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Muscarinic Receptors in Porcine Caudate Nucleus

I. Enhancement by Nickel and Other Cations of [^{3}H]*cis*-Methyldioxolane Binding to Guanyl Nucleotide-Sensitive Sites

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SUMMARY

Muscarinic acetylcholine receptors in the synaptic membrane fraction of porcine caudate nucleus were characterized by using a radiolabeled agonist, [^{3}H]*cis*-methyldioxolane ([^{3}H]CD) and an antagonist, [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB). Scatchard analysis of the specific binding of [^{3}H]CD gave a single equilibrium dissociation constant of 8.1 nM when a concentration of less than 80 nM [^{3}H]CD was used. The binding capacity was 390 fmoles/mg of protein and corresponded to about 10% of the binding sites of [^{3}H]QNB. Agonist/[^{3}H]CD competition binding experiments indicated that [^{3}H]CD was selectively bound to the sites with a high affinity for agonists. [^{3}H]CD binding was inhibited by Na^+, K^+, Mg^{2+}, and Ca^{2+} with the half-maximal effect at 10–50 mM. Nickel ion showed biphasic effects on [^{3}H]CD binding: a 2- to 3-fold enhancement of binding at 0.1–10 mM and inhibition above 10 mM. Other cations, including Co^{2+}, Mn^{2+}, and Zn^{2+}, at 1 mM also increased [^{3}H]CD binding by a factor of 1.5–1.8. Among 18 cations examined, only Cd^{2+}, Hg^{2+}, and Cu^{2+} caused significant inhibition of [^{3}H]CD binding at 1 mM. [^{3}H]CD binding was decreased to about 20% of the control value in the presence of guanylyl-5′-imidodiphosphate (GppNHp), GTP, and GDP with the half-maximal effect at 1.3, 32, and 45 μM, respectively. [^{3}H]CD binding in the presence of Ni^{2+} was decreased by GppNHp to a level obtained in the presence of GppNHp alone. The increase caused by Ni^{2+} in [^{3}H]CD binding was due to the increase in the maximal binding capacity (B_{max}) without changes in the affinity for [^{3}H]CD. We conclude that Ni^{2+} increases the proportion of a muscarinic receptor subclass (or state) that is sensitive to guanyl nucleotide.

INTRODUCTION

The development of binding techniques utilizing high-affinity radiolabeled antagonists has allowed direct study of muscarinic acetylcholine receptors (1, 2) and provided important information on the number, distribution, pharmacological specificity, and regulation of muscarinic receptors. Concerning the interaction of muscarinic receptors with agonists, however, only indirect information has been obtained from these studies. In several respects, the binding of agonists appeared to be fundamentally different from that of antagonists. The muscarinic receptors have been shown to be composed of different subpopulations with the same affinity for antagonists but with different affinities for agonists (3). In addition, guanyl nucleotide has been shown to cause a decrease in the affinities for agonists but no change or a little increase in the affinities for antagonists (4–6).

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The effect of guanyl nucleotide on agonist binding was originally reported for glucagon and *beta*-adrenergic receptors and was revealed to reflect the interaction of the receptors with the GTP binding protein (7). Subsequently, a similar effect was also found on *alpha*$_2$-adrenergic, dopaminergic (D_2), and opiate receptors which were thought to be connected with the inhibition of adenylate cyclase (8). The effects of guanyl nucleotide on muscarinic receptors have been examined mainly on cardiac receptors. The effect on cerebral muscarinic receptors was much smaller than that on cardiac receptors. It was therefore difficult to analyze the effect quantitatively by the agonist/[^{3}H]antagonist competition study. An evaluation of the effect of guanyl nucleotide on muscarinic receptors other than cardiac receptors has been required to show whether the effect is general on muscarinic receptors or is restricted to cardiac receptors.

Magnesium ion has been reported to increase the high-affinity binding of agonists with *beta*-adrenergic receptors (9, 10). It appears that Mg^{2+} also plays a critical role in activation of the GTP binding protein (11). Reports

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on the effect of Mg^{2+} on cardiac muscarinic receptors are not consistent among authors (12, 13). In addition, several cations, including Ni^{2+}, Mn^{2+}, and Co^{2+}, have been shown to increase the affinity for agonists of muscarinic receptors in some regions of rat brain (14).

Ehlert *et al.* (15, 16) have developed a radiolabeled agonist, [^{3}H]CD,[1] and have demonstrated that [^{3}H]CD is specifically bound to the muscarinic receptors and is very useful for the characterization of different subclasses or states of the receptor. In the present study, the effect of guanyl nucleotide on muscarinic receptors in porcine caudate nucleus was examined quantitatively with the use of [^{3}H]CD. [^{3}H]CD was shown to bind selectively to 10% of total muscarinic receptors, and this small portion of the receptor was sensitive to guanyl nucleotide. Furthermore, the effects of various kinds of cations on [^{3}H]CD binding was examined and it was shown that nickel ion increased [^{3}H]CD binding to the guanyl nucleotide-sensitive sites by 2- to 3-fold.

EXPERIMENTAL PROCEDURES

Materials. GppNHp was obtained from Sigma Chemical Company (St. Louis, Mo.), and GTP was from P-L Biochemicals (Milwaukee, Wisc.). *cis*-Methyldioxolane (unlabeled CD) was a gift of Dr. D. J. Triggle (State University of New York at Buffalo). [^{3}H]CD (38.1 Ci/mmole) and [^{3}H]QNB (33.1 Ci/mmole) were supplied by New England Nuclear Corporation (Boston, Mass.). Other chemicals were obtained from commercial sources and were of analytical or reagent grade.

