Recognition of the muscarinic receptor by its endogenous neurotransmitter: Binding of [³H]acetylcholine and its modulation by transition metal ions and guanine nucleotides

(receptor heterogeneity/affinity state/guanosine triphosphate/cobalt and nickel/rat cerebral cortex)

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ABSTRACT   Agonist binding to the muscarinic receptor in rat cerebral cortex membranes was studied by using the neurotransmitter itself, [³H]acetylcholine ([³H]AcCho). By using 10 μM atropine or oxotremorine to define specific binding, it was possible to demonstrate specific binding of [³H]AcCho that was sensitive to muscarinic but not to nicotinic ligands. Equilibrium binding experiments with 5–240 nM [³H]AcCho indicated specific binding of the ligand to a saturable population of muscarinic receptors (361 ± 29 fmol/mg of protein; $K_d = 76 \pm 17$ nM). This value represented 25% of the available binding sites for a labeled antagonist in the same preparation and corresponds to the proportion of high-affinity agonist binding sites observed previously in competition experiments with labeled antagonists. Inclusion of transition metal ions (e.g., 2 mM Ni^{2+}) in the assay increased the equilibrium binding of [³H]AcCho (628 ± 38 fmol/mg of protein, $K_d = 86 \pm 21$ nM) but did not affect equilibrium binding of ³H-labeled antagonists, indicating conversion of low- into high-affinity muscarinic agonist binding sites. The increase developed slowly over 30 min of incubation at 25°C but could be reversed rapidly (≈2 min) by the chelating agent EDTA or by guanine nucleotides. These data directly reveal a slow though quickly reversible interconversion of low- into high-affinity muscarinic agonist binding sites.

The binding properties of antagonists to muscarinic acetylcholine (AcCho) receptors are well characterized. Saturable binding of ³H-labeled antagonists to a homogeneous receptor population has been demonstrated in numerous preparations (see ref. 1 for a recent review).

Because of the technical difficulties inherent in measuring binding of ³H-labeled agonists to the muscarinic receptor by direct means (2), attempts to further characterize the interaction of agonists with the muscarinic receptor have instead employed competition studies utilizing unlabeled agonists and ³H-labeled antagonists (2–12). The results of such experiments could be interpreted by assuming the existence of two or three populations of noninteracting binding sites for agonists. In cerebral cortex preparations most receptors (≈75%) exhibit low affinity towards agonists (5, 8).

More recent studies indicated that binding properties of muscarinic agonists can be modulated in vitro by transition metal ions (8), GTP and its analogs (6–10, 13, 14), and the islet-activating protein, pertussis toxin (12). The mode of these modulations can be studied by the use of ³H-labeled antagonist/agonist competition experiments. However, in the complex system of ³H-labeled antagonist, agonist, receptor, and modulator, the only directly measured probe is the labeled antagonist. Thus, studies on the mechanism of muscarinic receptor modulation are highly complicated. To sim-

plify the system, direct binding studies with labeled muscarinic agonists are required. Several groups have already demonstrated binding of ³H-labeled agonists to the high-affinity fraction of muscarinic receptors. The agonists employed were cis-[³H]methyldioxolane (13, 15) and [³H]methyloxotremorine (2, 14). The presence of low-affinity binding sites could not be demonstrated in these studies due to high nonspecific binding. Binding of [³H]AcCho to the muscarinic receptor in mouse brain has also been demonstrated (16), but the low specific radioactivity and the technique used in these studies (equilibrium dialysis) did not allow precise characterization of these interactions. However, it is highly important to establish the interaction between the muscarinic AcCho receptor and the neurotransmitter itself. We therefore undertook a study on the interactions of the radiolabeled AcCho with the muscarinic receptors in rat cerebral cortex membranes. In the present work we describe the direct binding of [³H]AcCho of high specific radioactivity to the muscarinic receptor, as well as changes in its binding induced by transition metal ions and by guanine nucleotides. A preliminary report of some of these findings has appeared (17).

MATERIALS AND METHODS

Materials. [³H]AcCho of high specific radioactivity (70–86 Ci/mmol, 97% purity; 1 Ci = 37 GBq) was purchased from Amersham. Its synthesis and purity determinations have recently been described in detail (18). The radiochemical was kept at −70°C in small aliquots in ethanol/water (1:1, vol/vol), which were dried by a gentle stream of nitrogen prior to assay. Three different batches of [³H]AcCho that were used in the course of this study yielded essentially the same results.

