10-ml volume of 10 mM Hepes, 5 mM Mg^{2+} and incubations were carried out at 37° for 90 min. Binding reactions were initiated by the addition of washed membranes and terminated by filtration through Schleicher and Schuell No. 30 glass fiber filters. Each filter was washed with 10 ml of 10 mM Tris (pH 7.5), 145 mM NaCl. Nonspecific binding was defined as the amount of radioligand bound in the presence of 1 μM atropine and represented <5% of the total radioligand retained by the filters. Conditions were modified so that <15% of the total added radioligand was bound in all experiments. All assays were performed in triplicate. Binding assays with the muscarinic receptor agonist [^{3}H]oxotremorine-M were carried out as we previously have described (29).

RESULTS

NG108-15 neuroblastoma × glioma cells express muscarinic cholinergic receptors that upon activation attenuate adenylate cyclase activity (2, 11, 21, 24). This effect apparently completely accounts for the cholinergic agonist-induced decrease in cyclic AMP levels in these cells (21, 24). As has been demonstrated by this (24) and other (11) laboratories, carbachol and other agonists inhibit radiolabeled antagonist binding to muscarinic receptors of washed membranes from NG108-15 cells with high affinity. The carbachol competition curve in NG108-15 membranes was markedly shifted to the right and steepened when binding assays were carried out in the presence of GTP (Fig. 1) or Gpp(NH)p (24). In agreement with the report of Kurose *et al.* (11), pretreatment of NG108-15 cells with pertussis toxin resulted in carbachol competition curves of low affinity (Fig. 1); guanine nucleotides had no effect on the apparent affinity of carbachol in membranes from toxin-pretreated cells. Similar results were obtained using the radiolabeled agonist [^{3}H]oxotremorine-M. That is, in control NG108-15 membranes, [^{3}H]oxotremorine-M bound with high affin-

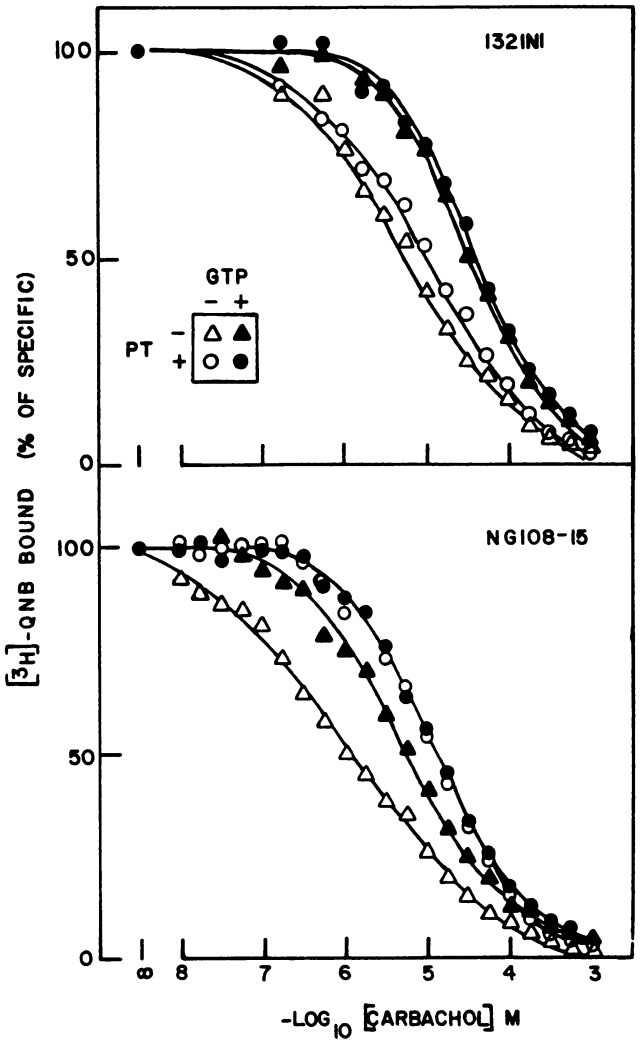

FIG. 1. *Effect of pertussis toxin on high affinity, guanine nucleotide-sensitive agonist binding to muscarinic cholinergic receptors in membranes from 1321N1 and NG108-15 cells*