Membrane preparation. Porcine brains were obtained at a slaughterhouse within 4 hr after sacrifice. Caudate nuclei (1.2 g/brain) were dissected and homogenized in 10 volumes of 0.32 M sucrose containing 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.5) and 50 μM $CaCl_2$ with the aid of a Potter-Elvehjem homogenizer. The synaptic membrane fraction was prepared according to the method of Jones and Matus (17) as follows. The homogenate was centrifuged at 800 × *g* for 20 min and the supernatant fluid was further centrifuged at 9,000 × *g* for 20 min. The pellet was washed once with the 0.32 M sucrose solution and then suspended in a hypotonic buffer (3.6 ml/g of original tissue) [5 mM Tris-HCl buffer containing 50 μM $CaCl_2$ (pH 8.1)], followed by incubation at 0° for 30 min. The suspension was made 1.14 M with respect to sucrose by the addition of an appropriate volume of 1.98 M sucrose. Ten milliliters of 0.93 M sucrose and then 2 ml of 0.32 M sucrose were carefully layered on 21.2 ml of the sample solution in a centrifuge tube, and six equivalent tubes were centrifuged at 60,000 × *g* for 110 min in a Beckman SW 27 rotor. The fraction from the interface between 0.93 M and 1.14 M sucrose was collected and centrifuged at 100,000 × *g* for 60 min after dilution with 2 volumes of water. The final pellet was suspended in 0.32 M sucrose to give a protein concentration of 7 mg/ml (4.6 mg protein/g of original tissue) and stored at −80°.

The concentration of muscarinic receptors per milligram of protein as assayed by [^{3}H]QNB binding was highest in the synaptic membrane fraction (3.0 pmoles/mg of protein), and the binding sites of [^{3}H]CD were also highest in this fraction (390 fmoles/mg of protein). The freezing of the synaptic membrane fraction did not affect the binding of [^{3}H]CD and [^{3}H]QNB. Determination of protein was performed by the method of Lowry *et al.* (18), using bovine serum albumin as standard.

Standard binding assay. The synaptic membranes (1 mg of protein per milliliter) were incubated with 5 nM [^{3}H]CD and 20 mM Tris-maleate buffer (pH 7.5) at 20° for 30 min in a total volume of 0.3 ml. The incubation was started by adding the membranes and was terminated by the addition of 3 ml of ice-cold 20 mM phosphate buffer (pH 7.0). The sample was filtered under suction through a Whatman GF/C glass filter disc within 5 sec. The filter was washed three times with ice-cold buffer (3 ml each). After being dried at 80° for 2 hr, the filter was placed in 4.5 ml of scintillation fluid consisting of Triton X-100 (3 liters), toluene (7 liters), 2,5-diphenyloxazole (PPO) (40 g), and dimethyl 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) (1 g) and counted for 10 min in an Aloka scintillation spectrometer with a counting efficiency of 38%. Nonspecific binding was defined as the binding of [^{3}H]CD in the presence of 10 μM atropine, and was subtracted from the total binding to yield the specific binding. Total [^{3}H]CD binding was about 2000 cpm under the above conditions, and nonspecific binding was about 25% of the total binding. The binding assay was carried out in duplicate, and each experiment was repeated more than twice. The binding assay of [^{3}H]QNB was carried out in the same way as that for [^{3}H]CD, except that the concentration of membranes was reduced to one-fifth of that for the [^{3}H]CD binding study and the incubation was carried out with 0.5 nM [^{3}H]QNB at 30° for 30 min in a total volume of 1.0 ml. Total [^{3}H]QNB binding was about 3000 cpm under these conditions, and nonspecific binding was below 10% of the total binding.

Statistics. Theoretical analysis of the displacement curve was carried out with a nonlinear least-squares regression analysis. The computer program was kindly supplied by Dr. Susan Yamamura (University of Arizona College of Medicine).

RESULTS

Characteristics of [^{3}H]CD binding to synaptic membranes of porcine caudate nucleus. The binding of various concentrations (0–80 nM) of [^{3}H]CD to synaptic membranes prepared from porcine caudate nuclei was determined (Fig. 1). The specific binding was a saturable process, and the Scatchard plot was linear (Fig. 1, *inset*). The receptor concentration (B_{max}) and the equilibrium dissociation constant (K_d) for [^{3}H]CD, obtained from the Scatchard analysis, were 390 fmoles/mg of protein and 8.1 nM, respectively. The [^{3}H]CD binding sites were about 10% of the [^{3}H]QNB binding sites (3.0 pmoles/mg of protein), which were assumed to represent the total receptor sites. The K_d for [^{3}H]QNB was 12 pM.

Hill coefficients and IC_{50} values for the muscarinic ligands were determined from the competition binding study between labeled ligands ([^{3}H]CD and [^{3}H]QNB) and various concentrations of unlabeled ligands (Fig. 2; Table 1). The Hill coefficient obtained from the antagonist/[^{3}H]QNB competition binding study was close to 1.0, but that for agonist/[^{3}H]QNB competition ranged between 0.4 and 0.6, as has been reported by others (2, 3). On the other hand, the Hill coefficient estimated from the agonist/[^{3}H]CD and antagonist/[^{3}H]CD competition experiments was close to 1.0 and ranged between 0.8 and 1.0.

The IC_{50} values obtained from the antagonist/[^{3}H]QNB and antagonist/[^{3}H]CD competition studies were of the same order. When the IC_{50} was estimated from the agonist/[^{3}H]CD competition, however, values were obtained 160–2000 times lower than those for agonist/[^{3}H]QNB competition.