Tissue Preparation. Cerebral cortex homogenates were prepared from four or five male rats (C-D strain) in 50 vol of 50 mM Tris·HCl buffer, pH 7.4, as described (3, 19). The homogenate was incubated for 30 min at 25°C with gentle shaking and then centrifuged at 30,000 × g for 15 min. This procedure was repeated twice. The final pellet was resuspended in modified Krebs buffer containing 25 mM Tris·HCl (pH 7.4, 25°C). A fresh solution of diisopropyl fluorophosphate (iPr₂P-F; Sigma lot 82F-0450) in water was added to the homogenate to achieve a concentration of 200 μM. The homogenate was incubated for a further 30 min at 25°C prior to binding assay. In some assays neostigmine or physostigmine was used instead of iPr₂P-F. Protein concentration was determined according to the Lowry method, using bovine serum albumin as a standard.

[³H]AcCho Binding Assay. Aliquots (20 μl) of homogenate (equivalent to 3–5 mg of original tissue weight) were added

Abbreviations: AcCho, acetylcholine; AcChoEase, acetylcholinesterase; iPr₂P-F, diisopropyl fluorophosphate; p[NH]ppG, guanylyl imidodiphosphate; 4NMPB, N-methyl-4-piperidyl benzilate.

to tubes containing 20 μl of modified Krebs buffer, 200 μM iPr$_2$P-F, and the indicated concentrations of [^{3}H]AcCho. After the indicated time of incubation with gentle shaking at 25°C, 4 ml of ice-cold modified Krebs buffer was added and the contents of the tubes were filtered under high pressure through GF/C filters (Whatman, 25-mm diameter). The filters were immediately washed with an additional 2 ml of buffer; the time that elapsed between the addition of buffer to the tube and the termination of filtration was 2–2.5 sec.

Specific binding was taken as the difference between the total binding to control membranes and the measured nonspecific binding—i.e., binding to membranes after adding 20 μM atropine during the last 10 min of the preincubation step. The same values for nonspecific binding were obtained when 20 μM oxotremorine was substituted for atropine. Under the experimental protocol described, there was no detectable specific binding to GF/C filters alone, or to membranes heated to 70°C for 10 min.

All determinations were carried out in quadruplicate, each one varying by <15%. Centrifugation assays were carried out by using a similar protocol, but bound and free ligand were separated by centrifugation in an Eppendorf microcentrifuge (10,000 × g, 2 min), followed by superficial washing of the membrane pellet three times with 1 ml of ice-cold buffer and determination of radioactivity in the pellets.

[^{3}H]4NMPB Binding. Aliquots (20 μl) of the membrane preparation were incubated for 60 min at 25°C with a saturating concentration (20 nM) of [^{3}H]4NMPB in 1 ml of modified Krebs buffer. Assays were terminated by filtration through GF/C filters and washing three times with 4 ml of ice-cold buffer. Nonspecific binding was determined with 10 μM atropine.

Data Analysis. Results of binding experiments are presented as mean values or means ± one standard deviation. Linear regression analysis of binding isotherms was utilized to obtain values for maximal binding capacity (B_{max}) and affinity (K_d).

RESULTS

Assay of [^{3}H]AcCho Binding to Muscarinic Receptors. Muscarinic AcCho receptors in rat cortex membranes have been assayed previously by using stable muscarinic antagonists and agonists (ref. 1 for review). Unlike these ligands, [^{3}H]AcCho is susceptible to hydrolysis by the enzyme acetylcholinesterase (AcChoEase) and may be isotopically diluted by endogenous AcCho. In order to avoid these difficulties, the membranes were prepared by hypotonic washing (see *Materials and Methods*). To prevent degradation of [^{3}H]AcCho, AcChoEase inhibitor was added to the washed membranes prior to the addition of [^{3}H]AcCho. Since these inhibitors may interact with the muscarinic receptor (13), we examined the effects of several inhibitors on the binding of [^{3}H]AcCho. Binding of [^{3}H]AcCho to the membrane preparation was critically dependent on both the concentration and the nature of the inhibitor (Fig. 1A). The inhibitor selected was iPr$_2$P-F, since [^{3}H]AcCho binding was unchanged at a wide iPr$_2$P-F concentration range of 100 μM to 2 mM, which completely inhibits AcChoEase (20). Physostigmine and neostigmine are less suitable, since they inhibit [^{3}H]AcCho binding at concentrations needed to inhibit AcChoEase. This is shown by the narrow range of maximal [^{3}H]AcCho binding, as well as by the reduced binding compared to that observed in the presence of iPr$_2$P-F (Fig. 1A). All subsequent binding experiments were therefore performed in the presence of 200 μM iPr$_2$P-F, which did not affect binding of the antagonist [^{3}H]4NMPB.