Cells were pretreated for 16 hr with vehicle or pertussis toxin (1 μg/ml). Washed membranes from each condition were incubated with [^{3}H]QNB (25 pM) and the indicated concentrations of carbachol in the absence and presence of 100 μM GTP.

ity ($K_d \approx 0.3$ nM) to muscarinic cholinergic receptors; GTP inhibited this binding by greater than 90%. Subsequent to pertussis toxin treatment, [^{3}H]oxotremorine-M binding was no longer detectable (data not shown). The time and concentration dependence of the effects of pertussis toxin on GTP-sensitive high affinity binding of agonist to muscarinic receptors in NG108-15 membranes was similar to the toxin concentration and time dependence for blockade of muscarinic receptor-mediated inhibition of adenylate cyclase activity in these cells (21). Both activities were completely lost subsequent to an overnight incubation with 100 ng/ml of toxin.

As described above, the evidence to date suggests that muscarinic receptors of 1321N1 cells do not couple to N_i (15, 21). Nonetheless, when carbachol competition curves were carried out using [^{3}H]QNB and washed 1321N1 membranes, high affinity binding of carbachol was observed and the apparent affinity of muscarinic receptors for carbachol was markedly reduced by GTP (Fig. 1). In the presence of guanine nucleotide, the competition curve more closely approximated that expected for the interaction of an agonist with a single affinity state of the receptor obeying law of mass action (i.e., Hill slopes of 0.9–1.0). In contrast to the results obtained with NG108-15 membranes, the negative heterotropic regulation by guanine nucleotides of carbachol binding to muscarinic receptors in membranes from 1321N1 cells was not affected by pretreatment of cells with 1 μg/ml of pertussis toxin (Fig. 1). Similarly, pretreatment of 1321N1 cells with cholera toxin (1 μg/ml, 4 hr) had no effect on the apparent affinity of carbachol for muscarinic receptors in the absence of guanine nucleotide or on the capacity of guanine nucleotides to regulate receptor affinity for carbachol (data not shown). Preliminary experiments demonstrated that cholera toxin at a concentration of 10 ng/ml maximally stimulated intracellular cyclic AMP accumulation in these cells.

The high affinity, GTP-sensitive, pertussis toxin-insensitive binding of carbachol to muscarinic receptors in washed membranes from 1321N1 cells was investigated further by examining the specificity and rank order of potency of the observed nucleotide effect. Thus, [^{3}H]QNB binding was measured in the presence of a fixed concentration of carbachol (10 μM) and various concentrations of nucleotides. Since guanine nucleotides had no effect on [^{3}H]QNB binding *per se*, the increase in [^{3}H]QNB binding observed in the presence of nucleotides is a consequence of the guanine nucleotide-induced decrease in the receptor affinity for agonist (Fig. 2). The order of potency for the decrease in apparent affinity of carbachol was GTPγS > Gpp(NH)p = GTP = GDP, an order similar to that observed for other guanine nucleotide-dependent processes (4, 7, 30). ATP had no effect on agonist or ^{3}H-antagonist binding.

We have previously demonstrated that 1321N1 astrocytoma cell membranes contain a $M_r = 41{,}000$ substrate (presumably the α-subunit of N_i) that is ADP-ribosylated by incubation with pertussis toxin and [^{32}P]NAD (21, 28). Since pertussis toxin failed to modify GTP-sensitive agonist binding to 1321N1 membranes, it was important to establish that the toxin nonetheless fully modified N_i in intact cells. Thus, 1321N1 cells were incubated with various concentrations of toxin for approximately 16 hr. Membranes were then prepared and adenylate cyclase activity was measured in the presence of 100 μM forskolin alone or in the presence of 100 μM forskolin plus 100 μM GTP (Fig. 3). In cells that had not been pretreated with toxin, GTP inhibited forskolin-stimulated enzyme activity by approximately 50%. Pretreatment of cells with increasing concentrations of toxin resulted in a concentration-dependent loss of the guanine nucleotide-mediated inhibition (Fig. 3). To correlate this loss of inhibition with the toxin-catalyzed covalent modification of the α-subunit of N_i in the intact cell, membranes from the same cells were purified and [^{32}P]ADP-ribosylated in the presence of pertussis toxin and [^{32}P]NAD. The relative intensity of the labeling of the $M_r = 41{,}000$ substrate was determined by densitometric analysis of the developed autoradiogram (Fig. 3). These data illus-

