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Regulation of Muscarinic Receptor Binding by Guanine Nucleotides and N-Ethylmaleimide

Frederick J. Ehlert, William R. Roeske, and Henry I. Yamamura

Departments of Pharmacology, Psychiatry, and Internal Medicine, University of Arizona Health Sciences Center, Tuscon, Arizona 85724

The regulation of muscarinic receptor binding by guanine nucleotides and N-ethylmaleimide (NEM) was investigated using the agonist ligand, [^{3}H] cis methyldioxolane ([^{3}H]CD). Characterization studies on rat forebrain homogenates showed that [^{3}H]CD binding was linear with tissue concentration and was unaffected by a change in pH from 5.5 to 8.0. The regional variation in [^{3}H]CD binding in the rat brain correlated generally with [^{3}H](−)3-quinuclidinyl benzilate ([^{3}H](−)QNB) binding, although the absolute variation in binding was somewhat less. At a concentration of 100 μM, the GTP analogue, guanyl-5′-yl imidodiphosphate [Gpp(NH)p], caused a 43–77% inhibition of [^{3}H]CD binding in the corpus striatum, ileum, and heart. The results of binding studies using several Gpp(NH)p concentrations demonstrated that the potency of this guanine nucleotide for inhibition of [^{3}H]CD binding was greater in the heart than in the ileum. In contrast to its effects on [^{3}H]CD binding, Gpp(NH)p caused an increase in [^{3}H](−)QNB binding in the heart heart and ileum and no change in [^{3}H](−)QNB binding in the corpus striatum. When measured by competitive inhibition of [^{3}H](−)QNB binding to the longitudinal muscle of the ileum, Gpp(NH)p (100 μM) caused an increase in the IC_{50} values of a series of agonists in a manner that was correlated with the efficacy of these compounds. The results of binding studies on NEM treated forebrain homogenates revealed an enhancement of [^{3}H]CD binding by NEM.

Key words: muscarinic receptor, [^{3}H] cis methyldioxolane, regulation, guanine nucleotides, N-ethylmaleimide

During the past decade, the development of specific [^{3}H] ligands for the muscarinic receptor has enabled a direct characterization of the nature of ligand binding to this receptor. The results of studies using [^{3}H] antagonists have shown that [^{3}H] antagonists bind to muscarinic receptors from neuronal tissue in a manner compatible with the law of mass action [1–6]. Similarly, studies of the competitive inhibition of [^{3}H] antagonist binding by antagonists have also yielded data consistent with the law of mass action [1, 7, 8]. In contrast to antagonists, the binding of agonists is incompatible with simple mass action behavior but can be rationalized in terms of a heterogeneous receptor popula-

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tion (superhigh, high, and low affinity receptors), which has uniform affinity for antagonists and differential affinity for agonists [9, 10].

Recently, we have characterized the binding of the muscarinic agonist, [^{3}H] cis methyldioxolane ([^{3}H]CD), and have obtained data demonstrating that [^{3}H]CD binds specifically to muscarinic receptors in the rat forebrain [11]. The results of pharmacological studies have shown that CD is a potent muscarinic agonist [12]. In this report additional characteristics of [^{3}H]CD binding are described, including the regulation of binding by guanine nucleotides and N-ethylmaleimide (NEM). We have also investigated the effects of guanine nucleotides on the competitive inhibition of [^{3}H](−)3-quinuclidinyl benzilate ([^{3}H](−)QNB) binding by a series of cholinergic analogues and have found that guanine nucleotides increase the IC_{50} of agonists in a manner that is correlated with the efficacy of the compound.

METHODS

Tissue Preparation

Binding assays were performed on tissues from 150–250 g male Sprague-Dawley rats. Ileal longitudinal muscle was obtained by mounting segments of the ileum on a 1 ml pipet and gently rubbing off the outer longitudinal muscle layer with a cotton swab. The whole heart was excised, and blood clots were removed by perfusion through the aorta with ice-cold 0.9% saline. Both the heart and the longitudinal muscle of the ileum were minced with scissors, homogenized with Potter Elvehjem homogenizer in ice-cold 50 mM sodium-potassium phosphate or Tris-HCl buffer, and rehomogenized on the polytron at setting 5 for approximately 15 sec. Except for experiments investigating the influence of pH on [^{3}H]CD binding, the pH of the buffers used was always 7.4 at the incubation temperature of the binding assay. The concentration of cardiac and ileal homogenates was either 40 or 100 mg original wet tissue weight per ml of buffer, depending upon whether [^{3}H] (−)QNB or [^{3}H]CD binding was measured, respectively.

[^{3}H]CD binding was also measured in P_2 membrane preparations of the rat forebrain (telencephalon and diencephalon). The forebrain was homogenized in 10 volumes of 0.32 M sucrose in a Potter Elvejhem homogenizer and centrifuged at 1,000g for 10 min. The pellet was discarded, and the supernatant was recentrifuged at 17,500g for 20 min. This supernatant was discarded, and the pellet (P_2) was resuspended with a polytron in 50 mM sodium-potassium phosphate buffer pH 7.4 to a concentration of 50 mg original wet tissue weight per milliliter of buffer. In other experiments, binding assays were performed on various rat brain regions. The cortex and corpus striatum were homogenized with a polytron at setting 5 for 15 sec in 50 mM sodium-potassium phosphate or Tris-HCl buffer. The final homogenate concentrations were 5 and 15 mg original wet tissue weight per ml of buffer for [^{3}H] (−)QNB and [^{3}H]CD binding assays, respectively. Homogenates of the hypothalamus, hippocampus, brainstem, and cerebellum were prepared in a similar fashion, except that the concentrations were greater by a factor of 2–4.

