Brain Research, 556 (1991) 101–107
 0006-8993/91/$03.50
ADONIS 0006899391168683

BRES 16868

Guanine nucleotide binding proteins and the regulation of cyclic AMP synthesis in NS20Y neuroblastoma cells: role of D_1 dopamine and muscarinic receptors

Timothy W. Lovenberg[1,2], David E. Nichols[6], Eric J. Nestler[4,5], Robert H. Roth[4,5] and Richard B. Mailman[1–3]

[1]Brain and Development Research Center, Departments of [2]Pharmacology and [3]Psychiatry, University of North Carolina School of Medicine, Chapel Hill, NC 27599 (U.S.A.), Departments of [4]Pharmacology and [5]Psychiatry, Yale University School of Medicine, New Haven, CT 06510 (U.S.A.) and [6]Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907 (U.S.A.)

(Accepted 19 March 1991)

Key words: Cyclic AMP; D_1 dopamine receptor; NS20Y neuroblastoma; Dihydrexidine; Guanine nucleotide binding protein; Pertussis toxin; Cholera toxin

D_1 dopamine receptors on NS20Y neuroblastoma cells stimulate adenylate cyclase activity, whereas muscarinic receptors on the same cells negatively regulate adenylate cyclase. To determine the mechanisms which underlie these processes, cyclic AMP accumulation was measured in intact cells following either cholera or pertussis toxin treatment. Pretreatment with pertussis toxin (100 ng/ml), which ribosylated >95% of inhibitory guanine nucleotide binding protein (G_i), caused the complete loss of muscarinic induced inhibition. Conversely, pertussis toxin did not affect the ability of dihydrexidine (1 μM, a full efficacy D_1 agonist), PGE_1 (100 nM), or forskolin (1 μM, a direct activator) to stimulate cAMP accumulation. Both the dihydrexidine-induced stimulation and the carbachol-induced inhibition of cyclic AMP accumulation were unaffected by either removal of extracellular calcium, or increased intracellular calcium caused by the addition of the calcium ionophore A23187. Cholera toxin dose- and time-dependently induced large accumulations of cAMP. At low cholera toxin concentrations, the effects of dihydrexidine (300 nM) were additive with those of cholera toxin. At cholera toxin concentrations >100 ng/ml, dihydrexidine became ineffective in stimulating further cAMP synthesis. Conversely, forskolin (1 μM) still caused marked increases in cAMP accumulation after all cholera toxin treatments. Dihydrexidine-stimulated cAMP accumulation was additive with forskolin-stimulated cAMP accumulation at low forskolin concentrations (10 nM–3 μM), but synergistic at high concentrations (3–100 μM). Additionally, forskolin was much more potent after cholera toxin treatment, suggesting that an activated stimulatory guanine nucleotide binding protein (G_s) may be required for full activation of adenylate cyclase by forskolin in this cell type. Together, these data suggest that adenylate cyclase activity in NS20Y cells is regulated by both stimulatory G-protein(s) linked to D_1 dopamine receptors and inhibitory G-protein(s) linked to muscarinic receptors.

INTRODUCTION

Guanine nucleotide binding proteins (G-proteins) are known to transduce both stimulatory and inhibitory signals elicited by receptors for a wide variety of hormones, neurotransmitters, and other messenger molecules (for review see ref. 15). The demonstration that a particular receptor interacts with, or is dependent on, G-proteins can yield a great deal of information about how that receptor can be regulated as well as the messenger systems with which that receptor can interact. It has been hypothesized that in the mammalian brain D_1 receptor activation of adenylate cyclase is mediated by a stimulatory G-protein. This hypothesis is based on two findings. The first is that GTP is required for activation of adenylate cyclase in washed brain membrane preparations[6,27,28]. The second is that, in brain membranes, GTP shifts D_1 agonist binding to lower affinity, suggesting an interaction with a G-protein[12,16,29]. While both lines of evidence support the contention that D_1 receptors interact with stimulatory G-protein (G_s), there are, however, other considerations.

It has been proposed that there are multiple subtypes of D_1 receptors which have similar ligand binding domains, but have different coupling to effector systems. For example, D_1 binding sites in the amygdala demonstrate no coupling to adenylate cyclase[23]. Interestingly, the presence of GTP has been reported to shift agonist binding in human amygdala from high to low affinity[12]. In an opposite manner, D_1 receptors stimulate adenylate

Correspondence: R. Mailman, CB 7250, Biological Sciences Research Center, University of North Carolina School of Medicine, Chapel Hill, NC 27599-7250, U.S.A.

cyclase in the frontal cortex[20], whereas GTP does not shift agonist affinity in membranes from this structure[10,11]. Therefore, it would be useful to understand the mechanism of activation of adenylate cyclase by D_1 receptors in an isolated system, such as a cultured cell line. An isolated system can be manipulated to duplicate and identify the causes of heterogeneous types of GTP/receptor interactions in different brain tissues, given that the receptors themselves are similar.