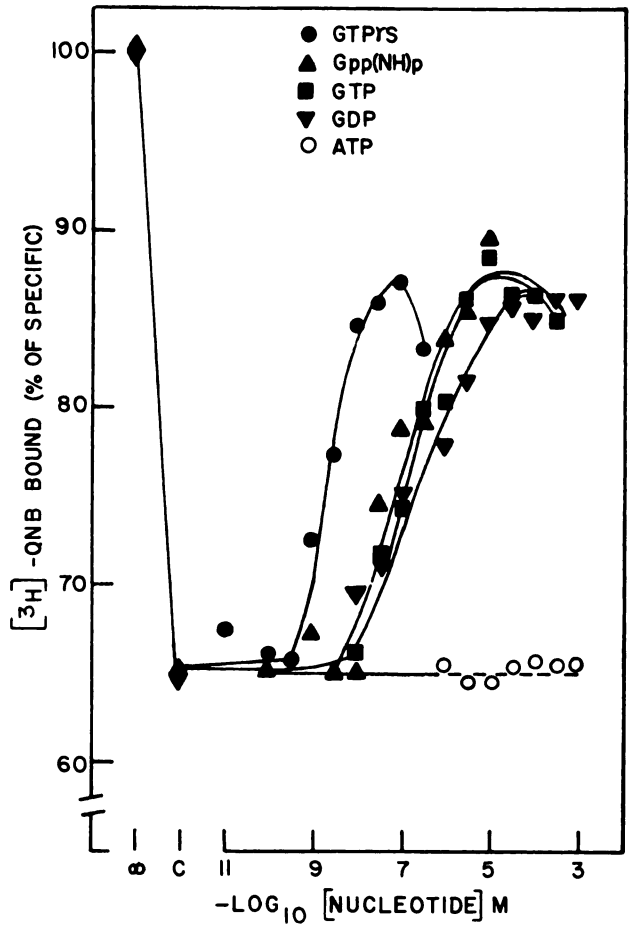

FIG. 2. *Specificity of the nucleotide-induced decrease in affinity of carbachol in 1321N1 cell membranes*

The binding of [^{3}H]QNB (25 pM) to a washed membrane preparation of 1321N1 astrocytoma cells was inhibited by a fixed concentration of carbachol (*C*, 10 μM). The increase in the binding of the radiolabeled antagonist, which results from the nucleotide-induced decrease in the receptor affinity for the agonist, was measured as a function of the indicated concentration of a series of nucleotides.

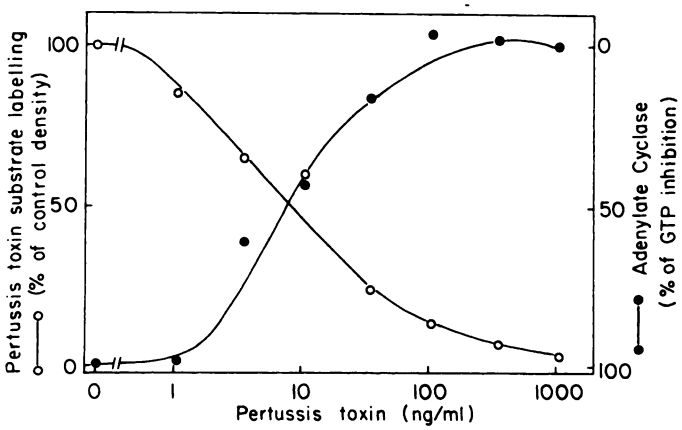

FIG. 3. *Effect of pertussis toxin pretreatment on guanine nucleotide-mediated inhibition of forskolin-stimulated adenylate cyclase is inversely correlated with toxin-catalyzed ADP-ribosylation of the M_r = 41,000 protein*