Binding Assays

The specific binding of [^{3}H] (−)QNB (44.0 Ci/mmole, Amersham) was determined according to the rapid filtration method of Yamamura and Snyder [6] with minor modifications. Briefly, 100 μl of tissue homogenate were incubated with [^{3}H] (−)QNB

for 2 h at 25°C in a final volume of 2 ml containing 50 mM sodium-potassium phophate or Tris-HCl buffer pH 7.4. In general, the concentration of [^{3}H] (−)QNB was 0.4 nM unless indicated otherwise in the text. Assays were carried out in triplicate, and specific binding was defined as the difference between measurements made in the presence and absence of 10 μM atropine. All binding data in the text refer to specific binding.

[^{3}H]CD was custom tritiated by Dr. Richard Young of New England Nuclear (Boston, MA) to a specific activity of 36.1 Ci/mmole. Specific [^{3}H] CD binding was measured as described previously [11]. Briefly, 200 μl of tissue homogenate were incubated with [^{3}H]CD in final volume of 2 ml containing 50 mM sodium-potassium phosphate or Tris-HCl buffer pH 7.4 at 0°C. Binding in the presence of 10 μM atropine was defined as nonspecific. After a 15-min preincubation at 37°C, the assay tubes were placed in an ice bath for 10 min. Subsequently, the tubes were centrifuged at 27,000g for 10 min at 0°C. The pellets were washed superficially with two 3 ml aliquots of ice-cold 0.9% saline. Thus, the specific [^{3}H]CD binding measurements in this text refer to equilibrium binding measurements made at 0°C. After the pellets were washed, the amount of bound or entrapped [^{3}H]CD in the pellets was determined as described previously [11]. Protein was measured by the method of Lowry et al [13], with bovine serum albumin as standard.

RESULTS

Characteristics of [^{3}H]CD Binding

Specific [^{3}H]CD binding was linear with tissue concentration up to 0.9 mg protein when the total [^{3}H]CD concentration varied between 1.0 and 15 nM. The results of tissue linearity studies on P_2 membrane fractions of the rat forebrain using [^{3}H]CD concentrations of 2.5 and 5.0 nM are shown in Figure 1. The linear relationship between binding and tissue concentration is readily apparent from that figure.

The influence of pH on the binding of [^{3}H]CD to rat forebrain homogenates is illustrated in Figure 2. For these experiments, [^{3}H]CD binding measurements were made in 50 mM citrate buffer, pH 4.0–6.0 (Figure 2A), and 50 mM sodium-potassium phosphate buffer, pH 6.0–8.0 (Figure 2B). Citrate buffer itself caused an inhibition of [^{3}H]CD binding, since binding in the presence of 50 mM citrate buffer, pH 6.0, was only one-third of that measured in the presence of 50 mM sodium-potassium phosphate buffer, pH 6.0. Nevertheless, the data in Figure 2 show that [^{3}H]CD binding is unaffected by a change in pH from 5.5 to 8.0 but decreases rapidly as the pH declines from 5.5 to 4.5.

In a previous study [11], it was noted that [^{3}H]CD binding was readily displaced by several muscarinic agonists and antagonists, whereas specific nicotinic and noncholinergic drugs were ineffective in inhibiting [^{3}H]CD binding. The results of competitive inhibition experiments on forebrain homogenates using QNB, CD, and physostigmine are illustrated in Figure 3. As shown in Figure 3, QNB and CD readily inhibited specific [^{3}H]CD binding with IC_{50} concentrations of 0.3 and 14 nM, respectively. Interestingly, the anticholinesterase agent, physostigmine, was moderately potent as an inhibitor of binding and had an IC_{50} concentration of approximately 8.0 μM.

Regional Variation of [^{3}H]CD Binding in Rat Brain

The regional variation of [^{3}H]CD binding in the rat brain was compared with that of [^{3}H]QNB, and the results of this analysis are shown in Table 1. The greatest variation in binding was observed with the antagonist, [^{3}H](−)QNB, which displayed a 14-fold

difference between binding values measured in the cerebral cortex and cerebellum. The rank order for [^{3}H]QNB binding was: cerebral cortex $\geqslant$ corpus striatum $>$ hippocampus $>$ brainstem $\geqslant$ hypothalamus $>$ cerebellum. A generally similar rank order for [^{3}H]CD binding was observed: cerebral cortex $>$ corpus striatum $\geqslant$ hippocampus $\geqslant$ hypothalamus $\geqslant$ brainstem $>$ cerebellum. The variation in [^{3}H]CD binding was somewhat less; there being only a 7-fold difference between binding values in the cerebral cortex and cerebellum. Also, [^{3}H]CD binding in the hippocampus, hypothalamus, and brainstem was approximately the same.

Influence of Gpp(NH)p on Muscarinic Receptor Binding

The effects of guanine nucleotides on [^{3}H]CD binding in the corpus striatum, ileum, and heart of the rat were investigated using the non-hydrolyzable GTP analogue, guanyl-5′-yl imidodiphosphate [Gpp(NH)p]. In these experiments, homogenates of the striatum, longitudinal muscle of the ileum, and heart were prepared in 50 mM Tris-HCl pH 7.4, and [^{3}H]CD binding was determined at a concentration of 5 nM in the presence and absence of 100 μM Gpp(NH)p. In the absence of Gpp(NH)p, [^{3}H]CD binding was 85 ± 5.0, 41 ± 3.3, and 23 ± 2.7 fmoles/mg protein in the corpus striatum, ileum, and heart, respectively. In the presence of Gpp (NH)p, the reduction in [^{3}H]CD binding was 43 ± 8%, 60 ± 4%, and 77 ± 4%, respectively.

