MOLECULAR PHARMACOLOGY, 21:570–580

Differential Modification of the Interaction of Cardiac Muscarinic Cholinergic and *Beta*-Adrenergic Receptors with a Guanine Nucleotide Binding Component(s)

T. KENDALL HARDEN,[1] ANTHONY G. SCHEER, AND MCHARDY M. SMITH

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

Received October 1, 1981; Accepted January 18, 1982

SUMMARY

The interactions of muscarinic cholinergic and *beta*-adrenergic receptors with a guanine nucleotide binding component(s) have been compared using rat heart membranes. The apparent affinity of oxotremorine, carbachol, and methacholine but not antagonists at muscarinic receptors as measured by their capacity to inhibit [^{3}H]quinuclidinyl benzilate binding was reduced in the presence of GTP. Similarly, the apparent affinity of (−)-isoproterenol but not (−)-propranolol as measured by its capacity to inhibit [^{125}I]hydroxybenzylpindolol binding to *beta*-adrenergic receptors was reduced in the presence of GTP. Concentration-effect curves generated for the effects of various nucleotides on agonist affinity at muscarinic and *beta*-adrenergic receptors indicated that the relative affinity for the effect of a given nucleotide was similar, although not identical, for each receptor type. The rank order of affinities for nucleotides determined in the absence of Mg^{2+} was guanosine 5′-*O*-3-thiotriphosphate ≈ guanosine 5′-*O*-2-thiodiphosphate ≈ GDP > GTP > 5′-guanylyl imidodiphosphate [Gpp(NH)p] > ITP ≈ UTP. GMP, CTP, and ATP were not active at high concentrations. In the presence of added Mg^{2+}, the $K_{0.5}$ for Gpp(NH)p was markedly reduced; the apparent $K_{0.5}$ values of GTP and GDP were markedly increased in the presence of Mg^{2+} due to degradation of nucleotide. Pretreatment of membranes with *N*-ethylmaleimide (NEM) caused a shift to the right of the competition binding curve for oxotremorine and increased the Hill slope of competition curves from 0.66 to 0.89. The apparent affinity of oxotremorine for muscarinic receptors in NEM-pretreated membranes was similar to the apparent affinity of oxotremorine in the presence of GTP in control membranes. GTP had little effect on the competition binding curve for oxotremorine in NEM-pretreated membranes. In contrast to the effects of NEM on agonist interaction at muscarinic receptors, pretreatment of membranes with NEM at concentrations as high as 1 mM had no effect on agonist interaction at *beta*-adrenergic receptors. The effects of NEM pretreatment on the interaction of muscarinic receptors with adenylate cyclase were also studied. Oxotremorine inhibited both basal and isoproterenol-stimulated adenylate cyclase activities in a concentration-dependent manner. Pretreatment of membranes with NEM under conditions which reduced the apparent binding affinity of oxotremorine and reduced the effects of GTP on agonist affinity also significantly reduced the efficacy of oxotremorine for inhibition of basal or isoproterenol-stimulated adenylate cyclase activity. These results indicate that, although the properties of interaction of muscarinic cholinergic and *beta*-adrenergic receptors with a guanine nucleotide binding component(s) are similar, this interaction can be differentially modified by alkylation of membrane proteins with NEM.

INTRODUCTION

Catecholamines activate adenylate cyclase through a series of steps that requires the presence of GTP (1, 2). A variety of approaches has led to the identification of a guanine nucleotide-binding protein which couples occupation of *beta*-adrenergic receptors by agonists to activation of the catalytic unit of adenylate cyclase (3–6). Indeed, it is now reasonably clear that a high-affinity complex involving the association of agonist, receptor, and guanine nucleotide-binding protein is obligatory for activation of the enzyme by catecholamines (see ref. 2). This agonist-induced interaction between the receptor and guanine nucleotide-binding protein is apparently

This work was supported by National Institutes of Health Grant GM/HL 29536.

[1] Recipient of an Established Investigatorship of the American Heart Association.

0026-895X/82/0570-11$02.00/0

dependent on the presence of magnesium (7, 8) and is reflected in radioligand binding studies by a decrease in the apparent affinity of agonists in the presence of guanine nucleotides (9, 10).

In contrast to the effects of catecholamines, several laboratories have confirmed that adenylate cyclase activity is inhibited in broken-cell preparations by agonists acting at muscarinic cholinergic (11–14), *alpha*$_2$-adrenergic (15), opiate (16, 17), and adenosine (18) receptors. As with activation of adenylate cyclase by catecholamines, inhibition of enzyme activity by agonists acting at muscarinic (13, 14) and other receptors (15, 17, 18) is dependent on the presence of GTP. In competition binding experiments with membrane preparations the apparent affinity of agonists interacting with these inhibitory receptors is decreased in the presence of guanine nucleotides (19–21). It has not been unequivocally demonstrated that this effect of nucleotides on agonist binding is related to the GTP-dependent attenuation of adenylate cyclase activity mediated through these receptors, nor is it known whether a common nucleotide binding protein is involved in the coupling of both stimulatory and inhibitory receptors to the catalytic protein.

In the present paper we report results from experiments that have been initiated with the goal of contributing to our understanding of the mechanism through which muscarinic cholinergic receptors inhibit adenylate cyclase. The properties of the interaction of muscarinic versus *beta*-adrenergic receptors with a guanine nucleotide binding component(s) have been compared, and an attempt has been made to alter differentially with a group-specific reagent the function of these two receptor systems. A preliminary description of portions of this work has been reported (22).

METHODS

Membrane preparation. Male or female Sprague-Dawley rats (150–225 g) were killed, and whole hearts were dissected free of vascular and connective tissue. The hearts were homogenized in 20–30 volumes of 10 mM Tris (pH 7.5 at 0°) and 1 mM EDTA/g of tissue (wet weight) using a Brinkman Polytron tissue disruptor (setting 6.0 for 15 sec). The homogenate was filtered through two layers of cloth gauze and centrifuged at 40,000 × *g* for 10 min. The resultant pellet was resuspended in 10 mM Tris (pH 7.5 at 0°) and 1 mM EDTA. This centrifugation step was repeated two more times.

NEM treatment. Membranes [6 mg (wet weight) per milliliter] were incubated in 10 mM Tris (pH 7.5), 1 mM EDTA, and various concentrations of NEM.[2] Incubations were usually carried out on ice for 25–30 min. The reaction was stopped with a concentration of dithiothreitol equivalent to that of NEM, and the samples were centrifuged at 40,000 × *g* for 15 min. Final resuspension for assays was in 10 mM Tris (pH 7.5 at 30° or 37°).

Muscarinic cholinergic receptor assay. Binding assays were carried out in 1 or 4 ml of 10 mM Tris (pH 7.5 at 37°) containing [^{3}H]QNB at a concentration of 125–400 pM and heart membranes (150–225 μg of protein). Under these conditions less than 10% of the added radioligand was bound at equilibrium. Incubations were usually carried out for 60 min at 37°. The reaction was terminated by adding 10 ml of wash buffer consisting of 10 mM Tris (pH 7.5 at 37°) and 0.145 M NaCl, pouring the samples over glass-fiber filters (Schleicher and Schuell No. 30), and washing the filters with an additional 10 ml of 37° wash buffer. Radioactivity retained by the filters was counted at an efficiency of 33%. All experimental points were carried out in triplicate. Nonspecific binding was defined as the amount of radioactivity bound in the presence of 1 μM atropine. Specific binding usually comprised 90–95% of the total counts retained by the filter. Specific [^{3}H]QNB binding was proportional to added membrane protein up to a concentration of 350 μg of protein per ml. The calculated rate constants for association (5.4×10^8 M^{-1} min^{-1}) and dissociation (0.01 min^{-1}) of [^{3}H]QNB were similar to those previously reported (23, 24). The kinetically derived K_D (18 pM) was in good agreement with the K_D (14 pM) determined by Scatchard analysis of equilibrium binding data. The density of binding sites determined by Scatchard analysis of saturation binding isotherms was 172 fmoles/mg of protein. Assays were typically carried out at concentrations of [^{3}H]QNB much higher than its K_D to facilitate more rapid attainment of steady-state binding during generation of competition binding curves. Thus, in most experiments greater than 80% of the total muscarinic receptors were occupied by [^{3}H]QNB. The illustrated competition binding curves are shifted 7- to 20-fold to the right as a result of this high concentration of [^{3}H]QNB.

Beta-adrenergic receptor assay. HYP was iodinated and [^{125}I]HYP purified as previously described (25). In a typical assay, heart membranes (50–225 μg of protein) and [^{125}I]HYP (40–90 pM) were incubated for 60 min at 37° in 10 mM Tris (pH 7.5 at 37°). The assay volume was 1.0 ml. The reaction was terminated as described for the [^{3}H]QNB binding assay, and radioactivity retained by the filters was counted in a gamma counter. Specific binding was defined as the amount of [^{125}I]HYP bound in the absence of competing ligand minus the amount of [^{125}I]HYP bound in the presence of 100 μM (−)-isoproterenol. Specific binding represented 75–90% of total binding. Experimental conditions were maintained whereby less than 5% of the added [^{125}I]HYP was bound. Assays were carried out in triplicate. The K_D of [^{125}I]HYP in this heart membrane preparation was approximately 35 pM and the density of binding sites was 30 fmoles/mg of protein.