1321N1 astrocytoma cells treated with various concentrations of pertussis toxin for 16 hr were divided into two groups. In the first group, forskolin (100 μM)-stimulated adenylate cyclase activity was assayed in the absence or presence of 100 μM GTP. The data are expressed as the percentage of maximal GTP-mediated inhibition observed in control membranes. Pertussis toxin pretreatment decreased the ability of forskolin to activate adenylate cyclase in these membranes. The enzyme activity (in picomoles/min/mg protein) in control membranes was 869 ± 48 (−GTP) and 440 ± 20 (+GTP). The respective values obtained in membranes pretreated with the highest concentration of toxin were 498 ± 49 and 519 ± 20. Membranes from the second group were purified on a 30–60% linear sucrose gradient, ADP-ribosylated by incubation with [^{32}P]NAD and pertussis toxin, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis as previously described (21, 28). Gels were fixed, stained with Coomassie Brilliant Blue, and dried, and autoradiograms were developed using Kodak XAR-5 film. The relative intensity of the labeling of the M_r = 41,000 substrate was assessed densitometrically and the value obtained for each toxin concentration is expressed as a percentage of that obtained for control, nontreated cells.

trate that pretreatment of intact cells with increasing concentrations of toxin resulted in a concentration-dependent inhibition of the capacity of the toxin to catalyze the ADP-ribosylation of the α-subunit of N_i in membranes prepared from these cells. This effect correlates well with the loss of guanine nucleotide-mediated inhibition of forskolin-stimulated adenylate cyclase activity (Fig. 3). These data also indicate that concentrations of pertussis toxin 5- to 10-fold less than those that failed to modify GTP-sensitive agonist binding to muscarinic receptors in 1321N1 cells prevented [^{32}P]ADP labeling of the M_r = 41,000 substrate and completely blocked guanine nucleotide-mediated inhibition of forskolin-stimulated adenylate cyclase. As described above, muscarinic receptor-mediated inhibition of adenylate cyclase activity (21) and GTP-sensitive high affinity binding of agonists to muscarinic receptors are both lost in membranes from NG108-15 cells subsequent to treatment of cells with 100 ng/ml of pertussis toxin.

DISCUSSION

Muscarinic receptor-mediated attenuation of cyclic AMP accumulation in 1321N1 astrocytoma cells occurs through activation of a cyclic AMP phosphodiesterase (15, 21). Although muscarinic receptor-mediated inhibition of adenylate cyclase activity has not been detected in these cells, functional N_i apparently is present. That is, guanine nucleotides inhibit forskolin-stimulated adenylate cyclase activity in membranes from 1321N1 cells (21), and these membranes contain the M_r = 41,000 pertussis toxin substrate (21, 28). Furthermore, pretreatment of 1321N1 cells with pertussis toxin blocks the guanine nucleotide-mediated inhibition of forskolin-activated adenylate cyclase but has no effect on muscarinic receptor-mediated attenuation of cyclic AMP accumulation (21). This finding adds further support to the idea that cholinergic regulation of cyclic AMP metabolism in these cells does not occur through adenylate cyclase and that N_i is not involved in the cholinergic agonist-stimulated activation of phosphodiesterase. Thus, taken together, our data indicate that muscarinic receptors of 1321N1 cells do not interact with N_i. With this idea in mind, it was surprising to observe regulation of muscarinic receptor agonist binding by guanine nucleotides in washed 1321N1 membranes. However, again consistent with our earlier findings concerning the muscarinic receptor of 1321N1 cells, pertussis toxin at concentrations

that apparently fully ADP-ribosylate N_i in intact cells had no effect on this GTP-sensitive, high affinity cholinergic agonist binding.

If muscarinic receptors of 1321N1 cells do not interact with N_i, then how can the occurrence of GTP-sensitive, high affinity binding of cholinergic agonists be explained? Unfortunately, explanations at this time remain speculative. Two hypotheses can be entertained. First, muscarinic receptors might couple to N_s. Direct evidence for this possibility is not available. However, an analogous interaction between a stimulatory receptor and N_i has been demonstrated. That is, β-adrenergic receptors have been shown to stimulate the activation of purified N_i in model vesicles (31). In addition, both Murayama and Ui (12) and Jakobs *et al.* (32) have reported that stimulation of β-adrenergic receptors can result in inhibition of adenylate cyclase. These reports give credibility to the idea that similar interactions might occur between inhibitory receptors and N_s. However, the failure of cholera toxin pretreatment to modify the capacity of guanine nucleotides to regulate receptor affinity for carbachol suggests that this is not the case in 1321N1 cells.