NS20Y neuroblastoma cells have been shown to be an interesting model system for the study of D_1 dopamine receptors which are linked in a stimulatory manner to adenylate cyclase[3,24]. We have previously shown that these cells accumulate cAMP in response to D_1 receptor agonists[22], and that this response is inhibited by muscarinic receptor agonists. The present study was undertaken to define some of the regulatory mechanisms involved in the D_1 mediated activation of adenylate cyclase, and to provide data testing the hypothesis that D_1 receptors stimulate adenylate cyclase through a direct G_s-type mechanism. In addition, the work sought to determine if, in this system, muscarinic-induced inhibition of adenylate cyclase activity occurs directly via an inhibitory G-protein (G_i)-type mechanism, as has been shown in other systems.

MATERIALS AND METHODS

Materials

Dihydrexidine was synthesized as previously published[5]. NS20Y cells were generously provided by Dr. Marshall Nirenberg (NIH, Bethesda, MD). Pertussis toxin and cholera toxin were purchased from List Biological Labs (Campbell, CA). Carbamylcholine, forskolin, and isobutylmethylxanthine were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were from standard commercial sources.

Cell culture

Cells were grown and passaged in Dulbecco's Modified Eagles Medium supplemented with glucose (4.5 g/l), sodium pyruvate (1 mM), fetal bovine serum (10%), HEPES (10 mM), penicillin/streptomycin. The cells were maintained at 37 °C in 95% O_2/5% CO_2. For cAMP measurements, cells from passage 17–35 were plated and grown in 24-well plates (Corning) at a final density ca. = 3×10^5 cells/well.

cAMP accumulation

cAMP was measured by radioimmunoassay with kits from Incstar (Stillwater, MN) based on the procedure of Steiner et al.[31], or using a manual assay following the same procedure. Briefly, cells were incubated with assay buffer (HEPES, 25 mM; NaCl, 145 mM; glucose, 19 mM; KCl, 6 mM; $CaCl_2$, 1.8 mM; $MgSO_4$, 0.8 mM) for 30 min prior to drug treatments[13]. For experiments in which cholera toxin or pertussis toxin were used, the cells were incubated with the toxins for 2 h or 6 h, respectively, prior to drug treatments. The cells were then incubated for 2 min in the presence or absence of drugs which included the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 500 μM). Following the incubation, the media was removed and the cells were lysed with 0.5 ml ice-cold HCl (0.1 N). After 10 min on ice, cells were scraped and collected in microfuge tubes. The wells were rinsed with an additional 0.5 ml of HCl which was added to the first lysate. The lysates were pelleted in a Beckman microfuge, after which 20 μl of the supernatant was neutralized with 20 μl of 0.1 N NaOH and then diluted in 460 μl of sodium acetate (50 mM, pH 6.2). These samples were acetylated with 25 μl of triethylamine/acetic anhydride (2:1). An aliquot of the acetylated sample was then assayed by RIA with the kits.

Cells in control wells were detached with 0.1% trypsin-EDTA and viable cells were counted using the Trypan blue dye exclusion method. All results are expressed as either pmol cAMP/10^6 cells or % control where control equals the accumulation of cAMP in the absence of drugs. All experiments were performed in triplicate and data shown is representative of at least 3 individual experiments.

Biochemical measurement of pertussis toxin-catalyzed ADP ribosylation

Cells were grown to near confluence after which the media was replaced with media containing pertussis toxin (100 ng/ml) or sodium phosphate vehicle. The incubation was for 6 h at 37 °C and was followed by two washes with ice-cold PBS. Cells were then scraped in ice-cold PBS, pelleted and immediately frozen and stored at −80 °C until protein ADP ribosylation was measured.

Measurement of ADP ribosylation was done according to published procedures[25]. Briefly, cell pellets were sonicated in ice-cold 'homogenization buffer' (50 mM Tris, pH 8, 6 mM $MgCl_2$, 1 mM EDTA, 3 mM benzamidine, 1 mM dithiothreitol, 5% (w/v) sucrose, 1 μg/ml of soybean trypsin inhibitor). Homogenates were centrifuged for 10 min at 10,000 *g* at 4 °C in a microfuge. Pellets were resuspended by sonication in the original volume of ice-cold 'ADP ribosylation buffer' (100 mM Tris, pH 8, 10 mM thymidine, 10 mM isoniazide, 5 mM $MgCl_2$, 2.8 mM dithiothreitol, 2.4 mM benzamidine, 0.8 mM EDTA, 2.5 mM ATP, 2 mM GTP, 4% (w/v) sucrose, 0.8 μg/ml soybean trypsin inhibitor, 0.5% (v/v) Triton X-100). Aliquots (80 μl) of these suspensions were incubated with 1 μg of purified pertussis toxin and 10 μM [^{32}P]NAD (30 Ci/mmol) in 1.5 ml microfuge tubes for 1 h at room temperature in a final volume of 100 μl. Reactions were terminated with 0.75 ml of ice-cold homogenization buffer followed by centrifugation at 10,000 *g* for 10 min. The pellets were vigorously resuspended in 30 μl of a solution containing 40 mM Tris, pH 6.8, 1 mM dithiothreitol, 2% (w/v) SDS (sodium dodecyl sulfate). The samples were incubated for 5 min at 75 °C after which 20 μl of 100 mM *N*-ethylmaleimide were added. The samples were further incubated for 15 min at room temperature. Fifty μl of a solution containing 40 mM Tris, pH 7.8, 1% (w/v) SDS, 50% (v/v) glycerol and 6% β-mercaptoethanol were then added followed by 2 min of boiling. The samples were then subjected to one-dimensional SDS-polyacrylamide gel electrophoresis as described by Laemmli[21] with 9% (w/v) acrylamide-0.25% (w/v) bisacrylamide in the resolving gels. Gels were dried and autoradiographed; the amount of radioactivity incorporated into individual G-proteins was determined by a Betagen Betascope blot analyzer.