Adenylate cyclase assay. Adenylate cyclase activity was quantitated by a modification of the method of Salomon *et al.* (26). Each assay tube contained, in final concentrations, the following reagents: 0.1 mM [α-^{32}P] ATP (60 cpm/pmole), 1.0 mM ^{3}H-labeled cyclic AMP (30,000 cpm/assay), 8 mM creatine phosphate, creatine phosphokinase (6 units/assay), 1 μM GTP, 5 mM $MgSO_4$, 1.33 mM EGTA, 150 mM NaCl, and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5) in a final volume of 0.150 ml. Reactions were started by adding approximately 100 μg of membrane protein to a test tube

[2] The abbreviations used are: NEM, *N*-ethylmaleimide; QNB, (−)-quinuclidinyl benzilate; HYP, hydroxybenzylpindolol; Gpp(NH)p, 5′-guanylyl imidodiphosphate; GDPβS, guanosine-5′-*O*-2-thiodiphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); $K_{0.5}$, concentration required to obtain 50% of the maximal effect.

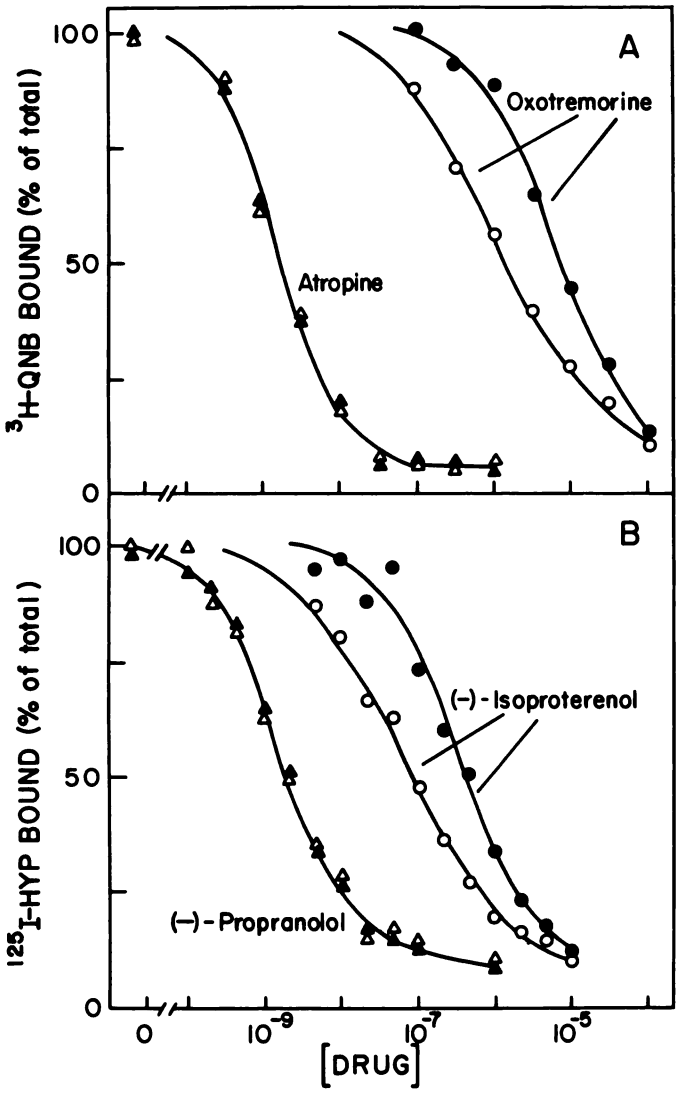

FIG. 1. *Effect of GTP on the interaction of agonists and antagonists with muscarinic cholinergic and beta-adrenergic receptors*

A. The capacity of atropine (△, ▲) and oxotremorine (○, ●) for inhibition of [^{3}H]QNB binding to heart membranes was measured in the absence (*open symbols*) or presence (*closed symbols*) of 100 μM GTP. Total [^{3}H]QNB binding in the absence of competing drug was 26 fmoles.

B. The capacity of (−)-propranolol (△, ▲) and (−)-isoproterenol (○, ●) for inhibition of [^{125}I]HYP binding to heart membranes was measured in the absence (*open symbols*) or presence (*closed symbols*) of 100 μM GTP. Total [^{125}I]HYP binding in the absence of competing drug was 3.5 fmoles.

The data are the average of three experiments in which [^{3}H]QNB (295 pM) and [^{125}I]HYP (40 pM) were incubated in a volume of 1 ml with the same heart membrane preparations (approximately 180 μg/ml). Replicate determinations of each data point varied by less than 7%. IC_{50} values in the absence of GTP for atropine, propranolol, oxotremorine, and isoproterenol were 2.8 ± 0.5 nM, 1.4 ± 0.1 nM, 1.6 ± 0.5 μM, and 0.08 ± 0.01 μM, respectively. IC_{50} values in the presence of GTP were 2.5 ± 0.3 nM, 1.4 ± 0.1 nM, 11.0 ± 2.0 μM, and 1.1 ± 0.2 μM, respectively, for the same four drugs. IC_{50} values for isoproterenol and oxotremorine in the presence of GTP were significantly different ($p < 0.01$) from the values in its absence. Hill coefficients in the absence of GTP for atropine, oxotremorine, propranolol, and isoproterenol were 1.01 ± 0.01, 0.69 ± 0.03, 1.03 ± 0.02, and 0.65 ± 0.02, respectively. Values for competition curves for the same compounds in the presence of GTP were 1.03 ± 0.02, 0.90 ± 0.04, 0.97 ± 0.04, and 0.97 ± 0.05, respectively.

containing all other reagents. Incubation was carried out for 12 min at 30° and the reaction was terminated with the addition 0.85 ml of 5% (w/v) trichloroacetic acid. [α-^{32}P]ATP was separated from ^{32}P-labeled cyclic AMP by sequential column chromatography over Dowex 50-X8 and neutral alumina. The procedure followed that suggested by Minneman *et al.* (27). Kontes columns (internal diameter 8 mm) were filled with 2.5 cm of Dowex or 600 mg of alumina. The 1-ml assay sample was applied to a Dowex column which was subsequently washed with 3 ml of water. The Dowex column was then placed over an alumina column, and 4 ml of water were applied to the Dowex column; the cyclic AMP eluted directly onto the alumina column. Cyclic AMP was eluted from the alumina column with 2.5 ml of 50 mM Tris (pH 8.0). Recovery of ^{3}H-labeled cyclic AMP was 60%, and assay blanks contained 50–60 cpm.

Chromatography of guanine nucleotides. The stability of [^{3}H]GTP and [^{3}H]GDP was assessed by incubation of labeled nucleotides in the presence of membranes (approximately 200 μg of protein per milliliter) for 60 min at 37° in a volume of 50 μl. The incubation was terminated with the addition of 10 μl of a solution containing 50 mM GTP, 50 mM GDP, and 50 mM GMP. A portion of the reaction mixture (1–2 μl) was spotted on cellulose MN 300 polyethyleneimine thin-layer plates (Macherey-Nagel) and chromatograms were developed in 1.0 M LiCl. R_F values for GTP, GDP, GMP, guanosine, and cyclic GMP in 1.0 M LiCl were 0.06, 0.16, 0.44, 0.58, and 0.58, respectively. Guanosine and cyclic GMP were differentiated by chromatography in water instead of 1.0 M LiCl. Guanosine ran with an R_F of approximately 0.6 in water while cyclic GMP remained at the origin.

Protein determination. Protein concentration was determined by the method of Lowry *et al.* (28), using bovine serum albumin as a standard.

Materials. ^{3}H-Labeled(−)-QNB (40.2 Ci/mmole), [^{3}H]GDP (6.1 Ci/mmole), [^{3}H]GTP (38.3 Ci/mmole), and [α-^{32}P]ATP (10–30 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.). ^{3}H-Labeled cyclic AMP (36.4 Ci/mmole) was obtained from ICN (Cleveland, Ohio). Gpp(NH)p was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), ICN, and Sigma Chemical Company (St. Louis, Mo.). GMP, GDPβS, and GTPγS were from Boehringer Mannheim Biochemicals. All other nucleotides were from Sigma Chemical Company. Other chemicals were from sources previously sited (29). Hydroxybenzylpindolol was a generous gift from Dr. Dan Hauser of Sandoz Pharmaceuticals (Basel, Switzerland).