The influence of various concentrations of Gpp(NH)p on [^{3}H]CD binding was investigated in the heart and longitudinal muscle of the ileum, and these results are shown in Figure 4A. It can be seen that Gpp(NH)p caused a dose-dependent inhibition

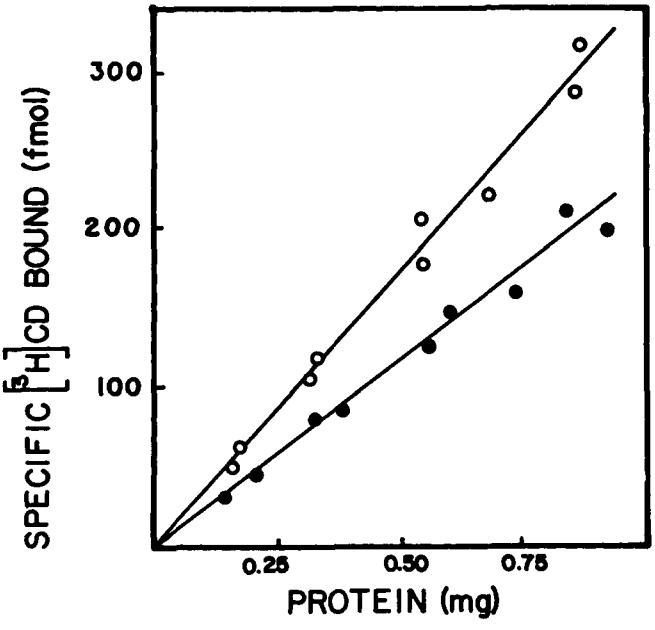

Fig. 1. Effect of tissue concentration on specific [^{3}H]CD binding to homogenates of the rat forebrain. The concentrations of [^{3}H]CD used in these experiments were 5 nM (○) and 2.5 nM (●). Triplicate binding assays were done using 50 mM sodium-potassium phosphate buffer pH 7.4 as the incubation medium.

of [^{3}H]CD binding in both the heart and the ileum and that the potency of Gpp(NH)p for influencing [^{3}H]CD binding is greater in the heart. Extrapolation of the inhibition curves to a high concentration of Gpp(NH)p suggests that [^{3}H]CD binding is not completely inhibited by Gpp(NH)p. If the IC_{50} concentration of Gpp(NH)p is defined as the concentration that produces half of the inhibition of binding caused by 100 μM, then the IC_{50} values of Gpp(NH)p are 0.68 μM and 6.2 μM in the heart and ileum, respectively. The calculated IC_{50} value of Gpp(NH)p in the ileum is probably less than the true IC_{50} value, since the data in Figure 4A suggest that maximum inhibition of [^{3}H]CD binding was not attained at 100 μM Gpp(NH)p.

In contrast to its effects on [^{3}H]agonist binding, Gpp(NH)p produced an increase in [^{3}H] antagonist binding in the heart and ileum (Figure 4B). In these experiments, [^{3}H] (−)QNB binding was measured at a subsaturating concentration of 0.1 nM in the presence and absence of various concentrations of Gpp(NH)p. As shown in Figure 4B, Gpp(NH)p produced a dose-dependent increase in [^{3}H] (−) QNB binding, with the ED_{50} for this effect being 0.21 and 1.0 μM in the heart and ileum, respectively. This increase in [^{3}H](−)QNB binding was due to an increase in the affinity of [^{3}H](−)QNB for muscarinic receptors, since the results of binding measurements made at several concentrations of [^{3}H](−)QNB showed that 10 μM Gpp(NH)p caused a significant reduction in the

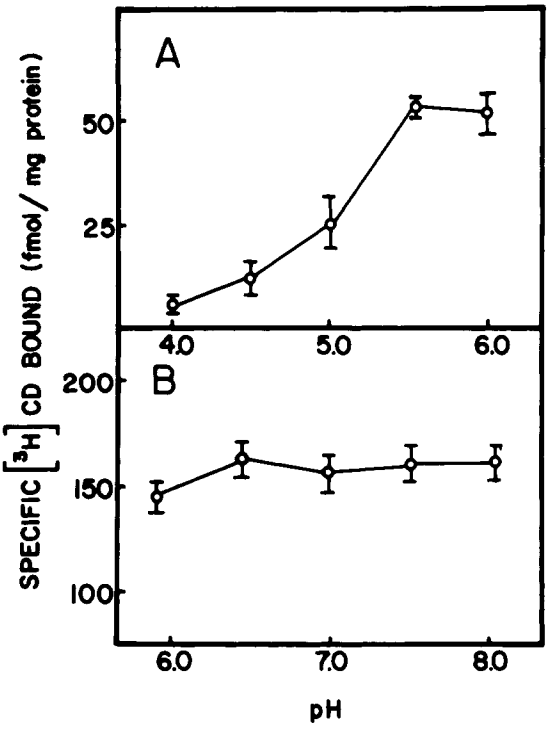

Fig. 2. Effect of pH on specific [^{3}H]CD binding to homogenates of the rat forebrain. Incubations were carried out in 50 mM citrate, titrated to the indicated pH values with Tris base, (A) and 50 mM sodium-potassium phosphate buffer (B). The concentration of [^{3}H]CD used in these experiments was 5 nM. Mean binding values ± SEM of three separate experiments, each done in triplicate, are shown.

dissociation constant of [^{3}H](−)QNB from 0.19 and 0.40 nM to 0.10 and 0.14 nM in the heart and ileum, respectively. In contrast to its effects on the peripheral tissues, Gpp(NH)p had no significant effect on striatal [^{3}H] (−)QNB binding (Figure 4B). A comparison of the data shown in Figure 4A and B shows that, in the ileum and heart, Gpp(NH)p has a greater potency for influencing [^{3}H]antagonist binding than [^{3}H] agonist binding.