Stimulation of muscarinic receptors of 1321N1 cells results in marked increases in phosphoinositide breakdown and Ca^{2+} mobilization (20). Could the effects of guanine nucleotides on agonist binding in membranes from these cells be a reflection of the interaction of muscarinic receptors and some unknown guanine nucleotide regulatory protein that couples receptor stimulation to phospholipid metabolism? Several recent reports present data that are potentially related to this hypothesis. In a preliminary report, Haslam and Davidson (33) have suggested the involvement of a guanine nucleotide binding protein in the thrombin-induced stimulation of inositol phospholipid hydrolysis in permeabilized human platelets, and Gomperts (34) has suggested the possibility that an unknown guanine nucleotide regulatory protein plays a role in the mobilization of Ca^{2+} by receptors. Although N_s or N_i potentially could be involved in receptor-mediated phosphoinositide/Ca^{2+} responses, our prejudice is that it is more likely that another guanine nucleotide regulatory protein is involved. The coupling of muscarinic receptors to the phosphoinositide system apparently does not involve N_i since pertussis toxin has no effect on phosphoinositide turnover in 1321N1 or chick heart cells.[4]

Substantial evidence is accumulating that suggests the existence of at least two muscarinic receptor subtypes (35), only one of which couples to the phosphoinositide system. Obviously, the muscarinic receptors of 1321N1 cells would be representative of the latter subtype and data indicate pharmacological differences between the muscarinic receptor populations of 1321N1 and NG108-15 cells (36). Thus, the difference in effects of pertussis toxin on the properties of the muscarinic receptors of NG108-15 versus 1321N1 cells could be considered further evidence for a difference in functionality of different muscarinic receptor subtypes on these two cell types. Boyer *et al.* (37) have presented data in a preliminary report that suggests that guanine nucleotide regulation of agonist binding at another receptor known to be linked to the phosphoinositide system is not affected by pertussis toxin. That is, although pertussis toxin blocked GTP-sensitive high affinity binding of agonists at α_2-adrenergic receptors of renal membranes, it had no effect on GTP-sensitive high affinity binding of agonists at α_1-adrenergic receptors.

Finally, as observed in other systems (22, 23), examination of the capacity of a series of muscarinic receptor agonists to enhance inositol phospholipid hydrolysis and promote Ca^{2+} mobilization in 1321N1 cells (20) has led to identification of "full" (e.g., carbachol and methacholine) and "partial" (e.g., oxotremorine and arecoline) agonists. Initial attempts to observe high affinity, GTP-sensitive binding of agonists to muscarinic receptors of 1321N1 cells were made using the "partial agonist" oxotremorine and only a very small nucleotide effect was observed. Preliminary experiments suggest that guanine nucleotide regulation of the binding of the "partial agonist" arecoline is similar to that observed for oxotremorine, and that for the "full agonist" methacholine is similar to that observed for carbachol.[5] The results suggest that the capacity of agonists to promote coupling of muscarinic receptors of 1321N1 cells with a guanine nucleotide-binding protein may be indicative of the full or partial nature of these agonists regarding stimulation of phosphoinositide breakdown and Ca^{2+} mobilization.

In summary, the current work adds further support to the idea that the muscarinic cholinergic receptor population of 1321N1 cells is incapable of coupling to components of the adenylate cyclase system. Nonetheless, an interaction of these receptors with a guanine nucleotide regulatory protein can be observed. The fact that this interaction is resistant to inactivation by pertussis toxin suggests that N_i is not involved. Current work in the laboratory is directed toward identifying this protein and determining the role, if any, of this muscarinic receptor-guanine nucleotide regulatory protein interaction in cholinergic agonist-stimulated increases in phosphoinositide breakdown and Ca^{2+} mobilization.

ACKNOWLEDGMENTS

We are indebted to Drs. Albert M. Collier and Ping-Chuan Hu for assistance in culturing *B. pertussis* and Angie Hodgin for preparing the manuscript.

REFERENCES

1. Murad, F., Y.-M Chi, T. W. Rall, and E. W. Sutherland. Adenyl cyclase. III. The effect of catecholamines and choline esters on the formation of adenosine 3′,5′-phosphate by preparations of cardiac muscle and liver. *J. Biol. Chem.* **237**:1223–1238 (1962).
2. Lichtshtein, D., G. Boone, and A. Blume. Muscarinic receptor regulation of NG-108-15 adenylate cyclase: requirement for Na^+ and GTP. *J. Cyclic Nucleotide Res.* **5**:367–375 (1979).
3. Jakobs, K. H., D. Aktories, and G. Schultz. GTP-dependent inhibition of cardiac adenylate cyclase by muscarinic cholinergic agonists. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **310**:113–119 (1979).
4. Ross, E. M., and A. G. Gilman. Biochemical properties of hormone-sensitive adenylate cyclase. *Annu. Rev. Biochem.* **49**:533–564 (1980).
5. Bokoch, G. M., T. Katada, J. K. Northup, E. L. Hewlett, and A. G. Gilman. Identification of the predominant substrate for ADP-ribosylation by islet activating protein. *J. Biol. Chem.* **258**:2072–2075 (1983).