RESULTS

The interaction of agonists and antagonists with cardiac *beta*-adrenergic and muscarinic cholinergic receptors was studied using [^{125}I]HYP and [^{3}H]QNB, respectively. GTP had no effect on the interaction of propranolol or [^{125}I]HYP alone with *beta*-adrenergic receptors but reduced the apparent affinity of isoproterenol for

The values for oxotremorine and isoproterenol in the presence of GTP were significantly different ($p < 0.01$) from those determined in its absence.

these binding sites (Fig. 1B). Similarly, while not affecting the interaction of [^{3}H]QNB alone or atropine with muscarinic cholinergic receptors, GTP caused a shift to the right of the competition binding curve of oxotremorine (Fig. 1A). Similar effects of GTP were also observed in competition binding curves with the agonists carbachol and methacholine (data not shown). These effects are similar to those previously reported by others for *beta*-adrenergic receptors (12, 30) and muscarinic cholinergic receptors (21, 31, 32) of mammalian heart.

Experiments were carried out to assess further the characteristics of the interaction of GTP and other nucleotides with the *beta*-adrenergic and muscarinic cholinergic receptor systems. Concentration-effect curves for nucleotides were generated by incubating [^{125}I]HYP or [^{3}H]QNB with various concentrations of nucleotide and with a single concentration of agonist which inhibited 40–75% of radiolabeled antagonist binding. GTP caused a concentration-dependent decrease (i.e., an increase in radioligand binding) in the capacity of oxotremorine (Fig. 2) or isoproterenol (data not shown) to inhibit antagonist binding to muscarinic cholinergic or *beta*-adrenergic receptors, respectively. From such curves a $K_{0.5}$ value for the effect of GTP was obtained. It should be pointed out that the effects of GTP on agonist affinity at *beta*-adrenergic and muscarinic receptors illustrated in Figs. 1 and 2 were obtained in binding assays carried out in the absence of added Mg^{2+} and with membranes that had been extensively washed with EDTA. This result is in contrast to the well-documented requirement for Mg^{2+} of high-affinity agonist binding and guanine nucleotide effects on agonist binding observed in a number of *beta*-adrenergic (7, 8) and muscarinic (32) receptor systems.

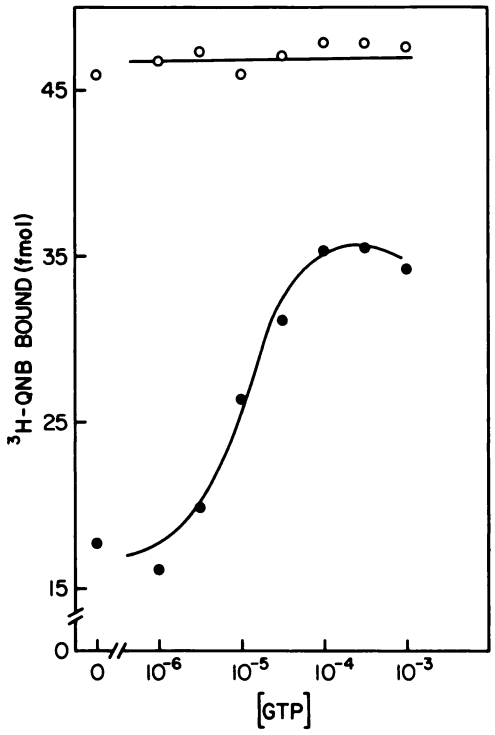

FIG. 2. *Concentration-effect curve for the reduction of oxotremorine affinity by GTP*

Cardiac membranes were incubated with 125 pM [^{3}H]QNB, 1 μM oxotremorine, and various concentrations of GTP in a volume of 4 ml. Total [^{3}H]QNB bound (○) and [^{3}H]QNB bound in the presence of 1 μM oxotremorine (●) are presented as a function of GTP concentration. The data are representative of 10 similar experiments.

One goal of these experiments was to compare the nucleotide selectivity of effects on muscarinic versus *beta*-adrenergic receptors. By using the approach illustrated in Fig. 2, $K_{0.5}$ values for the effects of a series of nucleotides were determined under similar assay conditions for both receptors. Concentration-effect curves for a number of nucleotides were generated both in the presence and in the absence of added Mg^{2+}. In general the effects of the nucleotides on agonist interactions with these two receptors were very similar (Table 1). The order of affinities of 10 nucleotides was the same for each receptor. The greatest difference in affinity was with GDP, which in the absence of Mg^{2+} was 4.5-fold more potent in the *beta*-adrenergic receptor system than the muscarinic receptor system; the affinities determined for GTP differed by 2-fold. In the absence of added Mg^{2+}, GDP was 2- to 3-fold more potent than GTP whereas the stable guanine nucleotide analogue, Gpp(NH)p, was 6- to 10-fold less potent than GTP. The effect of GDP on agonist affinity does not appear to be explained by transphosphorylation of GDP to GTP by myokinase in the preparation, since [^{3}H]GDP was recovered unchanged under the conditions of these assays (Fig. 3). Similarly, the apparent $K_{0.5}$ value for GTP appears to be reasonably valid, since more than 80% of added [^{3}H]GTP was recovered as authentic nucleotide (Fig. 3). Among the other nucleoside triphosphates examined, ITP was the most potent and ATP and CTP were without apparent effect at high concentrations (Table 1). However, the $K_{0.5}$ values for these nucleotides should be considered only in a relative sense, since the stability of these compounds has not been examined.

The addition of Mg^{2+} was not necessary to observe large effects of nucleotides on agonist affinities. As is illustrated in Table 1, the presence of Mg^{2+} had a negligible effect on the $K_{0.5}$ values determined for GDPβS but a somewhat larger effect on the $K_{0.5}$ for GTPγS. The presence of Mg^{2+} caused a marked shift of concentration-effect curves for GTP, GDP, and Gpp(NH)p (Table 1). The decrease in the apparent affinity of GTP and GDP can be explained by an enhanced degradation of these nucleotides in the presence of Mg^{2+} (Fig. 3); that is, little radioactivity was recovered chromatographing with authentic GTP or GDP following incubation in the presence of 5 mM $MgSO_4$. The apparent affinity of the stable analogue, Gpp(NH)p, was significantly increased in the presence of Mg^{2+}.

The binding affinity of agonists at muscarinic receptors was reduced approximately 10-fold by the addition of NaCl (data not shown; 21); addition of NaCl had no effect on the affinity of agonists at *beta*-adrenergic receptors in this membrane preparation (data not shown). Most of the assays in this study were carried out in the absence of NaCl. However, $K_{0.5}$ values ($n = 3$ or 4) determined for nucleotides were not changed by addition of NaCl to the assay. For example, $K_{0.5}$ values (micromolar) for Gpp(NH)p and GDPβS in the presence of 145 mM NaCl and 5 mM $MgSO_4$ were 2.4 ± 0.6 and 1.6 ± 0.9, respec-

TABLE 1

$K_{0.5}$ values for the effects of nucleotides on the apparent affinity of muscarinic and beta-adrenergic receptor agonists

Concentration-effect curves for the effects of nucleotides on the apparent affinity of oxotremorine for muscarinic receptors or isoproterenol for *beta*-adrenergic receptors were generated as is illustrated in Fig. 2 under identical conditions (equivalent tissue concentration and assay volume) for each receptor type. Assays were carried out with 3–10 μM oxotremorine and 0.3 μM isoproterenol. Assays were carried out in the absence or presence of 5 mM $MgSO_4$. The data are presented as means ± standard error of the mean of the indicated number of experiments.

Nucleotide	$K_{0.5}$ value			
	Beta-adrenergic receptor		Muscarinic cholinergic receptor	
	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$
	μM	μM	μM	μM
GTP	4.5 ± 0.8 (n = 11)	70.2 ± 16.2 (n = 4)$^{xx a}$	10 ± 1.3 (n = 10)$^{** b}$	113.9 ± 50.5 (n = 4)^{xx}
GDP	1.2 ± 0.2 (n = 10)	100.6 ± 36.0 (n = 4)^{xx}	5.9 ± 1.7 (n = 11)*	155.1 ± 104 (n = 4)^{xx}
Gpp(NH)p	43.3 ± 7.6 (n = 12)	3.2 ± 1.1 (n = 5)^{xx}	36.6 ± 7.4 (n = 14)	2.3 ± 1.0 (n = 4)^{xx}
GTPγS	1.4 ± 0.2 (n = 4)	0.6 ± 0.2 (n = 4)^{x}	2.5 ± 0.3 (n = 4)*	0.3 ± 0.1 (n = 4)^{xx}
GDPβS	1.7 ± 0.4 (n = 4)	1.4 ± 0.5 (n = 4)	2.3 ± 0.3 (n = 4)	1.4 ± 0.4 (n = 4)
GMP	>1000 (n = 3)		>1000 (n = 3)	
ITP	462 ± 152 (n = 4)		358 ± 125 (n = 4)	
UTP	638 ± 150 (n = 4)		>1000 (n = 4)	
CTP	>1000 (n = 4)		>1000 (n = 4)	
ATP	>1000 (n = 2)		>1000 (n = 2)	

[a] Significantly different (x = $p < 0.05$; xx = $p < 0.01$) from the value for the same receptor obtained in the absence of Mg^{2+}.

[b] Significantly different (* = $p < 0.05$; ** = $p < 0.01$) from the value determined for *beta*-adrenergic receptors under the same conditions.