Binding of [^{3}H]AcCho to cortical membranes could be detected by using either the centrifugation or the rapid filtration technique. In each case the bound ligand represented
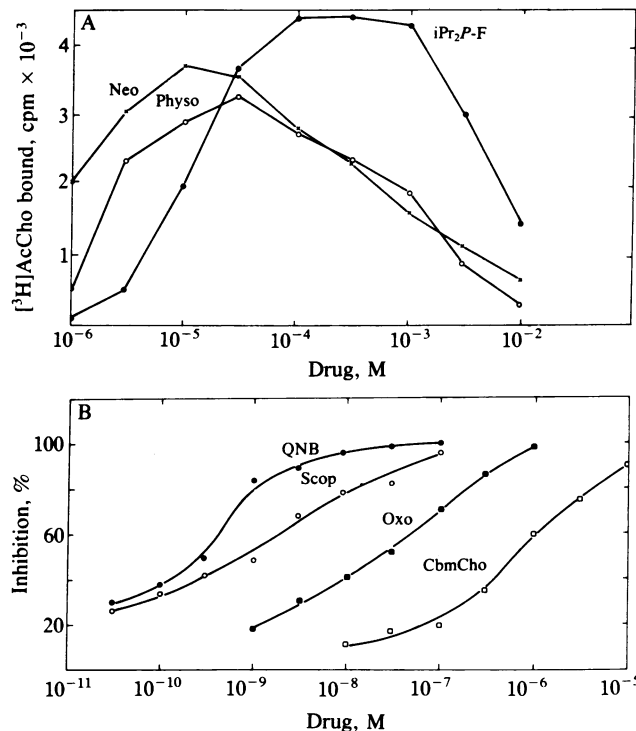

FIG. 1. (A) Specific binding of 45 nM [^{3}H]AcCho to rat cerebral cortex membranes in the presence of AcChoEase inhibitors at various concentrations. Membranes (0.48 mg of protein) were incubated for 1 hr at 25°C with 45 nM [^{3}H]AcCho and specific binding was determined. AcChoEase inhibitors present during incubation: ○, physostigmine; ×, neostigmine; ●, iPr$_2$P-F. Nonspecific binding was determined separately for each drug concentration. (B) Inhibition of [^{3}H]AcCho binding by muscarinic ligands. Membranes (0.07 mg of protein) were incubated for 60 min at 25°C with 46 nM [^{3}H]AcCho and various concentrations of (−)-quinuclidnyl benzilate (●), scopolamine (○), oxotremorine (■), or carbamoylcholine (□).

two components, one that could be inhibited by 10 μM atropine or 10 μM oxotremorine (specific binding), and one that could not be inhibited by these muscarinic drugs (nonspecific binding). Both techniques yielded similar specific binding of [^{3}H]AcCho, indicating that there was no significant loss of specifically bound ligand in the filtration process. Nonspecific binding, however, was much higher when measured by the centrifugation technique than by filtration. Results of a typical centrifugation experiment performed in sextuplet and employing 42 nM [^{3}H]AcCho were 10,450 ± 1255 cpm (total) and 7928 ± 1108 cpm (nonspecific). Filtration assay of the same membrane preparation with 42 nM [^{3}H]AcCho yielded 3943 ± 195 cpm (total) and 1376 ± 75 cpm (nonspecific). Further experiments were therefore carried out with the filtration method, because this afforded more accurate determinations of specific [^{3}H]AcCho binding. It should be noted that termination of the binding assay by rapid filtration with ice-cold buffer takes ≈2 sec, in contrast to the slower rate of [^{3}H]AcCho dissociation from its receptor at 0°C ($t_{1/2} \approx 90$ sec).