Using the IC_{50} value obtained from the agonist/[^{3}H]CD competition binding study, the K_d for agonists was calculated according to the method of Cheng and Prusoff (19). The values obtained were similar to those for the site with the super high affinity for agonists in rat

[1] The abbreviations used are: [^{3}H]CD, [^{3}H]*cis*-methyldioxolane; [^{3}H]QNB, [^{3}H]3-quinuclidinyl benzilate; GppNHp, guanylyl-5′-imidodiphosphate; AMPpNHp, adenylyl-5′-imidodiphosphate.

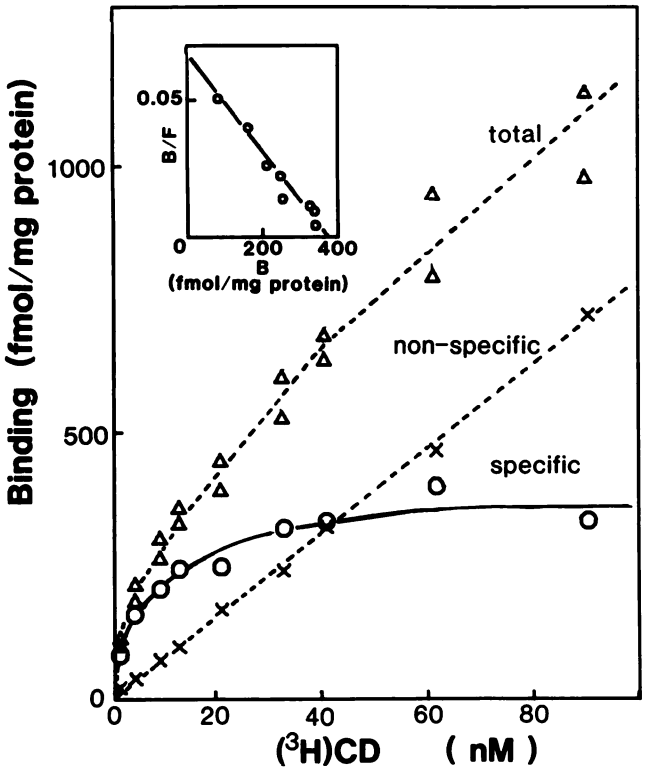

FIG. 1. *Specific and nonspecific binding of [³H]CD to synaptic membranes from porcine caudate nucleus as a function of [³H]CD concentration*

The binding assay was performed as described under Experimental Procedures except that the concentrations of [³H]CD was varied as indicated. Δ---Δ, ×---×, and O——O represent total, nonspecific, and specific binding, respectively. *Inset*, Scatchard plot analysis of specific [³H]CD binding. The experiment shown is a representative of five similar experiments.

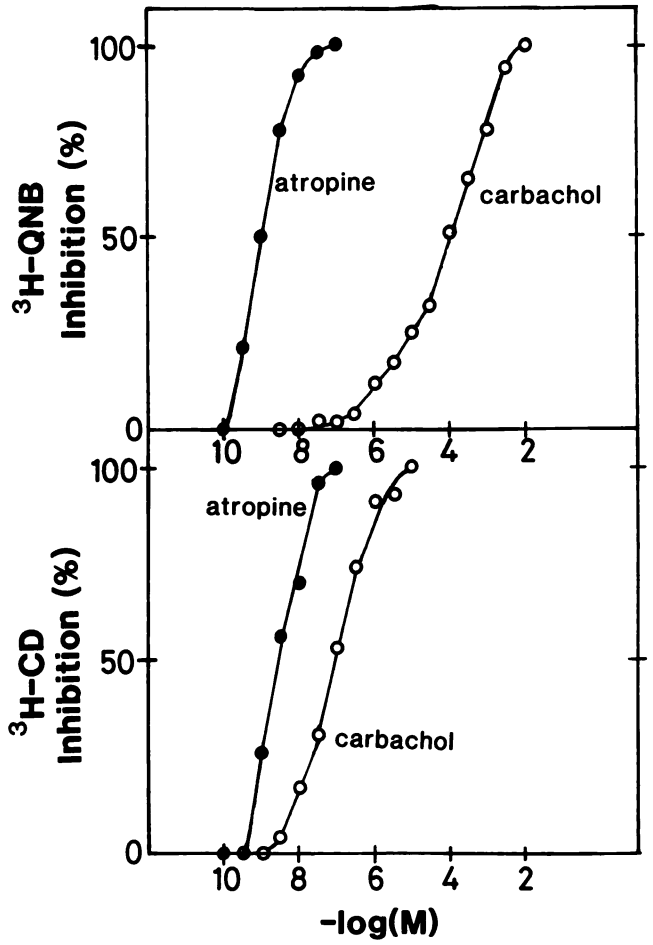

FIG. 2. *Displacement of specific [³H]QNB and [³H]CD binding by various concentrations of carbachol and atropine*

The binding assay was performed as described under Experimental Procedures in the presence of various concentrations of carbachol or atropine. *Top*, Displacement of specific [³H]QNB binding by carbachol (O——O) and atropine (●——●). *Bottom*, Displacement of specific [³H]CD binding by carbachol (O——O) and atropine (●——●). The experiment shown is representative of two to four similar experiments. Binding in the absence of carbachol and atropine was 2700 ± 40 cpm (n = 2) for [³H]QNB and 1681 ± 23 cpm (n = 4) for [³H]CD (mean ± standard deviation).

brain (3). All of these findings indicated that [³H]CD was bound mainly to a single class of noncooperative sites with high affinity for agonists.