Since Gpp(NH)p produced a preferential reduction in agonist binding while producing an increase or no change in antagonist binding, we investigated the influence of Gpp(NH)p on the binding of a series of cholinergic analogues to determine if there was a correlation between the guanine nucleotide effect and efficacy. For these experiments, a series of cholinergic analogues were tested for their ability to inhibit ileal [^{3}H](−)QNB binding in the presence and absence of 100 μM Gpp (NH)p. Figure 5 shows that Gpp (NH)p caused a 10-fold increase in the IC_{50} of carbachol when binding measurements were made after a 2 h incubation at 25°C. The guanine nucleotide effect was less at 37°C and was attenuated when ileal homogenates were washed by centrifugation. A similar attenuation of the guanine nucleotide-induced reduction in agonist affinity was observed when experiments were performed on washed rat heart and guinea pig ileum homogenates (data not shown). Careful inspection of the data in Figure 5 shows that the control carbachol competition curve is much flatter than a simple mass action curve. This observation is compatible with the hypothesis of a heterogeneous population of agonist binding sites (superhigh, high, and low affinity) that have equal affinity for antagonists [8–10].

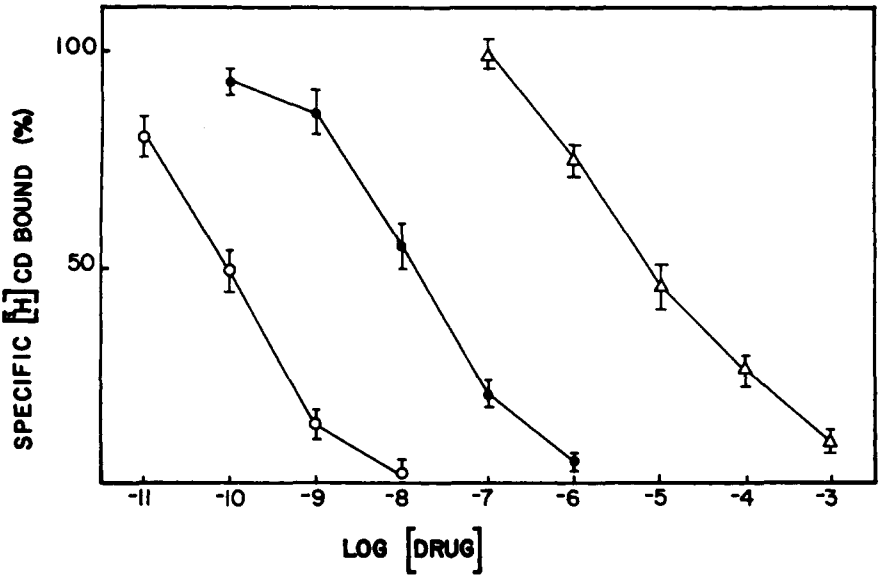

Fig. 3. Competitive inhibition of specific [^{3}H]CD binding by QNB (○), CD (●), and physostigmine (△). Binding assays were done on rat forebrain homogenates using 50 mM sodium-potassium phosphate buffer pH 7.4 as the incubation medium. Mean binding values ± SEM of at least three separate experiments, with each point measured in triplicate, are shown. The concentration of [^{3}H]CD was 5 nM.

In the presence of Gpp(NH)p, the carbachol competition curve shifts to the right and becomes steeper, compatible with the notion that guanine nucleotides produce a preferential reduction in agonist binding to superhigh and high affinity agonist binding sites. The data in Figure 5 also show that Gpp(NH)p produces only minimal effects on atropine and choline inhibition of [^{3}H](−)QNB binding. A summary of the data illustrated in Figure 5 and similar data with other cholinergic analogues is given in Table II. The greatest guanine nucleotide-induced reduction in affinity was seen with the highly efficacious agonists, carbachol and oxotremorine, whose IC_{50} values increased by a factor of 10 in the presence of Gpp(NH)p. A somewhat smaller effect was seen with the partial agonists, pilocarpine and pentyltrimethylammonium; their IC_{50} values were increased by factors of