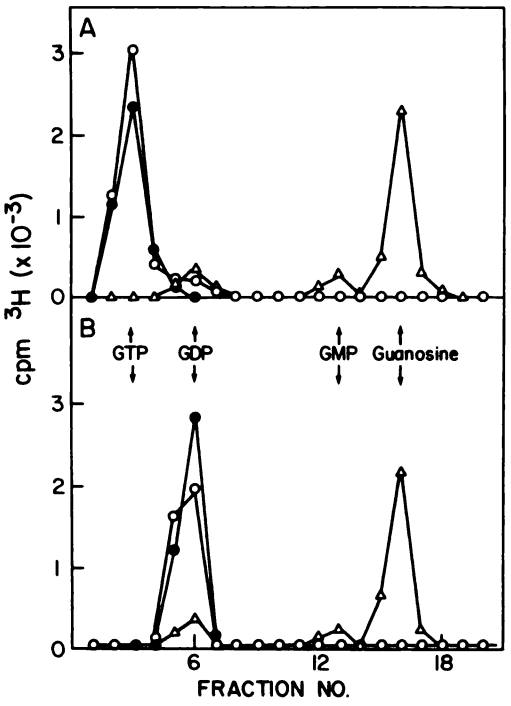

FIG. 3. *Chromatography of guanine nucleotides*

[^{3}H]GTP (A) or [^{3}H]GDP (B) was incubated at a concentration of 2 μM with 1 mM EDTA and 10 mM Tris (pH 7.5) (●) or with 1 mM EDTA and 10 mM Tris containing heart membranes (260 μg of protein per milliliter) in the absence (○) or presence (△) of 5 mM $MgSO_4$. After a 60-min incubation the reaction was stopped and guanine nucleotides were separated by thin-layer chromatography as described under Methods. Fraction numbers for which data points are not illustrated for an assay condition indicate portions of the chromatogram in which radioactivity was not greater than background. The data are presented as the counts per minute of radioactivity recovered in each fraction and are representative of four similar experiments.

tively, with *beta*-adrenergic receptors, and 3.2 ± 2.3 and 1.6 ± 1.0, respectively, with muscarinic receptors.

One approach for identifying differences in the properties of the interaction of nucleotides with their binding components in the muscarinic cholinergic versus *beta*-adrenergic receptor systems is through the utilization of group specific reagents. The reducing agent, dithiothreitol, had no effect at concentrations up to 1 mM on the interaction of either antagonists or agonists with muscarinic cholinergic receptors (data not shown). In contrast, preincubation of membranes with the alkylating agent NEM, while not affecting [^{3}H]QNB binding, produced marked effects on the interaction of agonists with muscarinic receptors. As is shown in Fig. 4, pretreatment of membranes with 60 μM NEM (25 min at 4°) caused a shift to the right of the concentration-effect curve for oxotremorine. This shift of the competition binding curve for oxotremorine was at least to the position of oxotremorine competition binding curves generated in the presence of GTP with control membranes. In a number of experiments the concentration-effect curve for oxotremorine in NEM-treated membranes was shifted as much as 2- to 3-fold to the right of that of oxotremorine in the presence of GTP in control membranes. As is also demonstrated in Fig. 4, the marked effect of GTP on oxotremorine affinity was lost in NEM-pretreated membranes. Not only did NEM pretreatment decrease the apparent affinity of oxotremorine for muscarinic receptors and block the effect of guanine nucleotides on affinity, but the competition binding curve more closely approximated that predicted by the law of mass action. Hill plots of these data are illustrated in Fig. 5. The concentration-effect curve for oxotremorine in control membranes deviates significantly from the law of mass action for the interaction of a ligand with a single site; the average Hill coefficient determined for 10 such curves was 0.66 ± 0.01.

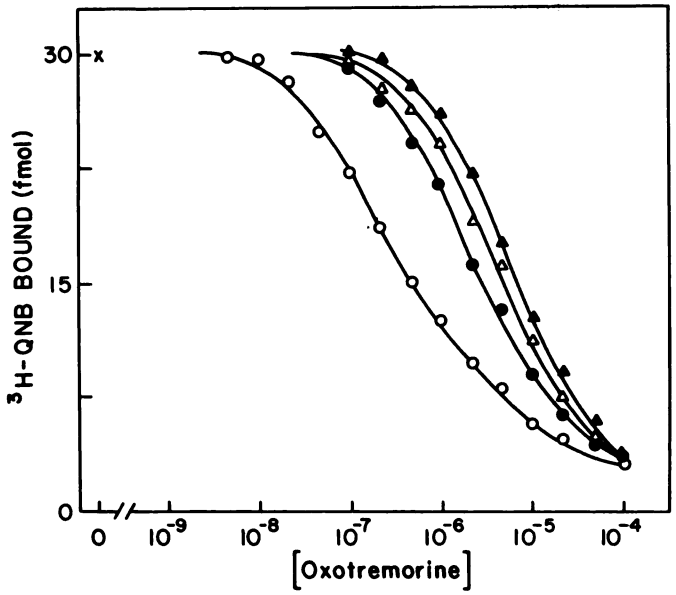

FIG. 4. *Effect of NEM pretreatment on competition binding curves for oxotremorine*

Cardiac membranes were treated with 60 μM NEM for 25 min on ice as is described under Methods. The capacity of oxotremorine to inhibit [^{3}H]QNB (155 pM in 4.0 ml) binding was measured as described under Methods in control (○, ●) or NEM-pretreated (△, ▲) membranes in the absence (*open symbols*) or presence (*closed symbols*) of 300 μM GTP. The data are representative of 14 similar experiments. IC_{50} values for oxotremorine taken from six experiments carried out with [^{3}H]QNB at a similar concentration (150 pM) were 0.88 ± 0.3 μM and 5.92 ± 1.7 μM for control membranes in the absence and presence of GTP and 7.14 ± 1.5 μM and 7.93 ± 1.8 μM for NEM-treated membranes in the absence and presence of GTP. The values for control membranes in the presence of GTP and for NEM-treated membranes under either condition were significantly different ($p < 0.01$) from control in the absence of GTP.

The Hill coefficients determined for oxotremorine concentration-effect curves with control membranes in the presence of GTP ($n_H = 0.86 \pm 0.03$; $n = 10$)[3] and in NEM-pretreated membranes in the absence ($n_H = 0.89 \pm 0.05$; $n = 8$) or presence ($n_H = 0.92 \pm 0.04$; $n = 8$) of 100 μM GTP were much closer to unity. These effects of NEM are not restricted to oxotremorine, since similar reductions in the apparent affinity and loss of guanine nucleotide effects on affinity were observed when carbachol or methacholine was used as the agonist (Table 2A).

The time dependence of the effect of NEM is illustrated in Fig. 6. Maximal effects of 60 μM NEM on oxotremorine affinity were observed within 25 min at 4°. The extent of effects of NEM during a 25-min incubation was dependent on the concentration of alkylating agent (Fig. 7). NEM pretreatment at 4° had no effect on [^{3}H]QNB binding alone. The $K_{0.5}$ value determined for the effects of NEM during a 25-min incubation varied somewhat with the batch of NEM used in the experiment and the age of the source of NEM. The average $K_{0.5}$ value for the effects of NEM during a 25-min incubation at 4° was 40 ± 20 μM ($n = 7$) in experiments carried out over a 12-month period. The experiments described here utilized an NEM preincubation of 25 min on ice. However, preincubation of membranes with NEM at 37° for 20 min also resulted in a decrease in apparent affinity of oxotremorine and a loss of the effects of guanine nucleotides on agonist affinity (data not shown). The $K_{0.5}$ for a 20-min incubation at 37° was approximately 5 μM. Incubation with NEM at 37° also resulted in a reduction in [^{3}H]QNB binding per se and an inactivation of adenylate cyclase (data not shown).

The receptor specificity of the effects of NEM was also examined. Pretreatment of membranes with NEM at concentrations up to 1 mM had no effect on the capacity of isoproterenol to inhibit [^{125}I]HYP binding or on the capacity of guanine nucleotides to decrease isoproterenol affinity in the absence of Mg^{2+} (Fig. 8). Concentration-effect curves for isoproterenol and oxotremorine with control and NEM (316 μM)-pretreated membranes are illustrated in Fig. 9. Although competition binding curves for isoproterenol in the absence or presence of Gpp(NH)p were unchanged by pretreatment with NEM, the oxotremorine-competition binding curve was shifted to the right and the effects of Gpp(NH)p on apparent affinity were lost. The effects of NEM do not appear to be