Effects of guanyl nucleotide on [³H]CD binding. [³H]CD binding was decreased in the presence of GppNHp, GTP, and GDP (Fig. 3; Table 2). The rank

TABLE 1

Inhibition of [³H]QNB and [³H]CD binding by muscarinic ligands

Displacement studies between radiolabeled ligands (0.5 nM [³H]QNB or 5 nM [³H]CD) and unlabeled muscarinic ligand were performed as described under Experimental Procedures. Hill plot analysis was carried out using the data from the displacement studies. IC_{50} values and Hill coefficients (n_H) were obtained from the Hill plot analysis. The data shown represent the mean ± standard deviation [carbachol (n = 4), CD (n = 5), atropine (n = 3)]. The other six data are the means of duplicate determinations from one experiment.

Ligand	[³H]QNB		[³H]CD	
	IC_{50}	n_H	IC_{50}	n_H
	μM		μM	
Agonists				
Carbachol	137 ± 24	0.44 ± 0.03	0.051 ± 0.018	0.85 ± 0.13
Arecoline	22	0.53	0.079	0.97
Oxotremoline	2.2	0.57	0.014	0.97
CD	42.5 ± 17	0.46 ± 0.1	0.018 ± 0.005	0.86 ± 0.08
	nM		nM	
Antagonists				
Atropine	1.2 ± 0.1	1.15 ± 0.05	2.7 ± 1.2	1.06 ± 0.17
Scopolamine	2.0	1.0	8.0	0.97

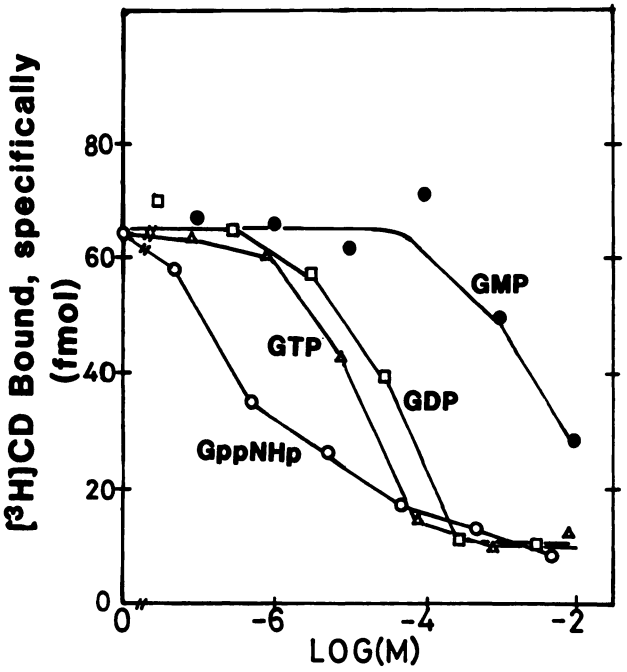

FIG. 3. *Effects of increasing concentrations of guanyl nucleotides on specific binding of [^{3}H]CD*

The binding assay was performed as described under Experimental Procedures in the presence of 2 mM $MgCl_2$ and various concentrations of GppNHp (○—○), GTP (△—△), GDP (□—□), or GMP (●—●). The values shown are the means of duplicate determinations in one of two similar experiments.

order of the inhibitory effect on [^{3}H]CD binding was GppNHp, GTP, and GDP with the half-maximal effect at 1.3, 32, and 45 μM, respectively. Inhibition by GMP was apparent only at concentrations 100 times higher than those for GDP. Among other nucleotides, ITP, CTP, and UTP also decreased the [^{3}H]CD binding, but at higher concentrations than GTP. ATP and many other nucleotides listed in Table 2 had no effect on [^{3}H]CD binding at 1 mM. [^{3}H]QNB binding was not affected by guanyl nucleotide. Inhibition of [^{3}H]CD binding by guanyl nucleotide was not complete, and approximately 20% of the binding remained insensitive even in the presence of 10 mM guanyl nucleotide. The B_{max} and K_d in the presence of 0.1 mM GppNHp were estimated to be roughly 150 fmoles/mg of protein and 22 nM, respectively, although accurate estimation was not possible because of the low ratio of specific to nonspecific binding. The K_d and B_{max} for [^{3}H]CD binding to the GppNHp-sensitive site was calculated by subtracting the data of the equilibrium study in the presence of GppNHp from those in its absence, and were 7.0 nM and 290 fmoles /mg of protein, respectively. [^{3}H]CD binding to the GppNHp-sensitive site was about 9% of the total receptor site.

Effects of Ni^{2+} and other cations on [^{3}H]CD binding. [^{3}H]CD binding was increased in the presence of various cations, including Ni^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+} at 1 mM, whereas [^{3}H]QNB binding was hardly affected by these ions (Table 3). Other cations tested (including Na^+, K^+, and Ca^{2+}) were not effective at 1 mM. Cd^{2+}, Hg^{2+}, and Cu^{2+} caused a considerable decrease in [^{3}H]CD binding. We studied the ion-specific enhancement of [^{3}H]CD binding in more detail using the Ni^{2+} ion. [^{3}H]CD binding was increased by 2- to 3-fold between 1 and 10 mM and was inhibited by a further increase in Ni^{2+} (Fig. 4). The latter inhibitory effect was not specific, and other monovalent (Na^+, K^+) and divalent cations (Mg^{2+}, Ca^{2+}) decreased [^{3}H]CD binding at almost the same concentration (Figs. 4 and 5). The degree of the increase caused by Ni^{2+} in [^{3}H]CD binding was not affected by the presence of Mg^{2+} or Ca^{2+} (Figs. 5 and 6). The increase in [^{3}H]CD binding caused by Mg^{2+} was not apparent but was man-

TABLE 2

Inhibition of [^{3}H]CD binding by guanyl and other nucleotides

Membranes (1 mg of protein per milliliter) were incubated with 5 nM [^{3}H]CD in the presence of 2 mM $MgCl_2$ and various concentrations of the nucleotides listed as described under Experimental Procedures. IC_{50} values are the concentrations of nucleotide which reduced [^{3}H]CD binding to 50%. The data shown are the means of duplicate determinations from two experiments.