[4] J. H. Brown, S. B. Masters, and T. K. Harden, unpublished observations.

[5] T. Evans, J. R. Hepler, S. B. Masters, J. H. Brown, and T. K. Harden, unpublished observations.

6. Manning, D., and A. G. Gilman. The regulatory components of adenylate cyclase and transducin: a family of structurally homologous guanine nucleotide binding proteins. *J. Biol. Chem.* **258**:7059–7063 (1983).
7. Gilman, A. G. G proteins and dual control of adenylate cyclase. *Cell* **36**:577–579 (1984).
8. Katada, T., J. K. Northup, G. M. Bokoch, M. Ui, and A. G. Gilman. The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase: subunit dissociation and guanine nucleotide-dependent hormonal inhibition. *J. Biol. Chem.* **259**:3578–3585 (1984).
9. Katada, T., and M. Ui. ADP ribosylation of the specific membrane protein of C6 cells by islet-activating protein associated with modification of adenylate cyclase activity. *J. Biol. Chem.* **257**:7210–7216 (1982).
10. Hazeki, O., and M. Ui. Modification by islet-activating protein of receptor-mediated regulation of cyclic AMP accumulation in isolated rat heart cells. *J. Biol. Chem.* **256**:2856–2862 (1981).
11. Kurose, H., T. Katada, T. Amano, and M. Ui. Specific uncoupling by islet-activating protein, pertussis toxin, of negative signal transduction via α-adrenergic, cholinergic, and opiate receptors in neuroblastoma × glioma hybrid cells. *J. Biol. Chem.* **258**:4870–4875 (1983).
12. Murayama, T., and M. Ui. Loss of the inhibitory function of the guanine nucleotide regulatory component of adenylate cyclase due to its ADP-ribosylation by islet-activating protein, pertussis toxin, in adipocyte membranes. *J. Biol. Chem.* **258**:3319–3326 (1983).
13. Hsia, J. A., J. Moss, E. L. Hewlett, and M. Vaughan. ADP-ribosylation of adenylate cyclase by pertussis toxin: effect on inhibitory agonist binding. *J. Biol. Chem.* **259**:1086–1090 (1984).
14. Kuno, T., O. Shirakawa, and C. Tanaka. Selective decrease in the affinity of D_2 dopamine receptor for agonist induced by islet-activating protein, pertussis toxin, assoicated with ADP-ribosylation of the specific membrane protein of bovine striatum. *Biochem. Biophys. Res. Commun.* **115**:325–330 (1983).
15. Meeker, R. B., and T. K. Harden. Muscarinic cholinergic receptor-mediated activation of phosphodiesterase. *Mol. Pharmacol.* **22**:310–319 (1982).
16. Meeker, R. B., and T. K. Harden. Muscarinic cholinergic receptor-mediated control of cyclic AMP metabolism: agonist-induced changes in nucleotide synthesis and degradation. *Mol. Pharmacol.* **23**:384–392 (1983).
17. Tanner, L. I., and T. K. Harden. Muscarinic cholinergic receptor-mediated reduction in cyclic AMP accumulation occurs by two mechanisms in a single cell. *Soc. Neurosci. Abstr.* **9**:88 (1983).
18. VanSande, J., C. Erneux, and J. E. Dumont. Negative control of TSH action by iodide and acetylcholine: mechanism of action in intact thyroid cells. *J. Cyclic Nucleotide Res.* **3**:335–345 (1977).
19. Barber, R., K. P. Ray, and R. W. Butcher. Turnover of adenosine 3′,5′-monophosphate in WI-38 cultured fibroblasts. *Biochemistry* **19**:2560–2567 (1980).
20. Masters, S. B., T. K. Harden, and J. H. Brown. Relationships between phosphoinositide and calcium responses to muscarinic agonists in astrocytoma cells. *Mol. Pharmacol.* **26**:149–155 (1984).