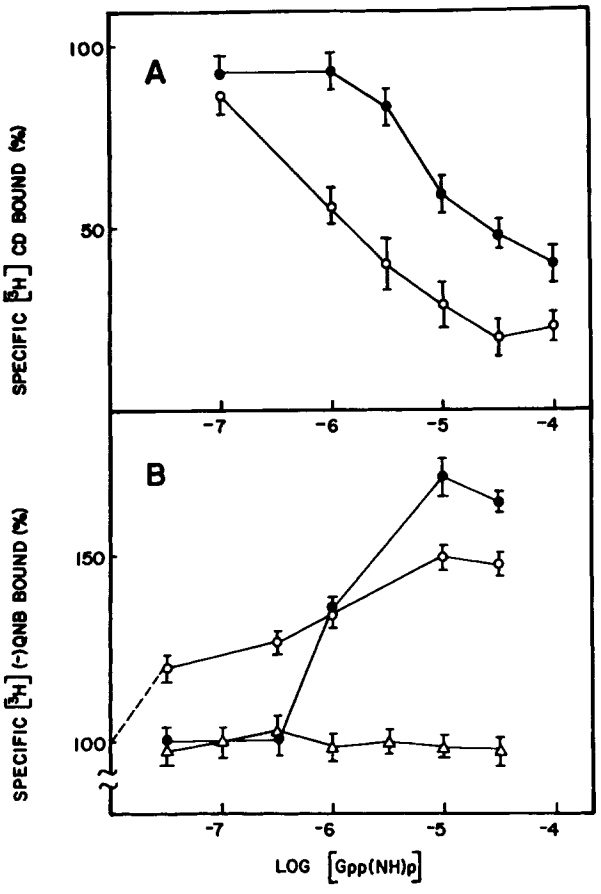

Fig. 4. Effect of Gpp(NH)p on the specific binding of [^{3}H] CD and [^{3}H] (−)QNB. A: Specific [^{3}H]CD binding was measured in homogenates of the heart (○) and ileum (●) in the presence of various concentrations of Gpp(NH)p. Binding assays were run in 50 mM Tris-HCl buffer pH 7.4. Mean binding values ± SEM of five separate experiments, each done in triplicate, are shown. The concentration of [^{3}H]CD was 5 nM. B: Specific [^{3}H] (−)QNB binding was measured in homogenates of the heart (○), ileum (●), and striatum (△) in the presence of various concentrations of Gpp(NH)p. Incubations were carried out for 2 h at 25°C in 50 mM Tris-HCl buffer pH 7.4. Mean binding values ± SEM of three separate experiments, each done in triplicate, are shown. The [^{3}H](−)QNB concentration was 0.1 nM.

4.9 and 5.5, respectively. In contrast, Gpp(NH)p produced only small effects on the binding of choline and atropine. Interesting results were obtained with the oxotremorine analogue, N-(5-pyrrolidino-3-pentynyl)-succinimide (BL14), which differs from oxotremorine in that it is a succinimide containing an additional methylene group in the alkyne chain. Pharmacological experiments have demonstrated that BL14 has antagonist-like properties [14]. The results of our binding experiments are consistent with the pharmacological data, since Gpp(NH)p caused a small reduction in the affinity of BL14. As described above, this behavior was typical of other compounds having little efficacy.

Influence of N-Ethylmaleimide on [^{3}H] CD Binding in Rat Brain

In studies of the effects of sulfhydryl reagents on muscarinic receptor binding, it was noted that treatment of brain homogenates with NEM caused an increase in agonist affinity for the muscarinic receptor while producing minimal effects on antagonist binding [15, 16]. Thus, it was of interest to determine the effects of NEM on [^{3}H] CD binding. Figure 6 shows the effects of NEM treatment on the binding of [^{3}H] CD to rat forebrain homogenates. For these experiments, rat forebrain homogenates were incubated with 1 mM NEM for 20 min at 37°C and washed twice by centrifugation. Control forebrain homogenates were prepared in a similar fashion except for exposure to NEM. The results in Figure 6 show that NEM treatment causes an increase in [^{3}H] CD binding when measured at concentrations between 0.5 and 64 nM. These increases in binding were significant ($P < .02$) at [^{3}H] CD concentrations between 0.5 and 8 nM. Weighted, nonlinear, least-squares regression analysis of the control [^{3}H] CD binding values showed that the data were adequately described by a two-site mass action equation with dissociation constants of 0.59 nM (95% confidence limits: 0.061–1.12 nM) and 37 nM (95% confidence

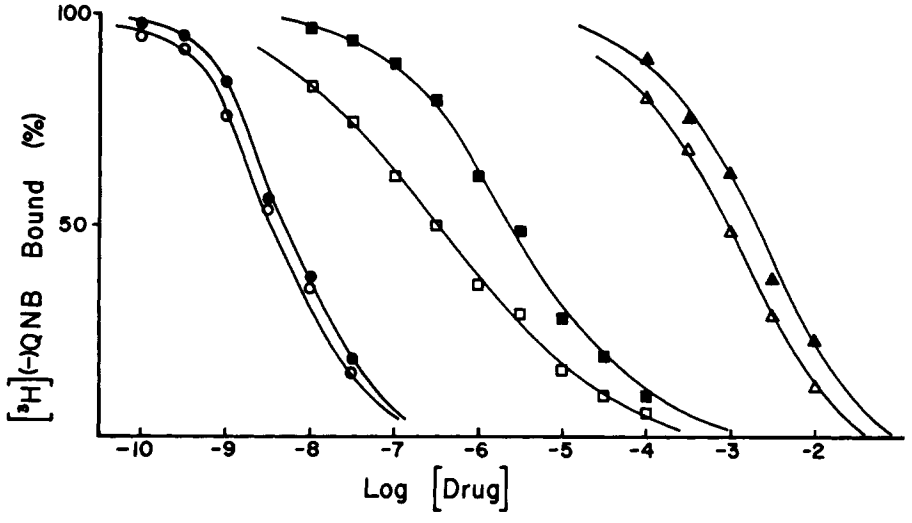

Fig. 5. Competitive inhibition of [^{3}H](−)QNB binding in the ileum by atropine (○), carbachol (□), and choline (△) in the presence (closed symbols) and absence (open symbols) of 100 μM Gpp(NH)p. Incubations were carried out for 2 h at 25°C in 50 mM Tris-HCl buffer pH 7.4. Mean binding values of at least four separate experiments, each done in triplicate, are shown. The [^{3}H](−) QNB concentration was 0.4 nM.