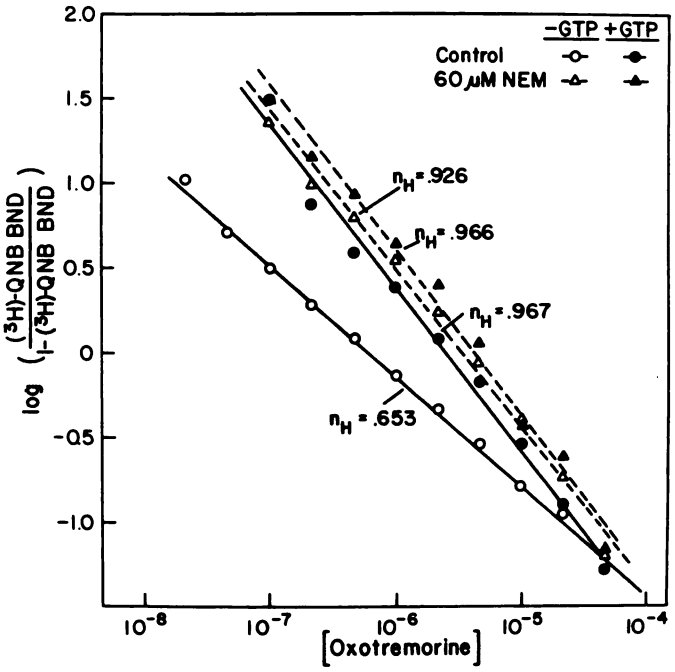

FIG. 5. *Hill plots of oxotremorine competition curves with control and NEM-pretreated heart membranes*

The data from the experiment described in Fig. 4 were utilized to generate Hill plots. The concentration of oxotremorine (abscissa) is plotted versus the log of the quotient of [^{3}H]QNB specifically bound at each oxotremorine concentration divided by 1 minus the amount of [^{3}H]QNB specifically bound. Data from control (○, ●) and NEM-treated membranes (△, ▲) in the absence (*open symbols*) or presence of 300 μM GTP (*closed symbols*) are plotted.

[3] All of the values for n_H with control membranes in the presence of GTP and with NEM-treated membranes in the absence or presence of GTP were significantly different ($p < 0.01$) from the value for control membranes in the absence of GTP. There was no significant difference ($p > 0.05$) between control membranes plus GTP and NEM-treated membranes in the absence or presence of GTP.

TABLE 2

Effect of NEM on the interaction of agonists with the muscarinic cholinergic receptor/adenylate cyclase system

Membranes were treated with 100 or 316 μM NEM as described under Methods. A. [^{3}H]QNB binding assays were carried out at a radioligand concentration of 400 pM in the presence of the indicated agonists and in the absence or presence of 100 μM GTP. The data are presented as counts per minute of [^{3}H]QNB bound and are the means ± standard error of the mean of three determinations. B. Adenylate cyclase activity was measured in each membrane preparation in the presence of various agonists. The data are expressed as picomoles of cyclic AMP per minute per milligram of protein and are the means ± standard error of the mean of four determinations. The numbers in parentheses represent enzyme activity in the presence of the indicated agonist divided by activity measured in the absence of agonist.

A. [^{3}H]QNB binding

Addition	Control		100 μM NEM		316 μM NEM	
	−GTP	+GTP	−GTP	+GTP	−GTP	+GTP
None	554 ± 39	585 ± 26	553 ± 17	524 ± 18	560 ± 28	583 ± 15
Oxotremorine (10 μM)	313 ± 8	452 ± 17	407 ± 13	466 ± 21	466 ± 15	492 ± 12
Methacholine (30 μM)	241 ± 9	393 ± 9	386 ± 8	427 ± 9	409 ± 8	441 ± 11
Carbachol (30 μM)	238 ± 1	427 ± 19	360 ± 5	431 ± 15	438 ± 12	444 ± 5

B. Adenylate cyclase activity

Addition	Control	100 μM NEM	316 μM NEM
None	15.4 ± 0.9	14.8 ± 0.7	11.8 ± 0.6
Oxotremorine (100 μM)	9.9 ± 0.4 (0.65)	13.2 ± 0.6 (0.89)	10.8 ± 0.6 (0.92)
Methacholine (100 μM)	10.5 ± 0.7 (0.69)	13.1 ± 0.3 (0.88)	10.6 ± 0.4 (0.90)
Carbachol (100 μM)	10.6 ± 0.4 (0.70)	12.8 ± 0.2 (0.86)	10.6 ± 0.1 (0.90)
Pilocarpine (100 μM)	12.4 ± 0.6 (0.81)	13.8 ± 0.4 (0.93)	10.8 ± 0.4 (0.92)
Propionyl choline (100 μM)	11.4 ± 0.4 (0.74)	13.6 ± 0.5 (0.92)	10.8 ± 0.4 (0.92)

dependent on the presence or absence of Mg^{2+} in the receptor assay, since the data presented in Figs. 4, 7, and 8 were obtained in the absence of added Mg^{2+} whereas the data in Fig. 9 were obtained in its presence.

The effects of NEM on the interaction of muscarinic

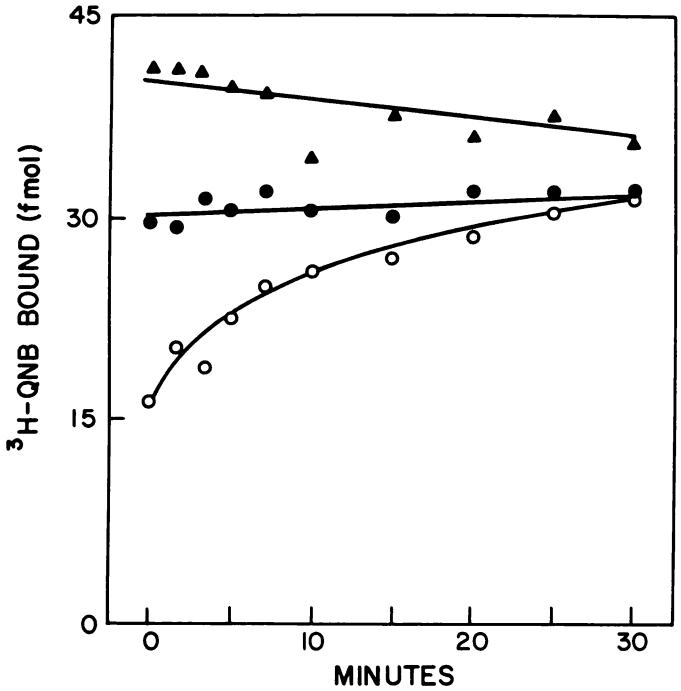

FIG. 6. *Time course for the effects of NEM on oxotremorine affinity*

Heart membranes were incubated with 60 μM NEM on ice for the indicated times. Subsequent to stopping the reaction as described under Methods, [^{3}H]QNB binding was measured in the absence (▲) of added drugs or in the presence of 1 μM oxotremorine (-○-,-●-) with (closed symbols) or without (open symbols) 300 μM GTP. The data are representative of three similar experiments.

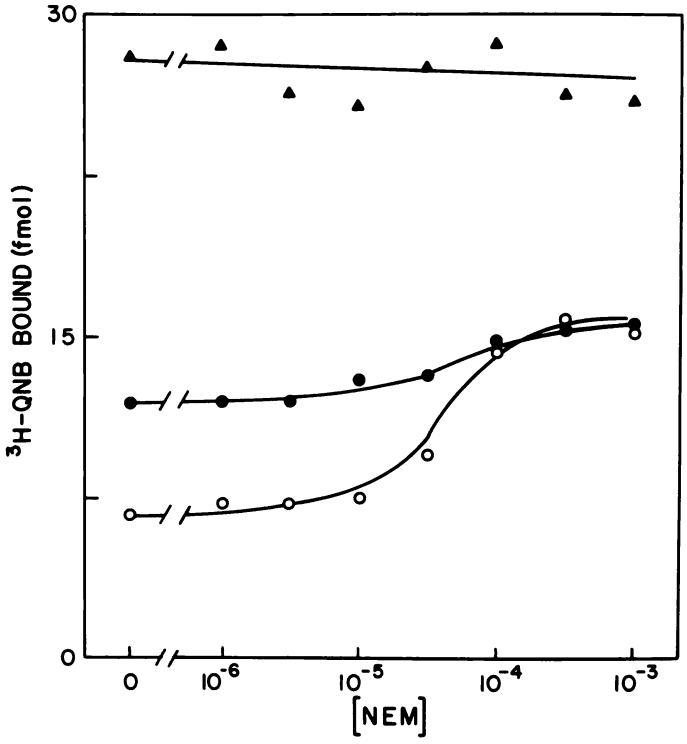

FIG. 7. *Concentration-dependent effect of NEM on agonist affinity at muscarinic receptors*

Heart membranes were incubated for 25 min on ice in the presence of the indicated concentrations of NEM. The reaction was stopped with an equal concentration of dithiothreitol. [^{3}H]QNB (300 pM) binding was measured in the NEM-treated membranes in the absence (▲) or in the presence (○, ●) of 1 μM oxotremorine. Assays containing oxotremorine were carried out in the absence (○) or in the presence (●) of 100 μM GTP. The data are representative of seven similar experiments.