Addition[a]	IC_{50}
	M
GppNHp	1.3×10^{-6}
GTP	3.2×10^{-5}
GDP	4.5×10^{-5}
GMP	5.6×10^{-3}
ITP	5.0×10^{-5}
CTP	1.8×10^{-4}
UTP	4.0×10^{-4}

[a] The following had no effects on [^{3}H]CD binding at 1 mM: AMP, ADP, ATP, AMPpNHp, cAMP, CMP, CDP, cGMP, IMP, UMP, UDP, NAD, NADH, NADP, NADPH.

TABLE 3

Effects of cations on [^{3}H]CD and [^{3}H]QNB binding at 1 mM (% of control binding)

Membranes (1 mg of protein per milliliter) were incubated with 5 nM [^{3}H]CD or 0.5 nM [^{3}H]QNB in the presence of 1 mM cations indicated. The specific binding of [^{3}H]CD or [^{3}H]QNB in the absence of cations was taken as the control binding (100%). All solutions and buffers used in the study were deionized by passage through a Chelex 100 column. The experimental apparatus for the binding assay was washed with 2 mM EDTA and then with deionized water which had been passed through a Chelex 100 column. Each metal chloride was dissolved in 0.1 N HCl to give a 100 mM solution. The solution was added to the reaction mixture for a final concentration of 1 mM immediately before the addition of membranes. The addition of 0.1 N HCl alone (final concentration of 1 mM) did not affect [^{3}H]CD binding. Values shown are the means of duplicate determinations from three experiments; 100% binding was 1300 ± 25 cpm for [^{3}H]CD and 2700 ± 20 cpm for [^{3}H]QNB (mean ± standard deviation).

Enhanced [^{3}H]CD binding
$NiCl_2$ (204), $MnCl_2$ (179), $CoCl_2$ (170), $ZnCl_2$ (145), $CrCl_2$ (145), $MgCl_2$ (126)
Inhibited [^{3}H]CD binding
$CdCl_2$ (30), $HgCl_2$ (7), $CuCl_2$ (52)
No effects on [^{3}H]CD binding
NaCl, KCl, LiCl, CsCl, RbCl, $CaCl_2$, $SrCl_2$, $PbCl_2$, $BaCl_2$
Effects on [^{3}H]QNB binding
$CdCl_2$ (19), $MnCl_2$ (97), $NiCl_2$ (97), $CoCl_2$ (102), $ZnCl_2$ (76), $CrCl_2$ (97), $SrCl_2$ (103)

ifested in the presence of 1 mM EDTA. EDTA decreased [^{3}H]CD binding by 50%, and this decrease was reversed by 1–10 mM $MgCl_2$ (Fig. 5). The effect of Mg^{2+} was also observed in the membranes that were pretreated with EDTA and then washed to remove the chelator. The degree of the increase caused by Ni^{2+} was not affected by the presence of or pretreatment with EDTA (Fig. 6). The Scatchard plot of the saturation binding in the presence of 1 mM Ni^{2+} demonstrated that the K_d was 9.1 nM and was very close to that in the absence of Ni^{2+}. The B_{max} was 890 fmoles/mg of protein and was 2-fold higher than that in the absence of the metal (Fig. 7). The enhancing effect of Ni^{2+} on [^{3}H]CD binding was completely lost in the presence of 0.1 mM GppNHp, and the characteristics of [^{3}H]CD binding in the presence of both Ni^{2+} and GppNHp were very similar to those in the presence of GppNHp alone (Fig. 7). This indicated that the number of [^{3}H]CD binding sites possessing GppNHp sensitivity was increased by Ni^{2+}. The K_d and B_{max} for [^{3}H]CD binding to GppNHp-sensitive sites in the presence of 1 mM $NiCl_2$ were calculated to be 7.8 nM and 720 fmoles/mg of protein, respectively.

Effects of GppNHp and $NiCl_2$ on competition between [^{3}H]QNB and CD. The displacement curve of [^{3}H]QNB binding by CD was shifted to the right in the presence of 0.1 mM GppNHp and to the left in the presence of 1 mM $NiCl_2$. The competition curves were fitted to a model of two or three classes of binding sites (Table 4). The curve in the presence of GppNHp was explained by a two-site model, and a three-site model did not further improve the curve fitting. The sites with the super high affinity for CD (SH sites) disappeared and the proportion of low affinity sites (L sites) was increased in the presence of 0.1 mM GppNHp, whereas the proportion of SH sites was increased by 2-fold in the presence of 1 mM $NiCl_2$.