limits: 16–58 nM) and binding capacities of 55 ± 10 and 654 ± 60 fmoles/mg protein, respectively. These two binding sites correspond to the superhigh and high affinity muscarinic receptor populations previously proposed for agonists [11]. Regression analysis of the data from NEM-treated homogenates gave dissociation constants of 0.41 nM (95% confidence limits: 0.02–1.03 nM) and 28 nM (95% confidence limits: 18–50 nM) and binding capacities of 110 ± 11 and 644 ± 35 fmoles/mg protein for the superhigh and high affinity receptors, respectively. Thus, these data demonstrate that alkylation of sulfhydryl groups causes an increase in the density of the putative superhigh affinity binding sites.

DISCUSSION

In a study investigating the influence of pH on the muscarinic activity of some tertiary amines and their quaternary ammonium analogues, it was demonstrated that the hydronium ions of the amines were responsible for their muscarinic activity [17]. This

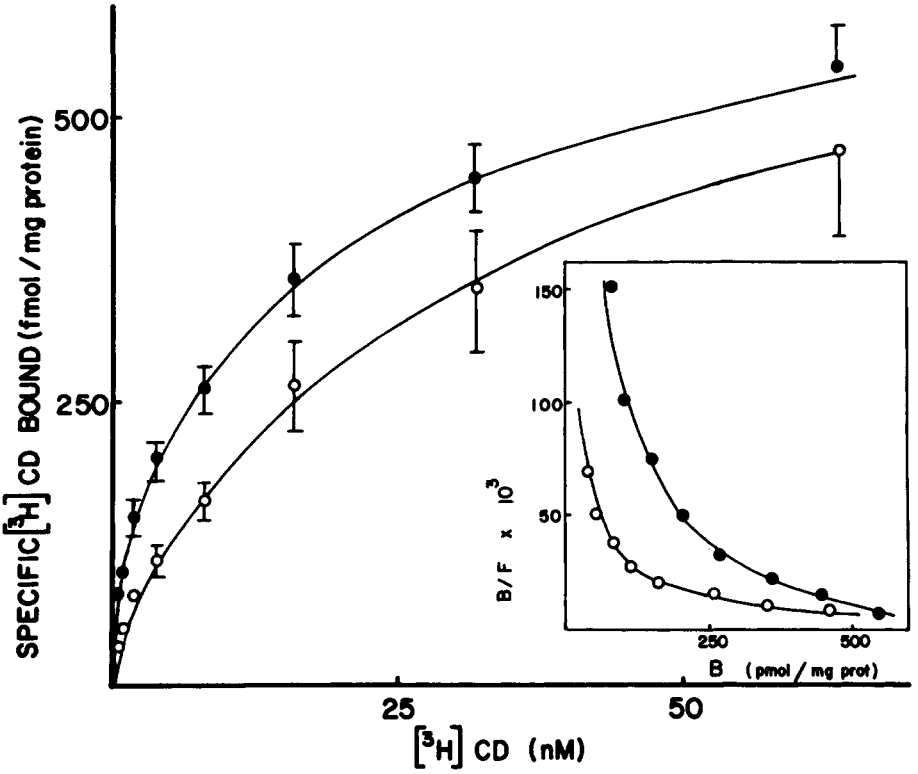

Fig. 6. Effect of NEM treatment on the specific binding of [^{3}H]CD. Specific [^{3}H]CD binding measurements were made in control (○) and NEM-treated (●) forebrain homogenates. NEM treatment was performed as described in the text. Binding assays were carried out in 50 mM sodium-potassium phosphate buffer pH 7.4. Mean binding values ± SEM of three separate experiments, each done in triplicate, are shown. The theoretical curves represent the weighted least-squares fit to the data, the points being weighted according to the reciprocal of the variance. The inset shows a Scatchard plot of the mean specific binding values.

TABLE I. Regional Distribution of [^{3}H]CD and [^{3}H](−)QNB Binding in Rat Brain

Brain region[a]	[^{3}H]CD bound[b] (fmoles/mg protein)	[^{3}H](−)QNB bound[c] (fmoles/mg protein)	[^{3}H]CD bound[d] / [^{3}H](−)QNB bound
Cerebral cortex	127 ± 9	1,728 ± 48	0.08
Corpus striatum	80 ± 4	1,567 ± 107	0.05
Hippocampus	53 ± 5	1,300 ± 41	0.04
Brainstem	50 ± 5	543 ± 26	0.09
Hypothalamus	52 ± 10	475 ± 28	0.11
Cerebellum	18 ± 4	120 ± 18	0.15

[a]Brain regions were homogenized in 50 mM sodium-potassium phosphate buffer pH7.4.

[b]The concentration of [^{3}H]CD used for binding measurements was 5.0 nM. Mean binding values ± SEM of 3 experiments each done in triplicate are shown.

[c]The concentration of [^{3}H](−)QNB used for binding measurements was 0.4 nM. Mean binding values ± SEM of 4 experiments each done in triplicate are shown.