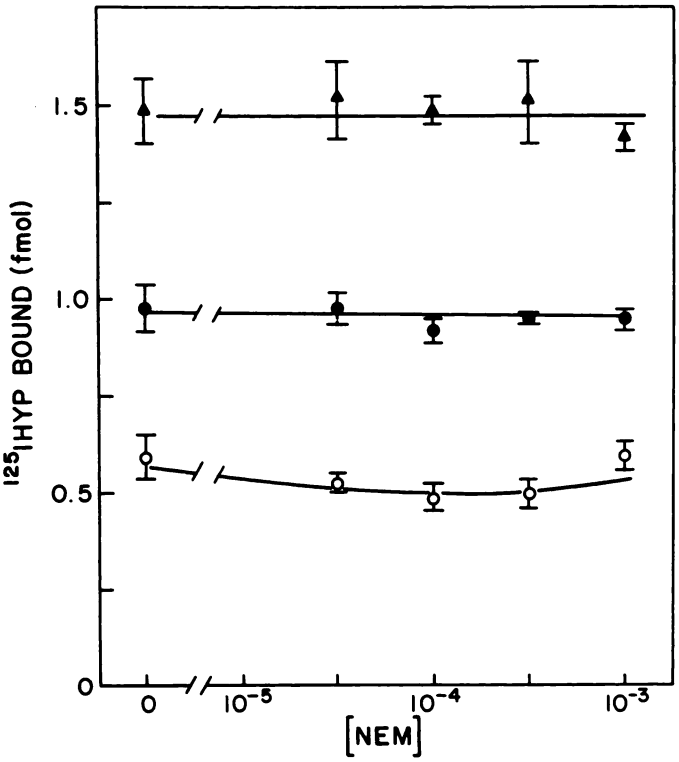

FIG. 8. *Effect of NEM on agonist affinity at β-adrenergic receptors*
Heart membranes were treated as described in Fig. 7. [^{125}I]HYP binding was measured in the NEM-pretreated membranes in the absence of competing drug (▲) or in the presence of 0.1 μM (−)-isoproterenol (○, ●). Assays containing isoproterenol were carried out in the absence (○) or the presence (●) of 100 μM GTP. The data are representative of four similar experiments.

versus *beta*-adrenergic receptors with adenylate cyclase were examined. Basal adenylate cyclase activity in the membrane preparation used in this study was 19.0 ± 1.0 pmoles/min/mg of protein (n = 21 preparations). A maximally effective concentration of isoproterenol (10 μM) markedly stimulated enzyme activity (48.3 ± 3.1 pmoles/min/mg of protein; n = 21 preparations). In contrast, in the presence of oxotremorine (100 μM), both basal (12.3 ± 0.7 pmoles/min/mg of protein; n = 21 preparations) and isoproterenol (10 μM)-stimulated enzyme activities (31.0 ± 2.0 pmoles/min/mg of protein; n = 21) were significantly ($p < 0.01$) reduced. These inhibitory effects of oxotremorine were blocked by atropine. As is illustrated in Fig. 10B, pretreatment of membranes with various concentrations of NEM for 25 min reduced the inhibitory effect of oxotremorine on isoproterenol-stimulated adenylate cyclase activity. Although the blockade of inhibitory activity of oxotremorine was complete at only one concentration of NEM (100 μM), these data suggest that pretreatment of membranes with NEM markedly interferes with the capacity of muscarinic receptors to inhibit adenylate cyclase. The higher concentrations of NEM caused some reduction of both basal and isoproterenol-stimulated activity (Table 2B; legend to Fig. 10). However, under these conditions isoproterenol still effectively activated the enzyme, and the fold stimulation of basal activity by catecholamine was the same as in control membranes. The capacity of oxotremorine to inhibit basal enzyme activity is also reduced by NEM pretreatment (Fig. 10A) in a manner similar to that observed with isoproterenol-stimulated enzyme activity. The effect of NEM pretreatment on inhibitory activity is not restricted to oxotremorine, since the activity of other muscarinic receptor agonists is affected in a similar manner (Table 2B). Concentration-effect curves for oxotremorine were also generated in control and NEM-treated membranes (Fig. 11). The effect of NEM pretreatment was to reduce the efficacy of oxotremorine as an inhibitory agonist.

DISCUSSION

The properties of interaction of nucleotides with the muscarinic cholinergic versus *beta*-adrenergic receptor system of rat heart are similar. As has been previously shown by others, guanine nucleotides reduced the appar-

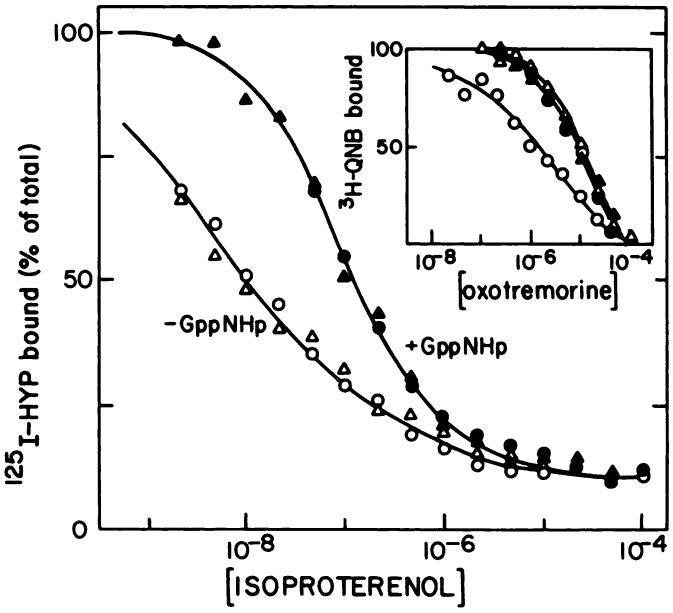

FIG. 9. *Competition binding curves for isoproterenol following NEM treatment*
Membranes were treated with 316 μM NEM as described. Competition binding curves were generated for isoproterenol with control (○, ●) and NEM-pretreated membranes (△, ▲) in the absence (○, △) or in the presence (●, ▲) of 30 μM Gpp(NH)p. All assays were carried out in the presence of 5 mM $MgSO_4$. The data are representative of the results obtained in three separate experiments.
Inset. Binding assays were carried out under identical conditions using [^{3}H]QNB and oxotremorine. Hill coefficients for the isoproterenol competition curves were 0.58 ± 0.05, 0.89 ± 0.02, 0.55 ± 0.05, and 0.90 ± 0.03 for control membranes, control membranes plus Gpp(NH)p, NEM-treated membranes, and NEM-treated membranes plus Gpp(NH)p, respectively. There was no statistical difference ($p > 0.1$) between competition curves of control and NEM-treated membranes in the absence of Gpp(NH)p or between competition curves for control and NEM-treated membranes in the presence of Gpp(NH)p. Hill coefficients for oxotremorine curves were 0.63 ± 0.05, 1.0 ± 0.09, 0.96 ± 0.06, and 0.94 ± 0.02 for control membranes, control membranes plus Gpp(NH)p, NEM-treated membranes, and NEM-treated membranes plus Gpp(NH)p. All of the values for the last three conditions were significantly different ($p < 0.01$) from control, but not ($p > 0.1$) from each other.

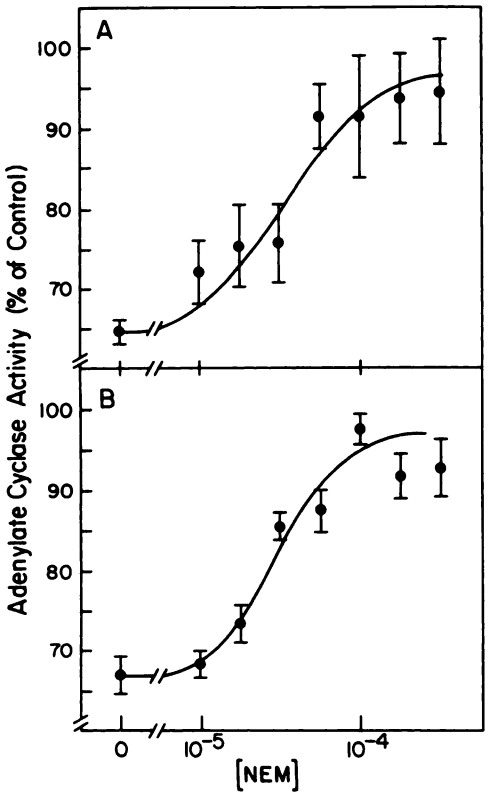

FIG. 10. *Loss of inhibitory effects of oxotremorine on adenylate cyclase following NEM pretreatment*

Membranes were treated with various concentrations of NEM as described under Methods. Adenylate cyclase activity was measured in the presence of oxotremorine (100 μM) and in the absence (A) or presence (B) of 10 μM (−)-isoproterenol. The data are plotted as the percentage of basal or isoproterenol-stimulated activity in the absence of oxotremorine in each NEM-treated membrane preparation. NEM had no significant effect on basal or isoproterenol-stimulated activity at concentrations less than 100 μM. Basal and isoproterenol-stimulated activities were reduced by 10–15, 20–25, and 40–50% by pretreatment of membranes with 100, 178, and 316 μM NEM, respectively. The data are means ± standard error of the mean of seven experiments for isoproterenol-stimulated activity (B) and five experiments for basal activity (A).

ent affinity of interaction of agonists with these receptors in heart membranes without significantly altering the interaction of receptor antagonists. Competition binding curves with agonists for either receptor exhibited Hill coefficients of 0.5–0.7, whereas coefficients for antagonist competition curves were approximately 1.0. In the presence of guanine nucleotides, the Hill coefficient for muscarinic or *beta*-adrenergic receptor agonist competition binding curves increased in value (although Hill coefficients of unity were not obtained). Finally, the order of potency of a series of nucleotides for altering interaction of agonists at muscarinic versus *beta*-adrenergic receptors was the same; although they were not identical, the calculated affinities of each nucleotide were very similar between the two receptors systems.