Biochemical measurement of cholera toxin-catalyzed ADP ribosylation

Cells were grown to near confluence after which the media was replaced with media containing cholera toxin (100 or 10,000 ng/ml) or sodium phosphate vehicle for 2 h at 37 °C. After two washes with ice-cold PBS, cells were scraped in ice-cold PBS, pelleted and immediately frozen and stored at −80 °C until protein ADP ribosylation was measured.

Measurement of ADP ribosylation was done according to Tamir and Gill[32] as modified by Saito et al.[26]. Briefly, cell pellets were homogenized in 10 vols of an ice-cold solution (50 mM Tris, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, 10% (w/v) sucrose). Homogenates were centrifuged for 10 min at 10,000 *g* at 4 °C in a microfuge. Pellets were rehomogenized in one half the original volume of the following buffer: 150 mM potassium phosphate, pH 7, 10 mM thymidine, 10 mM dithiotreitol, 20 mM isonicotinic acid hydrazide, 1 mM 3-acetylpyridine adenine dinucleotide, 0.1% (v/v) Triton X-100. Aliquots (80 μl) of the suspensions were incubated with 10

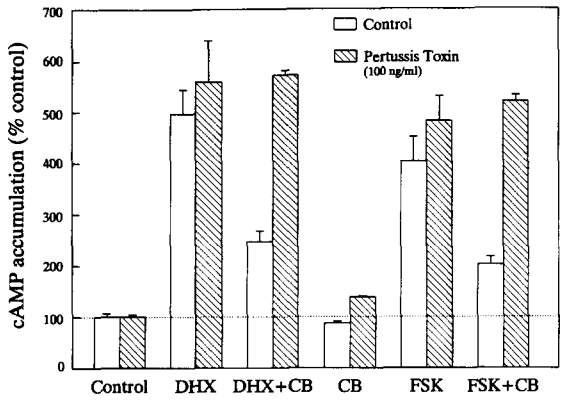

Fig. 1. Cells were incubated with pertussis toxin (100 ng/ml) or vehicle for 6 h in culture medium. Following a 30 min wash in assay buffer and a 2-min drug exposure, cellular cAMP was measured. Results are expressed as a percent of cAMP accumulation in the absence of drugs. DHX, dihydrexidine (1 μM); CB, carbachol (100 μM), FSK, forskolin (1 μM). Shown are the results of one of 3 identical experiments yielding similar results. Values are mean ± S.E.M. of triplicate samples in a single experiment.

μg of activated cholera toxin and 20 μM [^{32}P]NAD (30 Ci/mmol) in 1.5 ml microfuge tubes for 1 h at room temperature. Reactions were terminated and samples were prepared for electrophoresis as described above. The samples were then subjected to one-dimensional SDS-polyacrylamide gel electrophoresis as described by Laemmli[21]. ^{32}P-labeled $G_{s\alpha}$ subunits were identified by autoradiography, and quantified as described above.

RESULTS

Effects of pertussis toxin on muscarinic regulation of cAMP synthesis

NS20Y cells accumulated cAMP (5-fold and 4-fold, respectively) in response to the novel D_1 receptor agonist dihydrexidine (1 μM) or forskolin (1 μM). Carbachol (100 μM) inhibited these responses by about 65% (Fig. 1, open bars). After cells were treated with pertussis toxin for 6 h, the responses to dihydrexidine or forskolin are not changed, whereas the inhibitory effects of carbachol were completely reversed (Fig. 1, hatched bars). It was determined that the pertussis toxin treatment resulted in a greater than 95% ADP ribosylation of G_i, as determined by a 'back-ADP ribosylation' experiment.

Incubation of the cells with a buffer containing EGTA and no calcium had no effect on the ability of dihydrexidine to stimulate or carbachol to inhibit cAMP accumulation. The calcium ionophore, A23187 (1 μM), likewise had no effect on either dihydrexidine-stimulated- or carbachol-inhibited cAMP accumulation (data not shown).