The above results thus establish a simple assay for the binding of [^{3}H]AcCho to muscarinic receptors in membranes. Under the conditions specified, binding of the ligand showed linear dependence on protein concentration up to 0.6 mg of protein per assay. Binding reached equilibrium at about 30 min and remained unchanged even after 2 hr of incubation; binding studies at equilibrium were therefore conducted for 60 min at 25°C.

Characterization of [^{3}H]AcCho Binding. To characterize the nature of [^{3}H]AcCho binding to the muscarinic receptor in rat cortex membranes, we examined both the ability of

cholinergic drugs to affect the binding at equilibrium and the concentration dependence of the ligand binding. The specific binding sites for [^{3}H]AcCho are muscarinic cholinergic receptors, since muscarinic ligands proved to be potent inhibitors of [^{3}H]AcCho binding (Fig. 1*B*). Thus, apparent inhibition constants (nM) of 0.20, 0.25, 0.55, 0.60, and 3.0 were determined for the antagonists quinuclidinyl benzilate, *N*-methylscopolamine, 4 NMPB, scopolamine, and atropine, respectively. The apparent inhibition constants (nM) for agonists were 17, 320, 550, and 1100 for oxotremorine, carbamoylcholine, arecoline, and pilocarpine, respectively. These apparent inhibition constants (K_i) were calculated according to the equation $K_i = I_{50}/(1 + L/K_d)$, in which I_{50} is the drug concentration inhibiting half the specific binding of [^{3}H]AcCho (at concentration L) to the muscarinic receptor. On the other hand, nicotinic drugs such as nicotine, *d*-tubocurarine, and α-bungarotoxin did not inhibit binding of [^{3}H]AcCho (at 100 μM). In addition, muscarinic drugs at sufficiently high concentrations can completely inhibit the binding of [^{3}H]AcCho (Fig. 1*B*), in the order of potency expected for these drugs (2–4). Binding of [^{3}H]AcCho (5–240 nM) in the absence and in the presence of 10 μM atropine is shown in Fig. 2*A*. As expected, the nonspecific binding shows linear de-

FIG. 3. Scatchard plot of data from Fig. 2*B*. ○, No addition; ●, + 2 mM Ni^{2+}. Data are from a typical experiment, which yielded the following parameters: B_{max} = 410 and 690 fmol/mg of protein, and K_d = 95 and 77 nM, in the absence (control) and presence of 2 mM Ni^{2+}, respectively. The concentration of antagonist sites measured by [^{3}H]4NMPB in the same preparation was 1.68 pmol/mg of protein.
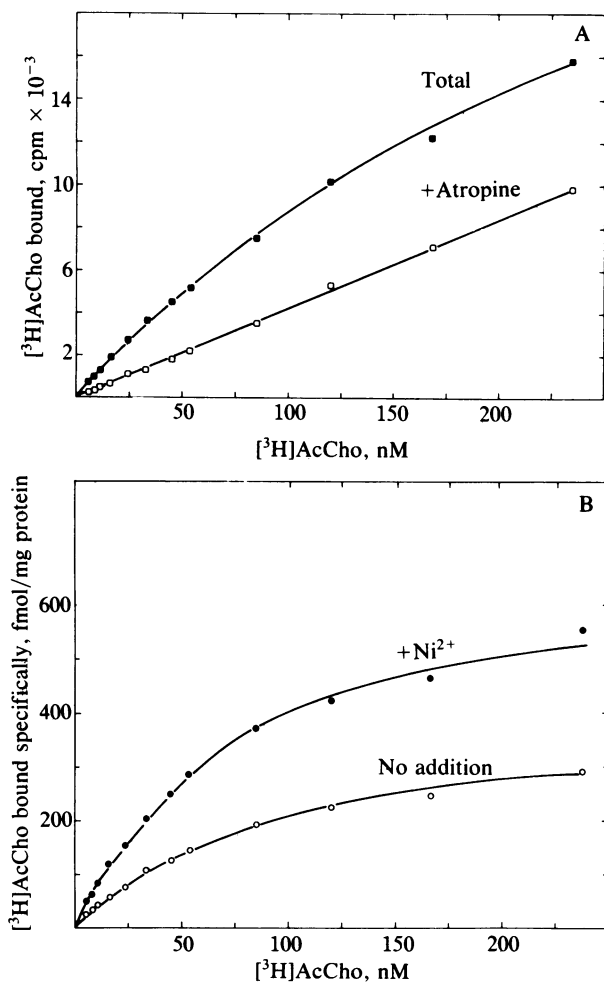

