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

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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 ($K_a = 86 ± 21$ nM) but did not affect equilibrium binding of $[^3H]$labeled antagonists, indicating conversion of low- to high-affinity muscarinic agonist binding sites. The experiments demonstrated that $[^3H]$AcCho is highly sensitive to the presence of divalent metal ions (Ni$^{2+}$) in the assay. 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 (iPr$_2$F-P; 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 iPr$_2$F-P. 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; iPr$_2$F-P, diisopropyl fluorophosphate; p[NH]ppG, guanylylimidodiphosphate; 4NMPb, N-methyl-4-piperidyl benzilate.

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to tubes containing 20 μl of modified Krebs buffer, 200 μM iPr2P-F, and the indicated concentrations of [3H]ACh. 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 non-specific binding—i.e., binding to membranes after adding 20 μM atropine during the last 10 min of the preincubation step. The same values for non-specific 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.

[3H]4NMPB Binding. Aliquots (20 μl) of the membrane preparation were incubated for 60 min at 25°C with a saturating concentration (20 nM) of [3H]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. Non-specific 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 (Bmax) and affinity (Kd).

**RESULTS**

**Assay of [3H]ACh Binding to Muscarinic Receptors.** Muscarinic ACh receptors in rat cortex membranes have been assayed previously by using stable muscarinic antagonists and agonists (ref. 1 for review). Unlike these ligands, [3H]ACh is susceptible to hydrolysis by the enzyme acetylcholinesterase (AChEase) and may be isotopically diluted by endogenous ACh. In order to avoid these difficulties, the membranes were prepared by hypotonic washing (see Materials and Methods). To prevent degradation of [3H]ACh, AChEase inhibitor was added to the washed membranes prior to the addition of [3H]ACh. Since these inhibitors may interact with the muscarinic receptor (13), we examined the effects of several inhibitors on the binding of [3H]ACh. Binding of [3H]ACh to the membrane preparation was critically dependent on both the concentration and the nature of the inhibitor (Fig. 1A). The inhibitor selected was iPr2P-F, since [3H]ACh binding was unchanged at a wide iPr2P-F concentration range of 100 μM to 2 mM, which completely inhibits AChEase (20). Phystostigmine and neostigmine are less suitable, since they inhibit [3H]ACh binding at concentrations needed to inhibit AChEase. This is shown by the narrow range of maximal [3H]ACh binding, as observed by the reduced binding compared to that observed in the presence of iPr2P-F (Fig. 1A). All subsequent binding experiments were performed in the presence of 200 μM iPr2P-F, which did not affect binding of the antagonist [3H]4NMPB.

Binding of [3H]ACh to cortical membranes could be detected by using either the centrifugation or the rapid filtration technique. In each case the bound ligand represented 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 [3H]ACh, 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 [3H]ACh were 10,450 ± 1255 cpm (total) and 7928 ± 1108 cpm (nonspecific). Filtration assay of the same membrane preparation with 42 nM [3H]ACh 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 [3H]ACh 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 [3H]ACh dissociation from its receptor at 0°C (t1/2 ≈ 90 sec).

The above results thus establish a simple assay for the binding of [3H]ACh 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 [3H]ACh Binding.** To characterize the nature of [3H]ACh 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 [3H]AcCho are muscarinic cholinergic receptors, since muscarinic ligands proved to be potent inhibitors of [3H]AcCho binding (Fig. 1B). 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 [3H]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 [3H]AcCho (at 100 μM). In addition, muscarinic drugs at sufficiently high concentrations can completely inhibit the binding of [3H]AcCho (Fig. 1B), in the order of potency expected for these drugs (2-4). Binding of [3H]AcCho (5-240 nM) in the absence and in the presence of 10 μM atropine is shown in Fig. 2A. As expected, the nonspecific binding shows linear dependence on [3H]AcCho concentration. Subtracting this binding from the total binding yielded a simple hyperbolic curve (Fig. 2B). Data replotted according to Scatchard yielded a straight line (Fig. 3). Mean binding parameters (five experiments) were: K_a = 76 ± 17 nM; B_max = 361 ± 29 fmol/mg of protein. In the same preparations binding capacity of the muscarinic antagonist [3H]4NMPB was 1430 ± 125 fmol/mg of protein. Thus, the ratio of high-affinity [3H]AcCho binding sites to muscarinic antagonist binding sites is 1:4.

Under the conditions employed, the concentration of [3H]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 [3H]AcCho due to (i) depletion of the free ligand and (ii) presence of endogenous unwashed AcCho. We therefore performed equilibrium binding studies with [3H]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 [3H]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 [3H]AcCho binding parameters at the concentration range indicated.

Kinetic experiments were carried out to further characterize the mode of interaction of [3H]AcCho with the muscarinic receptor. Binding of [3H]AcCho to muscarinic receptors is a rapid process (Fig. 4); the t_1/2 for the association of 36 nM [3H]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 binding.
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 $[^3H]$AcCho Binding. Inhibition by agonists of $[^3H]$-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 $[^3H]$AcCho.

The effect of Ni$^{2+}$ on $[^3H]$AcCho binding was examined with membranes incubated for 60 min at 25°C with 42 nM $[^3H]$AcCho and various concentrations of the metal ions. A concentration-dependent increase in the specific binding of $[^3H]$AcCho was observed (Fig. 5), with half-maximal increase occurring at $\approx$70 μM Ni$^{2+}$. No further increase in $[^3H]$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 Co$^{2+}$ or Mn$^{2+}$, which increased $[^3H]$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 $[^3H]$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 $[^3H]$AcCho is observed at all ligand concentrations studied. Repploting the data according to Scatchard yields two parallel lines (Fig. 3), indicating an increased capacity for $[^3H]$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 $[^3H]$4NMPB in the same preparations was not affected by 2 mM Ni$^{2+}$. Thus, the ratio of the number of $[^3H]$AcCho binding sites to the number of antagonist binding sites is increased in the presence of the metal ions from $\approx$0.25 to $\approx$0.45.

The increase in $[^3H]$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 $[^3H]$AcCho binding induced by the transition metal ions. The highest potency was observed with p[NH]ppG ($K_{ppG} = 2 \mu$M; Fig. 5). It is noteworthy that small decreases ($\approx$15%) in $[^3H]$AcCho binding could also be observed in the absence of transition metal ions. Inhibition of the Ni$^{2+}$-induced increase in $[^3H]$AcCho binding was restricted to the above-mentioned nucleotides; the following nucleotides were inactive at 200 μM: GMP, cGMP, cAMP, AMP, ADP, ATP, adenyllyl 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 $[^3H]$AcCho and 2 mM Ni$^{2+}$ for 30 min at 25°C. The basal $[^3H]$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 $[^3H]$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 $[^3H]$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. 1B), 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 \(^{[3]H}\)methylxoctremine (2) and cis-3-methylxidioxolane (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 \(\approx 75\%\) of the available antagonist binding sites in cerebral cortex membranes. In spite of their high proportion, attempts to demonstrate them directly by using \(^{[3]}\)HAcCho (in our experiments) and other labeled agonists (2, 13, 15) have failed. Binding of \(^{[3]}\)HAcCho to the putative low-affinity sites would occur at the micromolar concentration range (2–5). Under the assay conditions employed, nonspecific binding of \(^{[3]}\)HAcCho 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 \(^{[3]}\)HAcCho/\(^{[3]}\)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 \(^{[3]}\)H4NMPB 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).