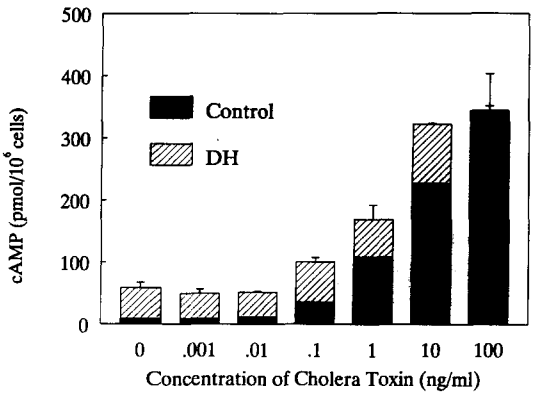

Fig. 2. Cells were incubated with cholera toxin (100 ng/ml) or vehicle for 2 h in culture medium. Following a 30 min wash in assay buffer and a 2 min drug exposure, cellular cAMP was measured. Results are expressed as cAMP accumulation during the 2 min treatment. Solid bars represent cAMP accumulation in the absence of dihydrexidine. Hatched bars represent additional cAMP accumulation due to addition of dihydrexidine. Shown are the mean ± S.E.M. of triplicate samples in a single experiment. Other identical experiments yielded similar results.

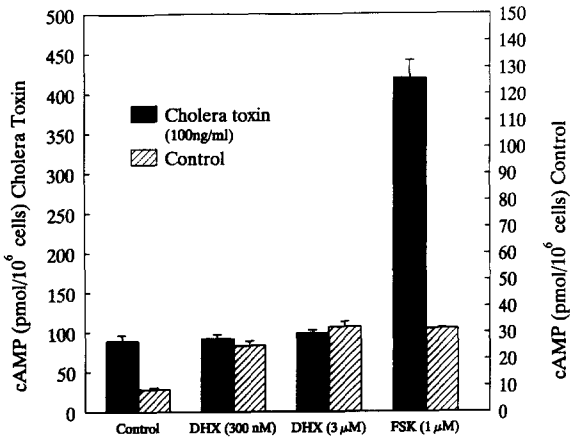

Fig. 3. Experimental design similar to Fig. 2. The left abscissa shows cAMP accumulation after cholera toxin treatment (solid bars). The right abscissa shows cAMP accumulation in control cells (hatched bars). Shown are the mean ± S.E.M. of triplicate samples in a single experiment. Other identical experiments yielded similar results.

Effects of cholera toxin on D_1 receptor-stimulated cAMP accumulation

Treatment of cells for 2 h with cholera toxin resulted in a dose-dependent increase in cAMP levels (Fig. 2, solid bars). Dihydrexidine stimulated additional accumulation of cAMP at all cholera toxin concentrations except 100 ng/ml (Fig. 2, hatched bars). As would be expected, additional experiments also demonstrated that dihydrexidine cannot cause further accumulation of cAMP at higher cholera toxin concentrations (i.e., 1000 ng/ml). 'Back-ADP ribosylation' experiments of control and cholera toxin-treated cell membranes show that at 100 ng/ml cholera toxin, only 18% of available sites were ADP ribosylated. This does not directly correlate with the observation that the dihydrexidine-stimulated response is completely lost at 100 ng/ml cholera toxin. In

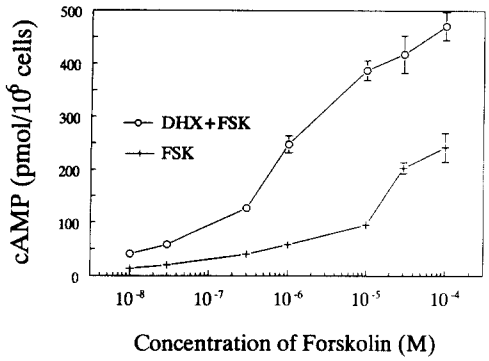

Fig. 4. Cells were incubated with forskolin for 30 min in assay buffer, followed by a 2 min drug exposure. Cellular cAMP was measured and expressed as pmol/10^6 cells. DHX, dihydrexidine (1 μM); FSK, forskolin. Shown are the results of one of three identical experiments yielding similar results. Values are mean ± S.E.M. of triplicate samples in a single experiment.