FIG. 2. (*A*) Equilibrium binding of [^{3}H]AcCho to rat cerebral cortex membranes. Membranes (0.35 mg of protein) were incubated for 1 hr at 25°C with the indicated concentrations of [^{3}H]AcCho alone (■) or [^{3}H]AcCho + 10 μM atropine (□). Results shown are means of quadruplicate determinations. (*B*) Specific binding of [^{3}H]AcCho to rat cerebral cortex membranes in the absence and presence of 2 mM Ni^{2+}. Membranes (0.35 mg of protein) were incubated for 1 hr at 25°C with the indicated concentrations of [^{3}H]AcCho in Krebs buffer alone (○) or Krebs buffer + 2 mM Ni^{2+} (●).
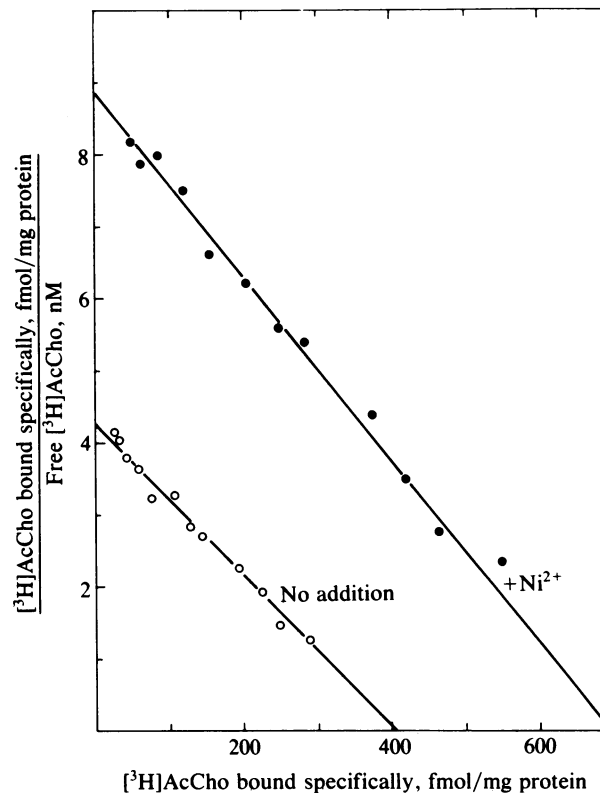
pendence on [^{3}H]AcCho concentration. Subtracting this binding from the total binding yielded a simple hyperbolic curve (Fig. 2*B*). Data replotted according to Scatchard yielded a straight line (Fig. 3). Mean binding parameters (five experiments) were: K_d = 76 ± 17 nM; B_{max} = 361 ± 29 fmol/mg of protein. In the same preparations binding capacity of the muscarinic antagonist [^{3}H]4NMPB was 1430 ± 125 fmol/mg of protein. Thus, the ratio of high-affinity [^{3}H]AcCho binding sites to muscarinic antagonist binding sites is 1:4.

Under the conditions employed, the concentration of [^{3}H]AcCho binding sites was found to be 3–4 nM. This high receptor concentration could lead to some inaccuracies in determination of the binding capacity and the dissociation constant of [^{3}H]AcCho due to (*i*) depletion of the free ligand and (*ii*) presence of endogenous unwashed AcCho. We therefore performed equilibrium binding studies with [^{3}H]AcCho after 1:5 dilution of the membrane. Such a dilution should decrease the concentration of any endogenous AcCho present. The specific binding capacity for [^{3}H]AcCho (404 ± 36 fmol/mg of protein) and its apparent dissociation constant (52 ± 19 nM) were similar to those observed under the standard assay conditions. This precludes the possibility of inaccurate determinations of [^{3}H]AcCho binding parameters at the concentration range indicated.