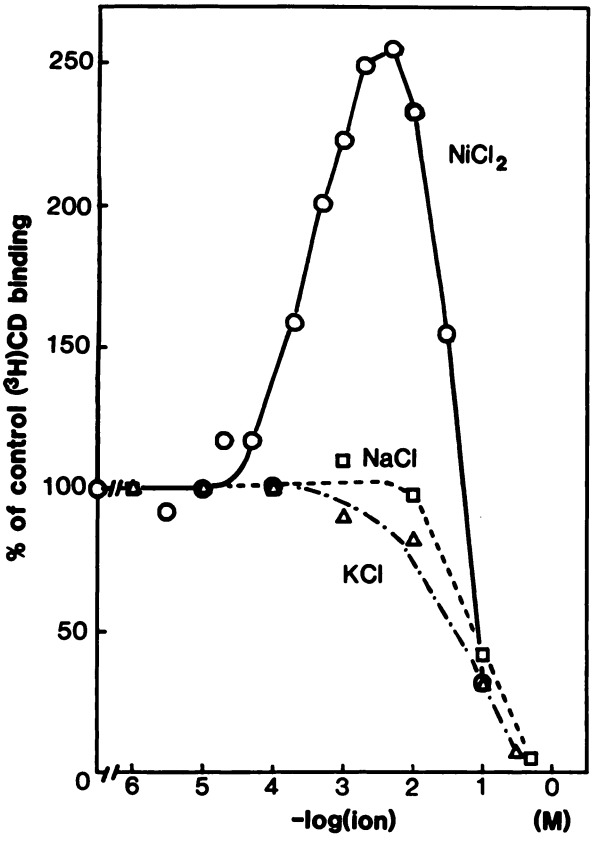

FIG. 4. *Effects of increasing concentrations of $NiCl_2$, NaCl, and KCl on the specific binding of [^{3}H]CD*

The binding assay was performed as described under Experimental Procedures in the presence of various concentrations of NaCl (□– –□), KCl (△– · –△), or $NiCl_2$ (○——○). The values shown are the means of duplicate determinations in one of two similar experiments; 100% binding was 1760 ± 56 cpm (mean ± standard deviation).

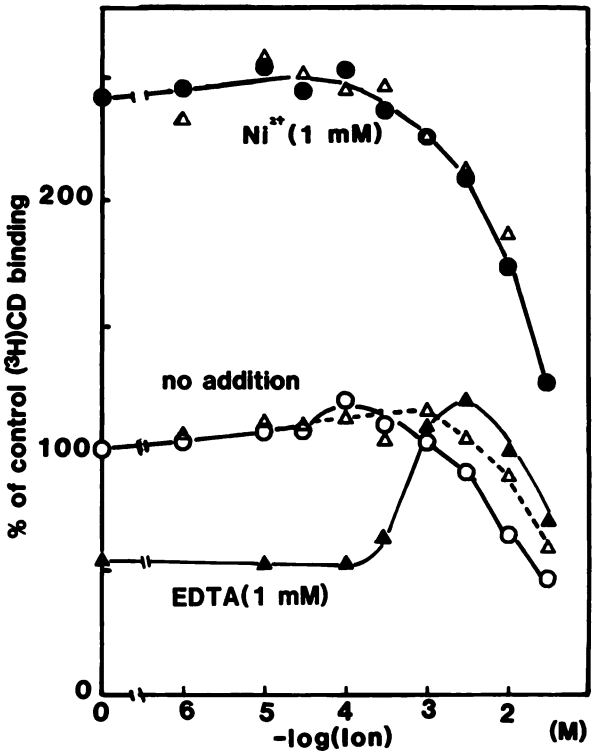

FIG. 5. *Effects of Mg^{2+} and Ca^{2+} on specific [^{3}H]CD binding in the presence or absence of Ni^{2+} and EDTA*

The binding assay was performed as described under Experimental Procedures except that the various concentrations of $MgCl_2$ or $CaCl_2$ were added in the presence or absence of 1 mM $NiCl_2$ and 1 mM EDTA. ○——○, △– –△, ●——●, △——△, and ▲——▲ represent the effects of $CaCl_2$, $MgCl_2$, $CaCl_2$ plus $NiCl_2$, $MgCl_2$ plus $NiCl_2$, and $MgCl_2$ plus EDTA, respectively. The values shown are the means of duplicate determinations in one of two similar experiments; 100% binding was 1680 ± 50 cpm (mean ± standard deviation).

DISCUSSION

The binding of [^{3}H]CD to synaptic membranes of porcine caudate nucleus was apparently homogeneous under the conditions employed and was characterized by a B_{max} of 390 fmoles/mg of protein and a K_d of 8.1 nM. When the binding was carried out in the presence of GppNHp, however, the heterogeneous nature of the binding became apparent. Approximately 20% of the [^{3}H]CD binding at 5 nM [^{3}H]CD remained insensitive to inhibition by GppNHp. The B_{max} and K_d of the guanyl nucleotide-sensitive [^{3}H]CD binding were estimated to be 290 fmoles/mg of protein and 7.0 nM, respectively. Thus, only about 10% of the [^{3}H]QNB binding sites (3 pmoles/mg of protein) had high affinity for CD and