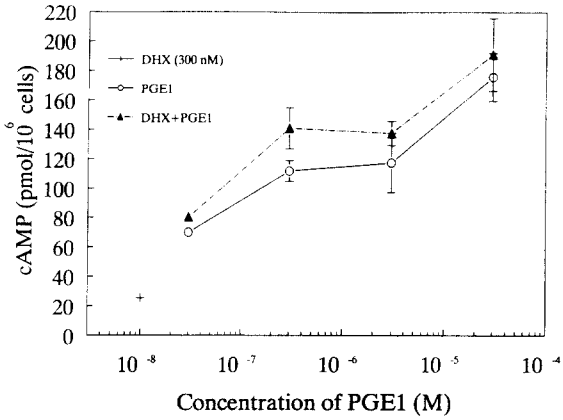

Fig. 6. Cells were incubated with for PGE_1 30 min in assay buffer, followed by a 2 min drug exposure. Cellular cAMP was measured and expressed as pmol/10^6 cells. PGE_1, prostaglandin E_1; DHX, dihydrexidine (300 nM). Values are mean ± S.E.M. of triplicate samples in a single experiment.

fact, even when cells were treated with 10,000 ng/ml cholera toxin, only 36% of available sites were ADP ribosylated.

Effects of cholera toxin on forskolin-stimulated cAMP accumulation

To show that the loss of stimulatory effect at high cholera toxin concentrations was not due to a loss of available substrate, forskolin was added to cells treated with cholera toxin (100 ng/ml). This resulted in a massive accumulation of cAMP which was much greater than the sum of either cholera toxin or forskolin alone. When added to control cells, forskolin (1 μM) induces accumulation of cAMP equal to that of dihydrexidine (Fig. 3, hatched bars). When added to cholera toxin-treated cells, however, forskolin induced a 16-fold greater than normal accumulation of cAMP (Fig. 3, solid bars).

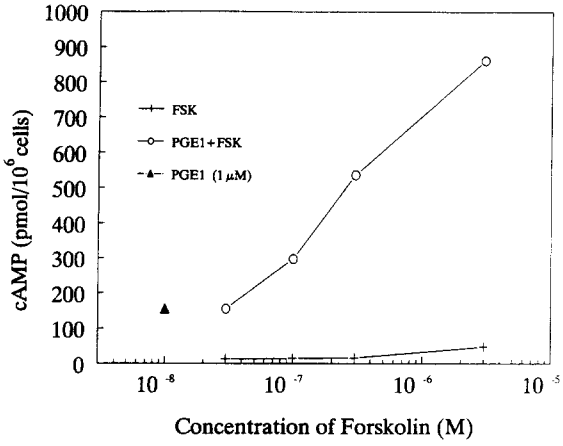

Fig. 5. Cells were incubated with forskolin for 30 min in assay buffer, followed by a 2 min drug exposure. Cellular cAMP was measured and expressed as pmol/10^6 cells. PGE_1, prostaglandin E_1 (1 μM); FSK, forskolin. Values are mean ± S.E.M. of triplicate samples in a single experiment.

Effects of forskolin on D_1 receptor-stimulated cAMP accumulation

Cells were incubated for 2 min with various concentrations of forskolin in the presence of either dihydrexidine or PGE_1. At high concentrations of forskolin, cells accumulated cAMP to a degree greater than the sum of either agent alone (Figs. 4 and 5). This synergistic action was similar to that seen between cholera toxin and forskolin. When dihydrexidine was added in combination with PGE_1, the accumulation of cAMP was additive (Fig. 6).

DISCUSSION

D_1 dopamine receptors have been shown to activate adenylate cyclase in NS20Y neuroblastoma cells[3,22]. In the present study, D_1 receptor activation and muscarinic receptor inhibition of adenylate cyclase in NS20Y cells was altered by pretreating cells with cholera toxin and pertussis toxin, respectively. The data presented are consistent with the hypothesis that D_1 receptors activate adenylate cyclase via a G_s-like protein and that muscarinic receptors inhibit adenylate cyclase through a G_i-like protein in NS20Y cells.

Although we have previously shown that muscarinic receptors present on NS20Y cells can inhibit D_1 receptor-stimulated cAMP accumulation, the mechanism of inhibition in this cell line was not known. Studies in cultured cells have shown that at least two mechanisms exist for the inhibition of cAMP accumulation by muscarinic receptors. Evans et al.[14] found that in 1321N1 astrocytoma cells, muscarinic receptor stimulation results

in the activation of calcium-dependent phosphodiesterase activity which results in the attenuation of cAMP levels. Conversely, in NG108-15 neuroblastoma cells, muscarinic receptors directly inhibit adenylate cyclase though a GTP-dependent mechanism which can be blocked by pertussis toxin[17].

In order to determine the mechanism present in NS20Y cells, we measured the effect of both pertussis toxin and calcium on muscarinic receptor-mediated inhibition of cAMP accumulation. We found that pretreatment of NS20Y cells with pertussis toxin blocked the ability of the muscarinic agonist, carbachol, to inhibit cAMP accumulation. The same concentration of pertussis toxin was found to ADP ribosylate >95% of G_i under the same experimental conditions. Conversely, we found that neither the removal of calcium from the extracellular media, nor the calcium ionophore A23187, had any effect on the stimulatory or inhibitory effects of dihydrexidine or carbachol, respectively. In addition, since all experiments were conducted in the presence of the phosphodiesterase inhibitor IBMX, it was not likely that activation of a phosphodiesterase was the mechanism by which the inhibitory effects of muscarinic agonists occurred. Therefore we conclude that the muscarinic induced inhibition of adenylate cyclase in NS20Y cells is through an inhibitory guanine nucleotide binding protein identical or similar to G_i.