21. Hughes, A. R., M. W. Martin, and T. K. Harden. Pertussis toxin differentiates between two mechanisms of attenuation of cyclic AMP accumulation by muscarinic cholinergic receptors. *Proc. Natl. Acad. Sci. U. S. A.* **81**:5680–5684 (1984).
22. Fisher, S. K., P. D. Klinger, and B. W. Agranoff. Muscarinic agonist binding and phospholipid turnover in brain. *J. Biol. Chem.* **258**:7358–7363 (1983).
23. Brown, J. H., and S. L. Brown. Agonists differentiate muscarinic receptors that inhibit cyclic AMP formation from those that stimulate phosphoinositide metabolism. *J. Biol. Chem.* **259**:2777–2781 (1984).
24. Smith, M. M., and T. K. Harden. Modification of receptor-mediated inhibition of adenylate cyclase in NG108-15 neuroblastoma × glioma cells by *N*-ethylmaleimide. *J. Pharmacol. Exp. Ther.* **228**:425–433 (1984).
25. Sekura, R. D., F. Fish, C. R. Manclark, B. Meade, and Y-L. Zhang. Pertussis toxin. Affinity purification of a new ADP-ribosyltransferase. *J. Biol. Chem.* **258**:14647–14651 (1983).
26. Johnson, R. A., and T. F. Walseth. The enzymatic preparation of [α-^{32}P]ATP, [α-^{32}P]GTP, [^{32}P]cAMP, and [^{32}P]cGMP, and their use in the assay of adenylate and guanylate cyclases and cyclic nucleotide phosphodiesterases. *Adv. Cyclic Nucleotide Res.* **10**:135–167 (1979).
27. Cassel, D., and T. Pfeuffer. Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. *Proc. Natl. Acad. Sci. U. S. A.* **75**:2669–2673 (1978).
28. Waldo, G. L., J. K. Northup, J. P. Perkins, and T. K. Harden. Characterization of an altered membrane form of the β-adrenergic receptor produced during agonist-induced desensitization. *J. Biol. Chem.* **258**:13900–13908 (1983).
29. Harden T. K., R. B. Meeker, and M. W. Martin. Interaction of a radiolabeled agonist with cardiac muscarinic cholinergic receptors. *J. Pharmacol. Exp. Ther.* **227**:570–577 (1983).
30. Harden, T. K., A. G. Scheer, and M. M. Smith. Differential modification of the interaction of cardiac muscarinic and beta-adrenergic receptors with a guanine nucleotide binding component(s). *Mol. Pharmacol.* **21**:570–580 (1982).
31. Asano, T., T. Katada, A. G. Gilman, and E. M. Ross. Activation of the inhibitory GTP-binding protein of adenylate cyclase, Gi, by β-adrenergic receptors in reconstituted phospholipid vesicles. *J. Biol. Chem.* **259**:9351–9354 (1984).
32. Jakobs, K. H., K. Aktories, M. Minuth, and G. Schultz. Inhibition of adenylate cyclase. *Adv. Cyclic Nucleotide Res. Protein Phosphor.* in press (1984).
33. Haslam, R. J., and M. M. L. Davidson. Roles of Ca^{2+}, GTP, diglyceride and protein phosphorylation in the secretion of 5-HT from permeabilized platelets. *Fed. Proc.* **43**:2641 (1984).
34. Gomperts, B. D. Involvement of a guanine nucleotide-binding protein in the gating of Ca^{2+} by receptors. *Nature* (*Lond*) **306**:64–66 (1983).
35. Hammer, R., and A. Giachetti. Muscarinic receptor subtypes: M_1 and M_2: biochemical and functional characterization. *Life Sci.* **31**:2991–2998 (1982).
36. Evans, T., M. M. Smith, L. I. Tanner, and T. K. Harden. Muscarinic cholinergic receptors of two cell lines that regulate cyclic AMP metabolism by different molecular mechanisms. *Mol. Pharmacol.* **26**:395–404 (1984).
37. Boyer, J. L., A. Garcia, C. Posadas, and J. A. Garcia-Sainz. Effect of pertussis toxin on renal alpha$_1$ and alpha$_2$-adrenoceptors. *Fed. Proc.* **43**:3451 (1984).

Send reprint requests to: Dr. T. K. Harden, Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC 27514.