Kinetic experiments were carried out to further characterize the mode of interaction of [^{3}H]AcCho with the muscarinic receptor. Binding of [^{3}H]AcCho to muscarinic receptors is a rapid process (Fig. 4); the $t_{1/2}$ for the association of 36 nM [^{3}H]AcCho at 25°C is 8–10 sec. Nevertheless, equilibrium was reached only after 14–30 min; this can be explained by the onset of a slower phase after the rapid early phase of
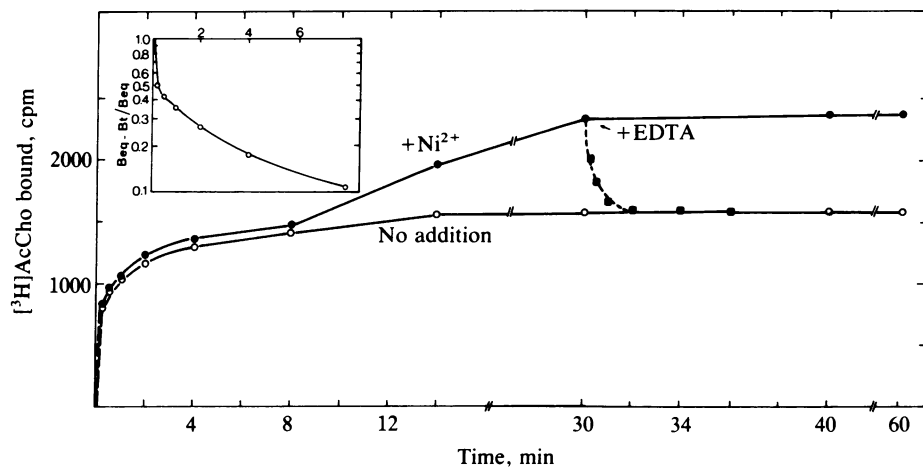
FIG. 4. Effect of 2 mM Ni^{2+} on specific [^{3}H]AcCho binding. Membranes (0.24 mg of protein) were incubated at 25°C with 36 nM [^{3}H]AcCho for the indicated periods, in the absence (○) or presence (●) of 2 mM Ni^{2+}. The results have been corrected for nonspecific binding determined separately for each incubation period. A parallel set of tubes was incubated at 25°C for 30 min with 36 nM [^{3}H]AcCho and 2 mM Ni^{2+}; 10 mM EDTA was then added and the reaction was terminated (■). (*Inset*) Pseudo-first-order kinetic plot of the association of specific [^{3}H]AcCho binding. The abscissa is time in min; the ordinate is $(B_{eq} - B_t)/B_{eq}$, in which B_{eq} is the amount of [^{3}H]AcCho bound specifically at equilibrium and B_t is the amount bound at the indicated time.

binding. Under the conditions employed here, the concentration of the ligand is >10-fold higher than that of its binding sites. Deviation of the pseudo-first-order curve from linearity therefore indicates that the reaction does not follow a simple bimolecular mechanism (Fig. 4 *Inset*). This could be a result of isomerization, as shown previously for receptor–antagonist complexes (3, 6).

Modulation of [^{3}H]AcCho Binding. Inhibition by agonists of ^{3}H-labeled antagonist binding to muscarinic receptors from rat cerebral cortex has been shown to be modulated conversely by transition metal ions and by guanine nucleotides (8). Since these modulators do not change the binding parameters of antagonists, it follows that they must modulate the binding of agonists. It is therefore expected that such changes should be detectable in agonist-receptor binding measured directly with [^{3}H]AcCho.

The effect of Ni^{2+} on [^{3}H]AcCho binding was examined with membranes incubated for 60 min at 25°C with 42 nM [^{3}H]AcCho and various concentrations of the metal ions. A concentration-dependent increase in the specific binding of [^{3}H]AcCho was observed (Fig. 5), with half-maximal increase occurring at ≈70 μM Ni^{2+}. No further increase in [^{3}H]AcCho binding could be achieved with Ni^{2+} concentrations higher than 2 mM. Nonspecific binding was not affected by these ions. A similar phenomenon was observed with
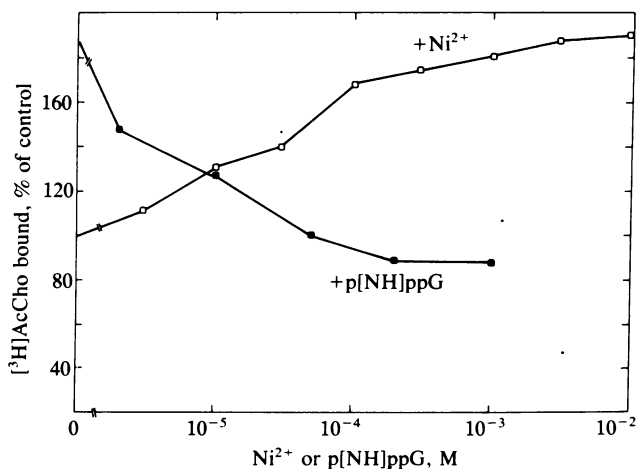