Monsma et al.[24] have shown that GTP shifts D_1 receptor agonist binding curves to the right in NS20Y cell membranes. This finding suggests that the agonist-occupied state of the D_1 receptor may be interacting with a G-protein. Since D_1 receptor activation results in the stimulation of adenylate cyclase, it is generally assumed that a stimulatory G-protein such as G_s is involved. We attempted to address this issue by measuring whole cell D_1 receptor function after chemically altering G_s with cholera toxin. When cells are treated with cholera toxin for 2 h, they accumulate a large amount of cAMP in a concentration-dependent fashion. We hypothesized that as the cholera toxin concentration was increased, more G_s would be activated, and therefore unavailable for interactions with D_1 receptors. When all of the G_s is activated, the effects of D_1 agonists should be ineffective if they are mediated by G_s. As expected, we found that when the cholera toxin concentration reached 100 ng/ml there was a complete loss of agonist efficacy. Since forskolin could still cause dramatic increases in cAMP accumulation, the cholera toxin treatments were neither causing a lack of availability of precursor ATP, nor inactivating the cyclase catalytic unit.

Interestingly, when cell membranes were measured for the ADP ribosylation state of G_s, it was found that at 100 ng/ml, cholera toxin resulted in only an 18% ADP ribosylation of G_s. While the state of ADP ribosylation does not correlate with the loss of agonist function, it may be explained by one of several alternative hypotheses. D_1 receptors may interact only with a subpopulation or pool of G_s in whole cells. Since the 'back-ADP ribosylation' experiments are carried out in broken cells, the preparation of the tissue may uncover G_s ADP ribosylation sites which were unavailable to the toxin in whole cells. Likewise, since the density of D_1 receptors is low in these cells, this response may be easily eliminated by concentrations of cholera toxin much lower than those necessary to ADP ribosylate all of the cellular G_s. Another intriguing hypothesis is that the presence of high levels of cAMP, presumably via cAMP-dependent protein phosphorylation, result in the loss of D_1 receptor function or coupling to the G-protein. One way to address this would be to duplicate the high levels of cAMP without altering G_s. Attempts to accomplish this using forskolin have proven difficult to interpret. Experiments in which cells were treated for 30 min with forskolin and then subsequently washed for 30 min to remove the forskolin still resulted in increased levels of cAMP (ca. 5-fold). Addition of dihydrexidine after the washout results in an increase in cAMP equal to that of dihydrexidine in control cells (data not shown). Initially, it would appear that the elevated cAMP levels did not affect the ability of dihydrexidine to activate adenylate cyclase. In light of the subsequently described experiments showing a synergism between forskolin and dihydrexidine, however, it would be hasty to make definitive conclusions. It should be noted that 10 ng/ml cholera toxin elicited significant increases in cAMP levels, yet did not result in a loss of agonist function. This observation supports the hypothesis that cAMP was not responsible for the lost agonist efficacy at 100 ng/ml cholera toxin.

As noted earlier, to demonstrate that cholera toxin was not preventing agonist-induced adenylate cyclase activation by depletion of substrate, we used forskolin to activate directly adenylate cyclase after treatment. Surprisingly, in cholera toxin-treated cells, forskolin elicited a massive increase in the accumulation of cAMP over a short period of time (2 min) compared to its effects in untreated cells. It has been previously hypothesized that an activated $G_{s\alpha}$-subunit is required to observe the full effects of forskolin[8,9,30]. In addition, synergism between forskolin and both hormone- and cholera toxin-stimulated adenylate cyclase in whole cells has been demonstrated previously[2,7,30]. The observation that dihydrexidine and forskolin are synergistic in a manner similar to cholera toxin and forskolin suggests that dihydrexidine and cholera toxin may act through a common mechanism, namely activating G_s. This observation is particularly interesting because it is similar to an earlier finding

in rat striatal membranes in which forskolin potentiated D_1 receptor-stimulated adenylate cyclase[4]. We have previously demonstrated that NS20Y cells have D_1 receptors which functionally resemble striatal D_1 receptors[22]. Thus, these results provide further evidence for the study of NS20Y cell D_1 receptors as a functional model for striatal adenylate cyclase-linked D_1 receptors.

To show that cholera toxin was not altering D_1 receptor response through direct interaction with the receptor, we tested the effects of cholera toxin on other receptor-linked adenylate cyclase systems. PGE_1, which is thought to stimulate adenylate cyclase through a G_s-dependent activation, exhibits a similar synergistic response with forskolin. Pretreatment of cells with cholera toxin also greatly dampens the adenylate cyclase response to PGE_1. The observation that dihydrexidine and PGE_1 are additive indicates that they are either acting through distinct mechanisms or through similar mechanisms in which neither is rate-limiting. For example, these compounds may either activate distinct pools of G_s, or the commonly available G_s may be sufficient to supply both receptor populations. The latter idea is supported by the observation that only a small amount of G_s is ADP ribosylated by high concentrations of cholera toxin and that this is enough to eliminate both dihydrexidine- and PGE-stimulated cAMP accumulation.