Beta-adrenergic receptor agonists induce the formation of a high-affinity complex of agonist, receptor, and guanine nucleotide binding protein (6, 33; see ref. 2). It has been proposed that Mg^{2+} is obligatory for the formation of this complex and that guanine nucleotides cause the complex to dissociate. Thus, in the absence of Mg^{2+}, high-affinity *beta*-adrenergic receptor agonist binding is not observed, and the effects of guanine nucleotides on agonist affinity are lost. A similar effect of Mg^{2+} on agonist interaction with muscarinic cholinergic receptors has been observed (32). In light of these observations, the paucity of effects of Mg^{2+} in the present study was surprising. Numerous attempts to observe Mg^{2+} effects on the affinity of both *beta*-adrenergic receptor and muscarinic receptor agonists in this membrane preparation have failed. The reasons for this result are unclear. However, the facts that (a) in the absence of added guanine nucleotide, isoproterenol and oxotremorine interact with *beta*-adrenergic and muscarinic receptors with affinities as high as those reported in the literature; (b) guanine nucleotides exhibit marked effects on apparent agonist affinities at both receptors; and (c) Hill slopes of competition curves of much less than 1 in the absence of guanine nucleotides and close to unity in their presence argue that the situation in the heart membrane preparation used in this study is similar to that observed previously in other systems. Indeed, the data suggest that, either due to tight binding of Mg^{2+} to the appropriate binding site or due to trapping of Mg^{2+} inside vesicles that are inaccessible to the EDTA wash, the addition of exogenous Mg^{2+} is inessential. Although the addition of Mg^{2+} did not affect the apparent affinity of agonists at either receptor, the apparent affinities of both GTP and GDP for decreasing agonist affinity were reduced. As was illustrated (Fig. 3), such a consequence can be explained by enhanced metabolism of nucleotides in the presence of Mg^{2+} and may relate to the Mg^{2+}

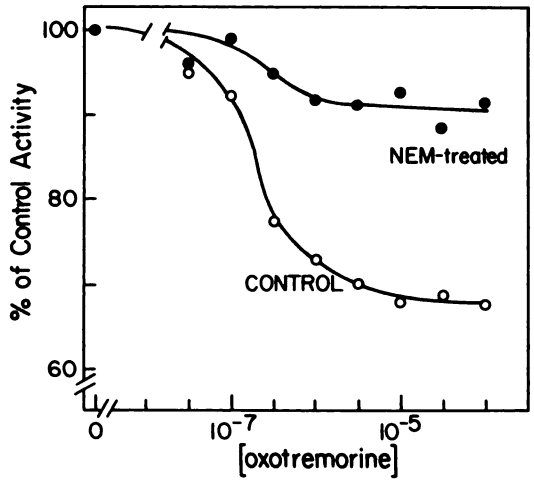

FIG. 11. *Concentration-effect curve for the inhibition of isoproterenol-stimulated adenylate cyclase activity by oxotremorine*

Adenylate cyclase activity was measured in the presence of 10 μM (−)-isoproterenol and the indicated concentrations of oxotremorine in control membranes (○) and in membranes that were pretreated with 100 μM NEM (●) for 25 min on ice. The data are plotted as the percentage of isoproterenol-stimulated enzyme activity in the absence of oxotremorine. Values for basal enzyme activity in control and NEM-treated membranes were 15.0 and 13.8 pmoles/min/mg, respectively. Values for isoproterenol-stimulated activity in control and NEM-treated membranes were 41.4 and 36.7 pmoles/min/mg, respectively. The data are means from six experiments with the standard error of the mean of each point less than 5% of the mean.

dependence of the nucleotide degrading enzymes. The marked increase in the affinity of Gpp(NH)p in the presence of Mg^{2+} is similar to that previously observed with [^{3}H]clonidine binding to *alpha*$_2$-adrenergic receptors in rat brain (34). More work is necessary to produce an explanation for this marked effect of Mg^{2+} on the interaction of Gpp(NH)p with membrane components. Nonetheless, it is clear from these studies that the interactions of a variety of nucleotides with the muscarinic cholinergic versus *beta*-adrenergic receptor systems share a number of common properties.

Very little is known about the components involved in the muscarinic receptor/adenylate cyclase system. In analogy with the *beta*-adrenergic receptor system, it is reasonable to propose that the effects of guanine nucleotides on agonist-muscarinic receptor interactions are mediated through a guanine nucleotide-binding protein rather than through a binding site on the muscarinic receptor per se and that these effects are reflective of fundamental events involved in the GTP-dependent attenuation of adenylate cyclase activity by muscarinic receptor agonists. However, these deductions have not been unequivocally demonstrated. In light of the great similarity of properties of the interaction of guanine nucleotides with receptor systems that both activate and inhibit adenylate cyclase, it is conceivable that a common nucleotide binding protein is involved with both stimulatory and attenuating receptor systems. However, it is clear from the present series of experiments that, if a common nucleotide binding protein is involved in the muscarinic and *beta*-adrenergic receptor systems, the interaction of this component with the muscarinic receptor system can be differentially altered by chemical modification; that is, preincubation of heart membranes with NEM results in a loss of effects of guanine nucleotides on muscarinic receptor agonist affinity while causing no measurable change in the *beta*-adrenergic receptor system. Although NEM has been shown to impede the interaction of *beta*-adrenergic receptors and the guanine nucleotide binding protein in other systems (35, 36), this effect has occurred at higher temperatures and concentrations of NEM than those used in this study. Whether the differential sensitivity of the muscarinic versus *beta*-adrenergic receptor systems is a reflection of different guanine nucleotide binding sites or proteins cannot be discerned from the present data. However, the data are clearly not inconsistent with such a conclusion. Indeed, the observations of Rodbell and co-workers have led to the proposal that separate nucleotide-binding proteins are involved in the coupling of stimulatory and inhibitory receptors to adenylate cyclase (37).

The data also support the idea that the effects of nucleotides on agonist interaction with muscarinic receptors are related to the interaction of muscarinic receptors with adenylate cyclase. Reductions in apparent affinity of oxotremorine and loss of guanine nucleotide effects in receptor binding experiments occurred in consonance with similar losses in the capacity of oxotremorine to inhibit adenylate cyclase. Such effects are similar to those observed in *beta*-adrenergic receptor systems that are uncoupled by a genetic deficiency in the guanine nucleotide-binding protein (38), by treatment with chemicals (33, 35, 36), or by short-term desensitization with catecholamines (39). Pretreatment of membranes did not consistently effect a complete block of the attenuating effects of oxotremorine on adenylate cyclase; it should be pointed out that a small percentage (5–10%) of the total guanine nucleotide effect on muscarinic agonist affinity was also resistant to NEM effects in a number of experiments (e.g., Fig. 4, Table 2).

While the manuscript was in preparation, a study (32) appeared which reports results comparable to those described in the present study; that is, it was demonstrated that pretreatment of rat atrial membranes with 2 mM NEM at 37° resulted in the conversion of competition binding curves (Hill coefficients ≈0.5) for carbachol to curves that more closely followed that predicted from the law of mass action for a single binding site (Hill coefficients close to 1.0). In addition, the capacity of GTP to cause a rightward shift and Mg^{2+} to cause a leftward shift of the competition binding curve for receptor agonists was lost in NEM-treated membranes. Interestingly, these changes induced by 2 mM NEM were predominantly manifested by an increase in the Hill coefficient of the competition binding curve for agonists, rather than the large rightward shift (and increased Hill coefficient) of agonist competition curves observed in the present study. These discrepancies cannot be explained by differences in tissue source, since we obtain the same results with NEM irrespective of whether atrium, ventricle, or whole heart is utilized. It has proven difficult for us to attain interpretable results under the conditions employed by Wei and Sulakhe (32), since in our hands large decreases in [^{3}H]QNB binding per se are observed when membranes are incubated with 2 mM NEM at 37°. Also, since Mg^{2+} has no effect on agonist affinity in the heart membrane preparation used in the present study, we have been unable to examine the effects of alkylation on Mg^{2+} interactions with this system. Nonetheless, it seems likely that we are both studying a similar phenomenon, and the discrepancies that are observed are likely to be dependent on the relatively large differences in the conditions for NEM pretreatment and differences in the membrane preparations used.

To date we have observed only a decrease in muscarinic receptor agonist affinity following NEM pretreatment of tissues where an effect of guanine nucleotides on agonist binding affinity is observed.[4] Thus, the phenomenon that we have reported would appear to be dependent on a functional interaction between muscarinic receptors and a guanine nucleotide binding component. We are currently attempting to utilize these NEM-induced alterations of muscarinic receptor properties to explore further the mechanism of transduction of signal between muscarinic and other inhibitory receptors and the catalytic component of adenylate cyclase.