[d]Represents the ratio of mean binding values shown in the first two columns.

cationic requirement is a well-known property of cholinergic ligands and suggests the existence of an anionic site on the muscarinic receptor. If the anionic site is formed by dissociation of an acidic hydrogen, then the binding of a quaternary ammonium compound like [^{3}H]CD should decline as the pH decreases in the region of the pK_A of the acidic moiety. In the present study, [^{3}H]CD binding was unaffected by a change in pH from 8.0 to 5.5 but decreased as the pH declined from 5.5 to 4.5, suggesting that the pK_A of the anionic site is close to 5.0. Thus, a likely candidate for the anionic site of the muscarinic receptor is a carboxylate group (pK_A = 4–5).

The results of equilibrium binding measurements using [^{3}H]CD concentrations between 0.5 and 64 nM were compatible with the hypothesis of superhigh and high affinity muscarinic receptors having dissociation constants of 0.59 and 37 nM and binding capacities of 55 and 654 fmoles/mg protein. Competitive inhibition of [^{3}H] CD binding by CD yielded an IC_{50} value of 14 nM, which can be rationalized in terms of the binding parameters and concentration of [^{3}H]CD (5 nM) used in the experiment. The results of physostigmine/[^{3}H]CD competitive inhibition experiments showed that this anticholinesterase agent was a moderately potent inhibitor of [^{3}H]CD binding (IC_{50} = 8.0 μM). These data suggest caution in the use of physostigmine as an inhibitor of cholinesterase in binding assays.

The regional distribution of [^{3}H]CD binding in the rat brain correlates generally with the distribution of [^{3}H](−)QNB binding. The most notable difference is the greater absolute variation in the magnitude of [^{3}H] QNB binding compared to [^{3}H]CD binding. This difference can be rationalized in terms of muscarinic receptor heterogeneity. At the ligand concentrations used in the present study of the regional variation in central muscarinic receptor binding, only superhigh and high affinity sites were labeled with [^{3}H]CD, whereas all the agonist binding sites were labeled with [^{3}H](−)QNB. Thus, the data described above suggest an inverse relationship between the total density of muscarinic receptors and the proportion of sites that are of superhigh and high affinity. A similar relationship has been reported by Birdsall et al [18].

Recent investigations concerning the influence of guanine nucleotides on muscarinic receptor binding in the rat forebrain, heart, and ileum have demonstrated that guanine nucleotides cause a selective reduction in agonist binding while producing only mini-

TABLE II. Effect of Gpp(NH)p on the Competitive Inhibition of [^{3}H(−)QNB Binding by Cholinergic Drugs in the Rat Ileum*

	IC_{50} Control (μM)	IC_{50} 100 μM Gpp(NH)p (μM)	IC_{50} Gpp(NH)p / IC_{50} control
Carbamylcholine	0.32	3.2	10 (8.0 – 12.6)[a]
Oxotremorine	0.045	0.43	9.6 (8.0 – 11.5)
Pilocarpine	3.5	17	4.9 (3.5 – 6.9)
Pentyltrimethylammonium	32	177	5.5 (3.5 – 8.6)
Choline	1,000	1,770	1.8 (1.4 – 2.6)
N-(5-pyrrolidino-3-pentynyl)-succinimide	2.2	3.2	1.5 (1.1 – 2.15)
Atropine	0.0010	0.0012	1.2 (1.06 – 1.3)

*Competitive inhibition experiments were performed on the longitudinal muscle of the rat ileum using a [^{3}H](−)QNB concentration of 0.4 nM. Incubations were carried out in 50 mM Tris-HCl buffer pH 7.4 for 2 h at 25°C. For each experiment, IC_{50} values were determined from 6 to 10 point ligand/[^{3}H](−)QNB competition curves, with each ligand concentration being geometrically spaced every 0.5 log molar units. Five separate experiments were done, with each point done in triplicate. The IC_{50} value represents the geometric mean of the individual experiments.

[a]95% confidence intervals.

mal effects on antagonist binding [19–22]. These findings are consistent with previous reports concerning the effects of guanine nucleotides on several neurotransmitter receptors [23–33]. The results of muscarinic agonist/[^{3}H]antagonist competition experiments have demonstrated that, in the presence of guanine nucleotides, there is both a shift to the right and a steepening of the agonist competition curve [22, 34]. Regression analysis of the data has shown that this effect is due to a reduction in the affinity [22] or, in some instances, the proportion, of high affinity agonist binding sites [34]. The results of our studies investigating the influence of guanine nucleotides on [^{3}H]CD binding are consistent with the effects of guanine nucleotides on agonist/[^{3}H]antagonist competition curves. Measurements of [^{3}H]CD binding in the presence of 100 μM Gpp(NH)p showed that this guanine nucleotide caused 77%, 60%, and 43% inhibition of [^{3}H]CD binding in the heart, ileum, and corpus striatum, respectively. In these experiments, a [^{3}H] CD concentration (5 nM) that labeled both superhigh and high affinity muscarinic receptors was used. Thus, the Gpp(NH)p-induced reduction in [^{3}H]CD binding is compatible with the concept that guanine nucleotides reduce the affinity of superhigh and high affinity muscarinic receptors or convert these higher affinity sites into low affinity sites.