The evidence presented here provides direct data in support of the hypothesis that D_1 receptor stimulated adenylate cyclase in NS20Y cells is mediated by the guanine nucleotide binding protein G_s. In addition, the data indicate that muscarinic receptors on NS20Y cells negatively regulate adenylate cyclase by a G_i-dependent mechanism. The D_1 receptors on NS20Y cells share many of the characteristics of D_1 receptors in the mammalian striatum as previously reported[22]. Since D_1 receptor function in rat striatum has been shown to be regulated by muscarinic receptors[18,19], it is tempting to speculate, in light of the current study, that NS20Y cells may be useful for elucidating one aspect of D_1-dopaminergic-cholinergic interactions. While it must be emphasized that NS20Y cells are not of striatal origin, they exhibit several properties associated with striatal cholinergic cells (e.g., high choline acetyltransferase and acetylcholinesterase activity)[1], and therefore may warrant attention as a model of striatal cells to study some of the more detailed biochemical interactions between D_1-dopamine and muscarinic cholinergic receptors. Taking into account the suggested association of D_1 receptors with aspects of the patch matrix architecture of the striatum, NS20Y cells may be a useful system for understanding aspects of this interaction.

Acknowledgements. This work was supported by PHS Grants MH40537 and MH42705, Program Grant ES01104, Center Grants HD03310, MH13127, Training Grant GM07040 (T.L.) and MH14902 (R.H.R.).

REFERENCES

1 Amano, T., Richelson, E. and Nirenberg, M., Neurotransmitter synthesis by neuroblastoma clones, *Proc. Natl. Acad. Sci. U.S.A.*, 69 (1972) 258–263.
2 Barovsky, K., Pedone, C. and Brooker, G., Distinct mechanisms of forskolin-stimulated cyclic AMP accumulation and forskolin potentiated hormone responses in C6-2B cells, *Mol. Pharmacol.*, 25 (1984) 256–260.
3 Barton, A.C. and Sibley, D.R., Agonist-induced desensitization of D1 dopamine receptor-coupled adenylyl cyclase activity in cultured NS20Y neuroblastoma cells, *Soc. Neurosci. Abstr.*, 15 (1989) 429.
4 Battaglia, G., Norman, A.B., Hess, E.J. and Creese, I., Forskolin potentiates the stimulation of rat striatal adenylate cyclase mediated by D-1 dopamine receptors, guanine nucleotides, and sodium fluoride, *J. Neurochem.*, 46 (1986) 1180–1185.
5 Brewster, W.K., Nichols, D.E., Riggs, R.M., Mottola, D.M., Lovenberg, T.W., Lewis, M.H. and Mailman, R.B., *trans*-10,11-Dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo[*a*]-phenanthridine: A highly potent selective dopamine D_1 full agonist, *J. Med. Chem.*, 33 (1990) 1756–1764.
6 Clement-Cormier, Y.C., Parrish, R.G., Petzold, G.L., Kebabian, J.W. and Greengard, P., Characterization of a dopamine sensitive adenylate cyclase in the rat caudate nucleus, *J. Neurochem.*, 25 (1975) 143–149.
7 Daly, J.W., Padgett, W. and Seamon, K.B., Activation of cyclic AMP generating systems in brain membranes and slices by the diterpine forskolin — augmentation of receptor mediated responses, *J. Neurochem.*, 38 (1982) 532–544.
8 Darfler, F.J., Mahan, L.C., Koachman, A.M. and Insel, P.A., Stimulation by forskolin of intact S49 lymphoma cells involves the nucleotide regulatory protein of adenylate cyclase, *J. Biol. Chem.*, 257 (1982) 11901–11907.
9 Darfler, F.J., Mahon, L.C., Koachman, A.M. and Insel, P.A., Stimulation by forskolin of intact S49 lymphoma cells involves the guanine nucleotide regulatory protein of adenylate cyclase, *J. Biol. Chem.*, 257 (1982) 11901–11907.
10 De Keyser, J., De Backer, J.-P., Ebinger, G. and Vauquelin, G., Coupling of D_1 dopamine receptors to the guanine nucleotide binding protein Gs is deficient in Huntington's disease, *Brain Research*, 496 (1989) 327–330.
11 De Keyser, J., Dierckx, R., Vanderheyden, P., Ebinger, G. and Vauquelin, G., D1 dopamine receptors in human putamen, frontal cortex and calf retina: differences in guanine nucleotide regulation of agonist binding and adenylate cyclase stimulation, *Brain Research*, 443 (1988) 77–84.
12 De Keyser, J., Walraevens, H., Ebinger, G. and Vauquelin, G., In human brain two subtypes of D_1 dopamine receptors can be distinguished on the basis of differences in guanine nucleotide effect on agonist binding, *J. Neurochem.*, 53 (1989) 1096–1102.
13 Evans, T., McCarthy, K.D. and Harden, T.K., Regulation of cyclic AMP accumulation by peptide hormone receptors in immunologically defined astroglial cells, *J. Neurochem.*, 43 (1984) 131–138.
14 Evans, T., Smith, M.M., Tanner, L.I. and Harden, T.K., Muscarinic cholinergic receptors of two cell lines that regulate cyclic AMP metabolism by different molecular mechanisms, *Mol. Pharmacol.*, 26 (1984) 395–404.
15 Gilman, A.G., G-proteins: transducers of receptor-generated signals, *Annu. Rev. Biochem.*, 56 (1987) 615–649.
16 Hess, E.J., Battaglia, G., Norman, A.B., Iorio, L.C. and