FIG. 5. Effect of Ni^{2+} and guanylyl imidodiphosphate (p[NH]-ppG) on specific [^{3}H]AcCho binding to rat cerebral cortex membranes. Membranes (0.41 mg of protein) were incubated with 42 nM [^{3}H]AcCho for 1 hr at 25°C. □, The indicated concentrations of Ni^{2+} were added to the incubation buffer. ■, Buffer contained 3 mM Ni^{2+} and the indicated concentrations of p[NH]ppG. Control specific binding was 126 fmol/mg of protein.

Co^{2+} or Mn^{2+}, which increased [^{3}H]AcCho binding to the same extent but at higher concentrations (half-maximal increase at 0.4 and 0.7 mM, respectively).

Binding isotherms describing the specific [^{3}H]AcCho binding to the muscarinic receptor in rat cerebral cortex membranes in the absence and presence of 2 mM Ni^{2+} are shown in Fig. 2B. A definite increase in the specific binding of [^{3}H]AcCho is observed at all ligand concentrations studied. Replotting the data according to Scatchard yields two parallel lines (Fig. 3), indicating an increased capacity for [^{3}H]AcCho binding with no substantial change in its affinity. The mean binding parameters (five experiments) are: B_{max} = 361 ± 29 and 628 ± 38 fmol/mg of protein and K_d = 76 ± 17 and 86 ± 21 nM, in the absence and presence of 2 mM Ni^{2+}, respectively. Under these conditions, binding of the muscarinic antagonist [^{3}H]4NMPB in the same preparations was not affected by 2 mM Ni^{2+}. Thus, the ratio of the number of [^{3}H]AcCho binding sites to the number of antagonist binding sites is increased in the presence of the metal ions from ≈0.25 to ≈0.45.

The increase in [^{3}H]AcCho binding induced by Ni^{2+}, Co^{2+}, or Mn^{2+} could be blocked by guanine nucleotides. At 100 μM, GDP, GTP, and its stable analog guanylyl imidodiphosphate (p[NH]ppG) completely inhibited the increase in [^{3}H]AcCho binding induced by the transition metal ions. The highest potency was observed with p[NH]ppG (K_{app} ≈ 2 μM; Fig. 5). It is noteworthy that small decreases (≈15%) in [^{3}H]AcCho binding could also be observed in the absence of transition metal ions. Inhibition of the Ni^{2+}-induced increase in [^{3}H]AcCho binding was restricted to the above-mentioned nucleotides; the following nucleotides were inactive at 200 μM: GMP, cGMP, cAMP, AMP, ADP, ATP, adenylyl imidodiphosphate, CTP, and UTP.

The time course of the onset of the Ni^{2+}-induced effect and its termination were examined. Reversal of the Ni^{2+} effect could be achieved by adding either 100 μM p[NH]ppG or 10 mM EDTA to membranes previously equilibrated with 36 nM [^{3}H]AcCho and 2 mM Ni^{2+} for 30 min at 25°C. The basal [^{3}H]AcCho binding level was reestablished within 2 min, with a $t_{1/2}$ of about 30–40 sec (Fig. 4). This rapid reversal was independent of the preincubation period in the range of 30–80 min and is almost as rapid as the dissociation rate of [^{3}H]AcCho–receptor complexes at 25°C ($t_{1/2}$ ≈ 20 sec; not shown). Unlike the rapid reversal of the Ni^{2+} effect, its onset was very slow. When membranes were incubated at 25°C in the presence of 36 nM [^{3}H]AcCho and 2 mM Ni^{2+}, increased binding was barely detectable in the first 5 min of incubation (Fig. 4); it developed slowly during the subsequent 15 min, and remained unchanged upon incubation for 30 min to 2 hr. This slow process was strongly dependent on temperature. Similar experiments carried out at 4°C indicat-

ed that the Ni^{2+} induced increase in [^{3}H]AcCho binding was apparent only after 3–5 hr of incubation, at which time the effect was equivalent in magnitude to that observed at 25°C after 30 min. It should be noted, however, that while no increase in [^{3}H]AcCho binding was evident after 1 hr at 4°C with 2 mM Ni^{2+}, binding in the absence of the metal ions was the same after 1 hr at either 4°C or 25°C.