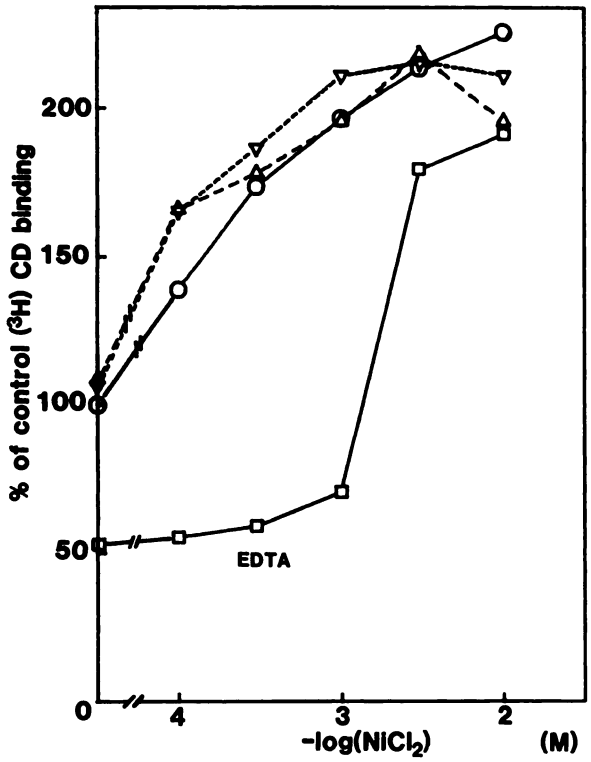

FIG. 6. *Effects of $NiCl_2$ on specific [^{3}H]CD binding in the presence or absence of $MgCl_2$ and EDTA*

The binding assay was performed as described under Experimental Procedures except that various concentrations of $NiCl_2$ were added in the absence (○——○) or presence of 1 mM $MgCl_2$ (▽- - -▽), 30 mM $MgCl_2$ (△- - -△), and 1 mM EDTA (□——□). The values shown are the means of duplicate determinations in one of two similar experiments; 100% binding was 1700 ± 38 cpm (mean ± standard deviation).

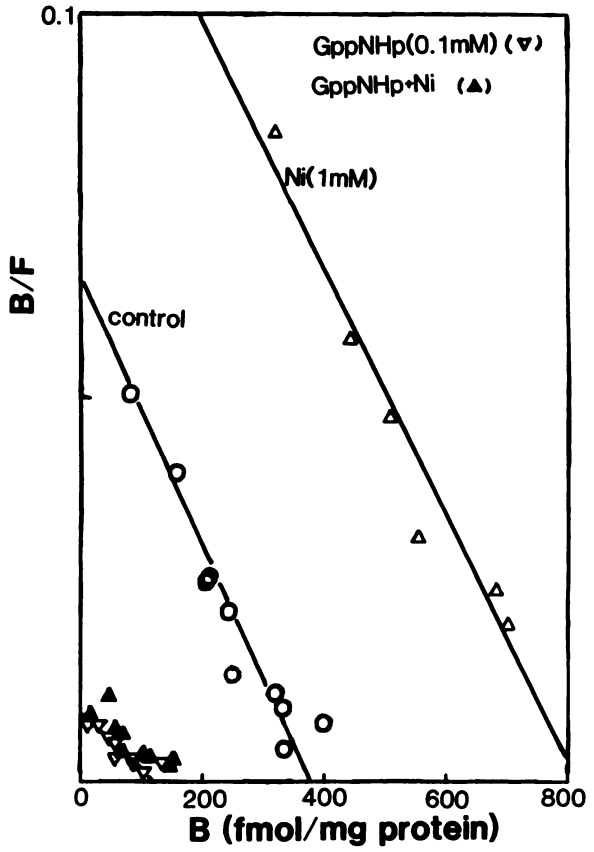

FIG. 7. *Scatchard plot of specific [^{3}H]CD binding in the absence or presence of $NiCl_2$ and GppNHp*

The binding assay was performed as described under Experimental Procedures except that the concentration of [^{3}H]CD was varied in the absence (○——○) or presence of 1 mM $NiCl_2$ (△——△), 0.1 mM GppNHp (▽) and both $NiCl_2$ and GppNHp (▲). The values shown are the means of duplicate determinations in one of three similar experiments.

[^{3}H]CD, in agreement with the results obtained from analysis of the displacement of [^{3}H]QNB binding by CD. The affinity for agonists of the [^{3}H]CD binding sites was very similar to that of SH sites in rat forebrain (3). These results suggested that [^{3}H]CD binding can be ascribed, for the most part, to SH sites and confirmed the usefulness of [^{3}H]CD as a probe of a single subclass of muscarinic receptors. Our results were essentially similar to those of Ehlert *et al.* (16) for [^{3}H]CD binding by rat forebrain muscarinic receptors, but there was a discrepancy concerning the contribution of GppNHp-insensitive binding to total [^{3}H]CD binding. Ehlert *et al.* reported a smaller effect of GppNHp (43% inhibition versus 80% at 5 nM [^{3}H]CD in the present study) and an apparently curved Scatchard plot. The greater contribution of GppNHp-insensitive binding in their experiment may be explained in part by the higher incubation temperature than that in the present experiment (37° versus 20°), as the GppNHp-sensitive site is much more labile to heat than the GppNHp-insensitive site (20).

The binding of [^{3}H]CD was depressed in the presence of GTP, GDP, UTP, CTP, and ITP as well as GppNHp but was not affected by ATP and other nucleotides. In

TABLE 4

Computer-derived parameters from curves of [^{3}H]QNB in competition with CD in the presence or absence of GppNHp or $NiCl_2$

The displacement curves by CD of [^{3}H]QNB binding (0.5 nM) in the presence or absence of 1 mM $NiCl_2$ or in the presence of 0.1 mM GppNHp plus 2 mM $MgCl_2$ were analyzed by a computer according to the two- or three-site model. The curves in the absence of GppNHp were best described by the three-site model, but the curve in the presence of GppNHp was best explained by the two-site model and the three-site model did not improve the curve fitting. The data shown represent means ± standard deviation [no addition (n = 4), GppNHp (n = 2), $NiCl_2$ (n = 2)].