Creese, I., Guanine nucleotide regulation of agonist interactions at [^{3}H]SCH23390-labeled D1 dopamine receptors in rat striatum, *Eur. J. Pharmacol.*, 121 (1986) 31–38.
17 Hughes, A.R., Martin, M.W. and Harden, T.K., Pertussis toxin differentiates between two mechanisms of attenuation of cyclic AMP accumulation by muscarinic cholinergic receptors, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 5680–5684.
18 Kelly, E. and Nahorski, S.R., Specific inhibition of dopamine D-1-mediated cyclic AMP formation by dopamine D-2, muscarinic cholinergic, and opiate receptor stimulation in rat striatal slices, *J. Neurochem.*, 47 (1986) 1512–1516.
19 Kelly, E., Rooney, T.A. and Nahorski, S.R., Pertussis toxin separates two muscarinic receptor-effector mechanisms in the striatum, *Eur. J. Pharmacol.*, 119 (1985) 129–130.
20 Krieger, N.R., Bunney, B.S. and Greengard, P., Localization of dopamine-sensitive adenylate cyclase activity within laminae of the rat prefrontal cortex, *Brain Research,* 179 (1979) 171–175.
21 Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature,* 227 (1970) 680–685.
22 Lovenberg, T.W., Roth, R.H., Nichols, D.E. and Mailman, R.B., D1 dopamine receptors of NS20Y neuroblastoma cells are functionally similar to rat striatal D1 receptors, submitted.
23 Mailman, R.B., Schulz, D.W., Kilts, C.D., Lewis, M.H., Rollema, H. and Wyrick, S., Multiple forms of the D1 dopamine receptor: its linkage to adenylate cyclase and psychopharmacological effects, *Psychopharm. Bull.*, 22 (1986) 593–598.
24 Monsma Jr., F.J., Brassard, D.L. and Sibley, D.R., Identification and characterization of D_1 and D_2 dopamine receptors in cultured neuroblastoma and retinoblastoma clonal cell lines, *Brain Research,* 492 (1989) 314–324.
25 Nestler, E.J., Erdos, J.J., Terwilliger, R., Duman, R.S. and Tallman, J.F., Regulation of G proteins by chronic morphine in the rat locus coeruleus, *Brain Research,* 476 (1989) 230–239.
26 Saito, N., Guitart, X., Hayward, M., Tallman, J.F., Duman, R.S. and Nestler, E.J., Corticosterone differentially regulates the expression of Gsa and Gia messenger RNA and protein in rat cerebral cortex, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 3906–3910.
27 Salomon, Y., Londos, C. and Rodbell, M., A highly sensitive adenylate cyclase assay, *Anal. Biochem.*, 158 (1974) 541–548.
28 Schulz, D.W. and Mailman, R.B., An improved, automated adenylate cyclase assay utilizing preparative HPLC: effects of phosphodiesterase inhibitors, *J. Neurochem.*, 42 (1984) 764–774.
29 Schulz, D.W., Stanford, E.J., Wyrick, S.W. and Mailman, R.B., Binding of [3H]SCH23390 in rat brain: regional distribution and effects of assay conditions and GTP suggest interactions at a D1-like dopamine receptor, *J. Neurochem.*, 45 (1985) 1601–1611.
30 Seamon, K.B. and Daly, J.W., Forskolin: a unique diterpine activator of cyclic AMP-generating systems, *J. Cycl. Nuc. Res.*, 7 (1981) 201–224.
31 Steiner, A.L., Parker, C.W. and Kipnis, D.M., Radioimmunoassay for cyclic nucleotides. I. Preparation of antibodies and iodinated cyclic nucleotides, *J. Biol. Chem.*, 247 (1972) 1106–1113.
32 Tamir, A., and Gill, M., ADP-ribosylation by cholera toxin of membranes derived from brain modifies the interaction of adenylate cyclase with guanine nucleotides and NaF, *J. Neurochem.*, 50 (1988) 1791–1797.