ACKNOWLEDGMENTS

The authors are indebted to Drs. Gene A. Scarborough and John P. Perkins for enlightening discussions and suggestions through the course of the study and to Brenda Brown and Angie Hodgin for excellent assistance in preparing the manuscript.

[4] T. K. Harden and A. G. Scheer, unpublished observations.

REFERENCES

1. Rodbell, M., L. Birnbaumer, S. L. Pohl, and H. M. J. Krans. The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. *J. Biol. Chem.* **246**:1877–1882 (1971).
2. Ross, E. M., and A. G. Gilman. Biochemical properties of hormone-sensitive adenylate cyclase. *Annu. Rev. Biochem.* **49**:533–564 (1980).
3. Pfeuffer, T. GTP-binding proteins in membranes and the control of adenylate cyclase activity. *J. Biol. Chem.* **252**:7224–7234 (1977).
4. Cassel, D. and Z. Selinger. Mechanism of adenylate cyclase activation by cholera toxin: inhibition of GTP hydrolysis at the regulatory site. *Proc. Natl. Acad. Sci. U. S. A.* **74**:3307–3311 (1977).
5. Ross, E. M., A. C. Howlett, K. M. Ferguson, and A. G. Gilman. Reconstitution of hormone-sensitive adenylate cyclase activity with resolved components of the enzyme. *J. Biol. Chem.* **253**:6401–6412 (1978).
6. Limbird, L. E., D. M. Gill, and R. J. Lefkowitz. Agonist-promoted coupling of the β-adrenergic receptor with the guanine nucleotide regulatory protein of the adenylate cyclase system. *Proc. Natl. Acad. Sci. U. S. A.* **77**:775–779 (1980).
7. Williams, L. T., D. Mullikin, and R. J. Lefkowitz. Magnesium dependence of agonist binding to adenylate cyclase-coupled hormone receptors. *J. Biol. Chem.* **253**:2984–2989 (1978).
8. Bird, S. J., and M. E. Maguire. The agonist-specific effect of magnesium ion on binding by β-adrenergic receptors in S49 lymphoma cells. *J. Biol. Chem.* **253**:8826–8834 (1978).
9. Maguire, M. E., P. M. Van Arsdale, and A. G. Gilman. An agonist-specific effect of guanine nucleotides on binding to the *beta*-adrenergic receptor. *Mol. Pharmacol.* **12**:335–339 (1976).
10. Lefkowitz, R. J., D. Mullikin, and M. G. Caron. Regulation of β-adrenergic receptors by guanyl-5′-yl imidophosphate and other purine nucleotides. *J. Biol. Chem.* **251**:4686–4692 (1976).
11. 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**:1233–1238 (1962).
12. Watanabe, A. M., M. M. McConnaughey, R. A. Strawbridge, J. W. Fleming, L. R. Jones, and H. R. Besch. Muscarinic cholinergic receptor modulation of β-adrenergic receptor affinity for catecholamines. *J. Biol. Chem.* **253**:4833–4836 (1978).
13. Lichtshtein, D., G. Boone, and A. Blume. Muscarinic receptor regulation of NG108-15 adenylate cyclase: requirement for Na^+ and GTP. *J. Cyclic Nucleotide Res.* **5**:367–375 (1979).
14. Jakobs, K. H., K. Aktories, and G. Schultz. GTP-dependent inhibition of cardiac adenylate cyclase by muscarinic cholinergic agonists. *Naunyn-Schmiedebergs Arch. Pharmacol.* **310**:113–119 (1979).
15. Jakobs, K. H., W. Saur, and G. Schultz. Inhibition of platelet adenylate cyclase by epinephrine requires GTP. *F. E. B. S. Lett.* **85**:167–170 (1978).
16. Sharma, S. K., M. Nirenberg, and W. A. Klee. Morphine receptors as regulators of adenylate cyclase activity. *Proc. Natl. Acad. Sci. U. S. A.* **72**:590–594 (1975).
17. Blume, A. J., D. Lichtshtein, and G. Boone. Coupling of opiate receptors to adenylate cyclase: requirement for Na^+ and GTP. *Proc. Natl. Acad. Sci. U. S. A.* **76**:5626–5630 (1979).
18. Londos, C., D. M. F. Cooper, W. Schlegel, and M. Rodbell. Adenosine analogs inhibit adipocyte adenylate cyclase by a GTP-dependent process: basis for actions of adenosine and methylxanthines on cyclic AMP production and lipolysis, *Proc. Natl. Acad. Sci. U. S. A.* **75**:5362–5366 (1978).
19. U'Prichard, D. C., and S. H. Snyder. Guanyl nucleotide influence on ^{3}H-ligand binding to α-noradrenergic receptors in calf brain membranes. *J. Biol. Chem.* **253**:3444–3452 (1978).
20. Blume, A. J. Interaction of ligands with the opiate receptors of brain membranes: regulation by ions and nucleotides. *Proc. Natl. Acad. Sci. U. S. A.* **75**:1713–1717 (1978).
21. Rosenberger, L. B., H. I. Yamamura, and W. R. Roeske. Cardiac muscarinic cholinergic receptor binding is regulated by Na^+ and guanyl nucleotides. *J. Biol. Chem.* **255**:820–822 (1980).
22. Harden, T. K. Interaction of cardiac neurotransmitter receptors with a guanine nucleotide binding site. *Soc. Neurosci. Abstr.* **6**:254 (1980).
23. Fields, J. Z., W. R. Roeske, E. Morkin, and H. I. Yamamura. Cardiac muscarinic cholinergic receptors: biochemical identification and characterization. *J. Biol. Chem.* **253**:3251–3258 (1978).
24. Wei, J-W., and P. V. Sulakhe. Properties of the muscarinic cholinergic receptors in rat atrium. *Naunyn-Schmiedebergs Arch. Pharmacol.* **309**:259–269 (1979).
25. Harden, T. K., B. B. Wolfe, and P. B. Molinoff. Binding of iodinated *beta*-adrenergic antagonists to proteins derived from rat heart. *Mol. Pharmacol.* **12**:1–15 (1976).
26. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
27. Minneman, K. P., L. R. Hegstrand, and P. B. Molinoff. The pharmacological specificity of *beta*-1 and *beta*-2 adrenergic receptors in rat heart and lung *in vitro*. *Mol. Pharmacol.* **16**:21–33 (1979).
28. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
29. Harden, T. K., S. J. Foster, and J. P. Perkins. Differential expression of components of the adenylate cyclase system during growth of astrocytoma cells in culture. *J. Biol. Chem.* **254**:4416–4622 (1979).
30. Hegstrand, L. R., K. P. Minneman, and P. B. Molinoff. Multiple effects of guanosine triphosphate on beta adrenergic receptors and adenylate cyclase activity in rat heart, lung and brain. *J. Pharmacol. Exp. Ther.* **210**:215–221 (1979).
31. Berrie, C. P., N. J. M. Birdsall, A. S. V. Burgen, and E. C. Hulme. Guanine nucleotides modulate muscarinic receptor binding in the heart. *Biochem. Biophys. Res. Commun.* **27**:1000–1005 (1979).
32. Wei, J.-W., and P. V. Sulakhe. Requirement for sulfhydryl groups in the differential effects of magnesium ion and GTP on agonist binding of muscarinic cholinergic receptor sites in rat atrial membrane fraction. *Naunyn-Schmiedebergs Arch. Pharmacol.* **314**:51–59 (1980).
33. DeLean, A., J. M. Stadel, and R. J. Lefkowitz. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J. Biol. Chem.* **255**:7108–7117 (1980).
34. Glossman, H., and P. Presek. Alpha noradrenergic receptors in brain membranes: sodium, magnesium, and guanyl nucleotides modulate agonist binding. *Naunyn-Schmiedebergs Arch. Pharmacol.* **306**:67–73 (1979).
35. Howlett, A. C., P. M. Van Arsdale, and A. G. Gilman. Efficiency of coupling between *beta*-adrenergic receptor and adenylate cyclase. *Mol. Pharmacol.* **14**:531–539 (1978).
36. Stadel, J. M. and R. J. Lefkowitz. Multiple reactive sulfhydryl groups modulate the function of adenylate cyclase-coupled *beta*-adrenergic receptors. *Mol. Pharmacol.* **16**:709–718 (1979).
37. Rodbell, M. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* (*Lond.*) **284**:17–22 (1980).
38. Ross, E. M., M. E. Maguire, T. W. Sturgill, R. L. Biltonen, and A. G. Gilman. Relationship between the β-adrenergic receptor and adenylate cyclase. *J. Biol. Chem.* **252**:5761–5775 (1977).
39. Harden, T. K., Y.-F. Su, and J. P. Perkins. Catecholamine-induced desensitization involves an uncoupling of beta-adrenergic receptors and adenylate cyclase. *J. Cyclic Nucleotide Res.* **5**:99–106 (1979).

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