DISCUSSION

The present work describes the atropine-sensitive binding of [^{3}H]AcCho to receptors in rat cerebral cortex membranes. Binding of [^{3}H]AcCho to these receptors is inhibited by muscarinic ligands with the expected rank-order potency, but not by nicotinic ligands (Fig. 1*B*), and thus represents high-affinity binding to putative muscarinic receptors in the membranes. The validity of the assay conditions has been verified by comparing the binding of [^{3}H]AcCho in the centrifugation and filtration methods, by comparing the binding data under the standard assay conditions with a 1:5-diluted preparation, and by examining the effect of long-term incubations (2 hr). All these tests yielded results similar to those obtained under the standard conditions, thus eliminating substantial interference of parameters such as dissociation of bound ligand, ligand degradation, or the presence of endogenous AcCho.

The equilibrium binding isotherms of [^{3}H]AcCho (5–240 nM) reflect an interaction with apparently homogeneous, noninteracting binding sites, as indicated by the linear Scatchard plot. These high-affinity binding sites represent about 25% of the sites available for the labeled antagonist [^{3}H]-4NMPB in the same preparation. This value is in close agreement with the proportion of high-affinity agonist binding sites in cerebral cortex as determined by competition experiments of unlabeled agonists with [^{3}H]4NMPB (8). It should be noted that previous studies employing other labeled muscarinic agonists, such as [^{3}H]methyloxotremorine (2) and *cis*-3-methyldioxolane (13, 15), indicated the existence of "super-high affinity" sites. Under our assay conditions there was also a hint for the existence of such sites for AcCho. However, further studies at the subnanomolar AcCho range are required to characterize this phenomenon.

Low-affinity agonist binding sites, evaluated from competition experiments with ^{3}H-labeled antagonists, represent ≈75% of the available antagonist binding sites in cerebral cortex membranes. In spite of their high proportion, attempts to demonstrate them directly by using [^{3}H]AcCho (in our experiments) and other labeled agonists (2, 13, 15) have failed. Binding of [^{3}H]AcCho to the putative low-affinity sites would occur at the micromolar concentration range (2–5). Under the assay conditions employed, nonspecific binding of [^{3}H]AcCho at such concentrations would be >10 times higher than its specific binding to muscarinic receptors, thus precluding reliable measurements and consequently preventing determination of the exact stoichiometry of [^{3}H]AcCho/^{3}H-labeled antagonist binding sites. We may safely conclude, however, that at the nanomolar concentration range and under the standard assay conditions employed, one AcCho molecule binds with high affinity to the muscarinic receptor for every four antagonist molecules. This stoichiometry can be increased to one agonist molecule bound for two antagonist molecules in the presence of transition metal ions. Together with the lack of change in [^{3}H]4NMPB binding in the presence of 2 mM Ni^{2+}, these findings provide direct evidence that interconversion between low- and high-affinity states of the muscarinic receptor towards agonists (8) involves an actual increase in the number of high-affinity sites, with no change in the total number of antagonist sites.

This demonstration that low-affinity agonist sites can be converted to high-affinity (or back to low affinity by either guanine nucleotides or EDTA) suggests that the same muscarinic receptors can exist either in low- or high-affinity states. As for the remaining 50% of the receptors, which apparently remained in the low-affinity form, there are two possible explanations: (*i*) They could represent a separate class of receptors that are incapable of interconversion. (*ii*) Alternatively, these receptors may undergo interconversion, but under conditions different from those employed here. The latter possibility would imply the existence of an equilibrium between low- and high-affinity states of the muscarinic receptors, which can be altered under various experimental conditions.

Mechanisms leading to such reversible transitions could involve conformational changes induced directly by the modulators or indirect modifications through the activation of specific enzymes (21). Possible targets for these changes are the muscarinic binding sites, a guanyl-nucleotide binding protein (12, 21, 22), or the coupling between these two. Involvement of a guanyl-nucleotide binding protein in the interconversion process is suggested by the fast reversal of the Ni^{2+} effect by GTP. These findings are in accord with the proposal that the binding of GTP to its binding protein induces the dissociation of the latter from the muscarinic receptor, transferring the receptor to a low-affinity state towards agonists (12, 21, 22).

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