The ability of guanine nucleotides to alter muscarinic receptor binding varies in different tissues. Investigations into the effects of several concentrations of Gpp(NH)p on [^{3}H]CD binding showed that the potency of Gpp(NH)p for inhibition [^{3}H]CD binding was greater in the heart than in the ileum. In a previous study, we noted that 30 μM Gpp(NH)p produced a greater reduction in agonist binding to muscarinic receptors in the ileum than in the forebrain, whereas no significant effects of guanine nucleotides on agonist binding were detected in the cerebellum and brainstem [22].

With regard to cardiac muscarinic receptors, the influence of guanine nucleotides on agonist binding appears to be related to adenyl cyclase activity. Muscarinic agonists have been shown to inhibit GTP-stimulated adenyl cyclase activity in the heart [32, 34–36], and the ED_{50} of carbachol for inhibiting the enzyme agrees quantitatively with

the affinity constant of the low affinity agonist binding site [34]. Thus, it appears that, in the heart, some of the physiolocial effects of muscarinic agonists are mediated through the low affinity agonist binding site. The reduction in agonist binding caused by guanine nucleotides may be a manifestation of an alteration in the coupling of cardiac muscarinic receptors to the cyclase. Whether muscarinic receptors are coupled to an adenyl cyclase system in the ileum and brain is unknown. It seems plausible that guanine nucleotides may alter the coupling of muscarinic receptors to another effector system in these tissues.

In contrast to its effects on [^{3}H] agonist binding, Gpp(NH)p caused an increase in [^{3}H](−)QNB binding in the ileum and heart. As observed in [^{3}H]CD binding experiments, the potency of Gpp(NH)p for influencing [^{3}H](−)QNB binding was greater in the heart than in the ileum. However, careful inspection of the data revealed that the potency of Gpp(NH)p for influencing [^{3}H](−)QNB binding in the heart and ileum was greater than that observed in [^{3}H]CD binding experiments. These data suggest the existence of separate guanine nucleotide binding sites that selectively alter either agonist or antagonist binding. A further extension of this hypothesis would suggest that striatal muscarinic receptors possess only GTP binding sites, which alter antagonist binding, since Gpp (NH)p produced a reduction in striatal [^{3}H]CD binding without a measurable change in [^{3}H](−)QNB binding. These results demonstrate that muscarinic receptors may be differentiated on the basis of guanine nucleotide influences on [^{3}H] antagonist binding. Recent experiments by Hammer et al [37] have shown that the antagonist pirenzepine has a 20-fold higher affinity for muscarinic receptors in some brain regions than for cardiac and ileal muscarinic receptors. This phenomenon is unique insofar as classical muscarinic antagonists have nearly equal affinity for muscarinic receptors in different tissues [10]. The results described above suggest that there may be an inverse correlation between the affinity of pirenzepine for muscarinic receptors and the ability of guanine nucleotides to alter the binding of classical [^{3}H]antagonist ligands.

In the present study, it was noted that washing ileal and cardiac homogenates caused an attenuation of the guanine nucleotide-induced reduction in agonist affinity. We also noticed that the effects of Gpp(NH)p on [^{3}H]CD binding were variable. In this study, Gpp(NH)p (100μM) caused 60% inhibition of ileal [^{3}H]CD binding, whereas in a previous study [38], 85% inhibition of binding was observed. These results suggest that GTP binding sites are labile and may be perturbed or solubilized during homogenization procedures.

The ability of guanine nucleotides to alter the affinity of cholinergic ligands appears to be correlated with the efficacy of the drug. When measured by competitive inhibition of [^{3}H](−)QNB binding to homogenates of the rat ileum, the IC_{50} values of the efficacious agonists carbachol and oxotremorine increased 10-fold in the presence of 100 μM Gpp(NH)p. Somewhat smaller reductions in affinity were observed for other compounds having little efficacy. It is of interest to note that the choline/[^{3}H](−)QNB competition curve was only slightly affected by Gpp(NH)p, suggesting that the efficacy of choline is very low. This conclusion is consistent with the relative pharmacological inactivity of choline relative to ACh [39, 40]. It has been suggested that the therapeutic efficacy of choline treatment in neurological disorders is due to a direct postsynaptic effect of choline on cholinergic receptors [40]. Indeed, iontophoretic application of choline excites cortical neurons with a potency only 1/8 that of ACh [41]. Our data suggest that choline must be present in high concentrations in the synapse if it is to have a postsynaptic agonistic effect, since it has low efficacy and affinity.

The small Gpp(NH)p-induced increase in the IC_{50} of the atropine/[^{3}H](−)QNB competition curve might seem anomalous in view of the fact that Gpp(NH)p caused an increase in the binding of the antagonist [^{3}H](−)QNB. However, the concentration of [^{3}H](−)QNB used in these competition experiments was nearly saturating (0.4 nM). Thus, the IC_{50} of atropine was perturbed from its intrinsic value by a factor of $(1 + [[^3H]\ (-)QNB]/K_{QNB})$. This factor increases in the presence of Gpp(NH)p, because there is a reduction in K_{QNB}, and thereby attenuates any increase in the potency of atropine.

The results of carbachol/[^{3}H](−)QNB competitive inhibition experiments on NEM-treated homogenates have provided evidence demonstrating that alkylation of sulfhydryl groups enhances the affinity of agonists by conversion of low affinity sites into high affinity sites [15, 16]. In the present study measurements of [^{3}H]CD binding in NEM-treated forebrain homogenates revealed that NEM treatment also leads to an increase in the proportion of putative superhigh affinity sites. This effect is opposite that of guanine nucleotides and suggests a possible relationship between the mechanism of action of the influence of NEM and guanine nucleotides on muscarinic receptor binding.

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