Addition	SH site		H site		L site	
	IC_{50}	%	IC_{50}	%	IC_{50}	%
	μM		μM		μM	
None	0.032 ± 0.035	9 ± 3	2.2 ± 1.5	28 ± 9	110 ± 57	63 ± 10
GppNHp			0.69 ± 0.2	26 ± 11	82 ± 37	74 ± 11
$NiCl_2$	0.027 ± 0.012	16 ± 4	1.5 ± 0.2	28 ± 9	72 ± 7	56 ± 1

addition, GTP accelerated the dissociation of bound [^{3}H]CD from membranes (data not shown). The specificity for nucleotides and the effect of GTP on the dissociation of bound agonist were very similar to the results obtained for cardiac muscarinic receptors (4, 21, 22) and other kinds of receptors (23–25). These similarities support the following scheme as a possible mechanism of the action of guanyl nucleotide:

$$RN \rightleftharpoons R + N \quad (1)$$

where *R, N,* and *RN* represent receptor, GTP binding protein, and receptor-GTP binding protein complex, respectively. The *RN* complex and *R* have high affinity and low affinity for [^{3}H]CD, respectively. GTP promotes the dissociation of the *RN* complex into *N* and *R*. This assumption was indirectly supported by the recent finding of Uchida *et al.* (26) that the effect of GppNHp on agonist binding is related to a larger size of molecules than the [^{3}H]QNB-binding component itself.

It is known that the effect of guanyl nucleotide on muscarinic receptors of cerebral cortex, including caudate nucleus, is much smaller than that on cardiac receptors when the effect is evaluated from the shift of the displacement curve between radiolabeled antagonist and nonradioactive agonist. In the present study, however, the effect of guanyl nucleotide on [^{3}H]CD binding of membranes from caudate nucleus was very similar to that on agonist binding of cardiac membranes. This indicated that the two tissues were different only in the proportion of the guanyl nucleotide-sensitive sites in total muscarinic receptors but not in the properties of the guanyl nucleotide-sensitive sites themselves. Under the assumption formulated in Eq. 1, the number of *RN* complexes, that is, the guanyl nucleotide-sensitive sites, should be determined from the equilibrium constant and the number of *R* and *N*. Enhancement of GppNHp-sensitive [^{3}H]CD binding by Ni^{2+} indicated that the *RN* complex was increased in the presence of Ni^{2+}. It follows that the proportion of *RN* complexes in the absence of Ni^{2+} was not limited by the availability of *N* or *R* but regulated by the equilibrium between them. Even in the presence of Ni^{2+}, however, the highest proportion of GppNHp-sensitive binding sites observed was 30% of the total receptors. This value may reflect the limitation in available *N* or in the proportion of the subclass of *R* which has the ability to bind with *N*. It is not known whether *R* in Eq. 1 represents total muscarinic receptors or a certain subclass. Analysis of the displacement of [^{3}H]QNB binding by CD suggested that the increase in SH sites by Ni^{2+} and their decrease by GppNHp were accompanied by a decrease and increase in L sites, respectively, but not in H sites. It is conceivable that *R* represents a part or all of the L sites and not the H sites.

To our knowledge, the physiological role of nickel ion has not been demonstrated in mammals. The concentration of metal required for enhancement of [^{3}H]CD binding is far above the concentration in tissues. It is possible, however, that Ni^{2+}-binding sites have a physiological role and that some other cations play a role. Magnesium and calcium have been reported to increase the affinity for agonists of muscarinic receptors in rat heart (13). In addition, opposing effects of Mg^{2+} and guanyl nucleotides on agonist binding of *beta*-adrenergic receptors have been reported (9, 10); the reported effects were apparently similar to the opposing effects of Ni^{2+} and guanyl nucleotides observed in the present study on [^{3}H]CD binding. In our experiment, Mg^{2+} slightly increased [^{3}H]CD binding but the effect was not prominent unless membranes were treated with EDTA. The sites that became appreciable in the presence of EDTA or after EDTA treatment appeared to have a high affinity for Mg^{2+}, because [^{3}H]CD binding in the presence of 1 mM EDTA was markedly increased by $MgCl_2$ between 0.1 and 1 mM. On the other hand, Ni^{2+}-binding sites seemed to have a much lower affinity for Mg^{2+} since $MgCl_2$ of 30 to 300 times higher concentration than $NiCl_2$ did not interfere with the effect of 0.1–1 mM Ni^{2+}. These considerations suggest that the two metal binding sites are independent.

Another possible binding site of Ni^{2+} is the Ca^{2+} channel, and it is interesting to note that the cations with the ability to enhance [^{3}H]CD binding, such as Co^{2+}, Mn^{2+}, and Ni^{2+}, are known as calcium antagonists. However, competition between Ca^{2+} and Ni^{2+} was not detected under the conditions used (Fig. 5). Calcium antagonists such as nifedipine, verapamil, nicardipine, and diltiazem did not affect the effect of Ni^{2+} on [^{3}H]CD binding (data not shown). Thus, the physiological role of Ni^{2+} binding sites remains to be elucidated.

In conclusion, high-affinity agonist binding was selectively regulated by guanyl nucleotide in porcine caudate muscarinic receptors as in cardiac muscarinic receptors, and cations such as Ni^{2+} and Mn^{2+} modulated regulation by guanyl nucleotide.

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