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High Affinity Binding of [^{3}H]Acetylcholine to Muscarinic Receptors

Regional Distribution and Modulation by Guanine Nucleotides

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SUMMARY

The interaction of [^{3}H]acetylcholine ([^{3}H]AcCh) with the muscarinic receptor was studied in seven distinct rat brain regions and in heart atrium by employing 10 μM atropine to define specific binding. The specific binding exhibited by the labeled neurotransmitter was found to be sensitive to muscarinic but not to nicotinic drugs. The muscarinic high affinity agonist-binding sites were characterized with respect to their binding properties, regional distribution, pharmacology, and modulation by guanyl nucleotides and by transition metal ions. In all tissues examined, specific binding of [^{3}H]AcCh was saturable over the range of 4–200 nM and occurred in a receptor population that was apparently homogeneous and had a dissociation constant of approximately 19–39 nM in most of the regions. The ratio of muscarinic receptors labeled by [^{3}H]AcCh to those labeled by the potent antagonist [^{3}H]*N-methyl*-4-piperidylbenzilate varied markedly among tissues, from 0.15 in the hippocampus to 0.71 in the atrium. This ratio was lower in brain regions rich in muscarinic receptors, where smaller sensitivity of [^{3}H]AcCh binding to guanyl nucleotides was also observed. In the presence of the latter [^{3}H]AcCh binding was decreased by 25 to 90% in different tissues, with the greatest decreases occurring in the atrium and brainstem. In the latter preparations, transition metal ions do not affect [^{3}H]AcCh binding, while in the other preparations studied they induce an increase in the binding capacity for the labeled neurotransmitter, which is sensitive to guanine nucleotides.

INTRODUCTION

Muscarinic receptors in various brain regions and peripheral tissues show up as an apparently homogeneous population of sites when examined with ^{3}H-labeled antagonists such as benzilate or tropate esters (Ref. 1 and references therein). In contrast, the interaction of agonists with these receptors involves multiple binding sites with differing affinities for the agonists (2–6). Two or three different populations of agonist-receptor complexes have been detected in studies employing [^{3}H]antagonists/agonist competition experiments, and in direct binding studies with the agonists [^{3}H]oxotremorine-M (2, 3, 7–9) and [^{3}H]*cis*-methyldioxolane (10, 11), These were termed superhigh, high, and low affinity agonist-binding sites (2).

We have recently introduced a sensitive and specific procedure to assay the binding of [^{3}H]acetylcholine to muscarinic receptors (12–14). In preliminary reports on the use of a similar procedure (15–17), results were in agreement with some of our own earlier findings (12–14). Like labeled OXO-M[1] and CD, [^{3}H]AcCh binds with high affinity to a small fraction ($\leq$25%) of antagonist binding sites in rat cerebral cortex membranes, but unlike these agonists, the labeled neurotransmitter recognizes a homogeneous population of sites (13).

In the present communication we describe (i) the technical considerations related to the success of the binding assay, (ii) the characterization and regional distribution of [^{3}H]AcCh binding to muscarinic receptors in rat heart and various brain regions, (iii) modulation of [^{3}H]AcCh binding by a guanyl nucleotide derivative and by transition metal ions.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]AcCh of high specific radioactivity (86 Ci/mmol, 98% purity) was purchased from Amersham Corp. Small aliquots of the radiochemical in ethanol/water (1:1, v/v) were kept at −70° and subjected to drying by a gentle stream of nitrogen prior to use. [^{3}H]4-NMPB (70 Ci/mmol, 97% purity) was prepared by catalytic tritium exchange as described elsewhere (18). Gpp(NH)p and other nucleotides were purchased from Sigma. Most of the drugs tested were purchased from Sigma, except (−)-QNB and (+)-QNB, which were prepared as described elsewhere (19), and pirenzepine which was generously donated by Dr. R. Hammer, Institute de Angeli, Milan, Italy.

Tissue preparation. Adult male rats of the CD strain were obtained from Levinstein's Farm (Yokeneam, Israel) and maintained in an air

[1] The abbreviations used are: OXO-M, oxotremorine-M; AcCh, acetylcholine; DFP, diisopropyl fluorophosphate; 4-NMPB, *N*-methyl-4-piperidylbenzilate; QNB, 3-quinuclidinyl benzilate; OXO, oxotremorine; CD, *cis*-methyldioxolane; Gpp(NH)p, guanylylimidodiphosphate.

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conditioned room at 24 ± 2° for 14 hr (0500–1900) under fluorescent illumination and in darkness for 10 hr. Food from Assia Maabarot Ltd. (Tel Aviv, Israel) and water were supplied *ad libitum*. Rats aged 3–4 months and weighing 190–250 g were decapitated (between 0900 and 1000 hr), and their brains and hearts were rapidly removed. Specific brain areas were immediately dissected out in a cold room after identification with the aid of a stereotaxic atlas and immersed in cold 50 mM Tris-HCl buffer, pH 7.4 (buffer A). For each experiment, tissues were pooled from 2–3 rats (olfactory bulb, cerebral cortex, hippocampus, brainstem, and cerebellum) or 6–8 rats (striatum, hypothalamus, and cardiac atrium). Brain tissues were homogenized in 50 volumes of buffer A, using a glass-Teflon homogenizer (10 strokes). The homogenates were incubated for 30 min at 25° in buffer A with gentle shaking and then centrifuged at 30,000 × *g* for 15 min. This procedure was repeated twice. Atrial tissue was washed of blood in buffer A, cut up finely, and homogenized at setting 7 on an Ultra-Turrax (Ika-Werk Instruments) with three 15-sec bursts separated by 30-sec pauses and then filtered through three layers of cheesecloth and subjected to the above procedure. The final pellet was resuspended in modified Krebs buffer containing 25 mM Tris-HCl, 118 mM NaCl, 5 mM KCl, 10 mM glucose, 2 mM $MgCl_2$, and 1.9 mM $CaCl_2$ (pH 7.4, 25°). A fresh solution of DFP (Sigma lot 93F-0101) in water was added to the homogenate to achieve a concentration of 200 μM. The homogenate was incubated for a further 20 min at 25° prior to binding assay. In some assays neostigmine or physostigmine was used instead of DFP. Protein concentration was determined according to the Lowry method (20), using bovine serum albumin as a standard.

[^{3}H]AcCh binding assay. Aliquots (20 μl) of freshly prepared membranes (equivalent to 3–5 mg of original tissue weight) were added to tubes containing 20 μl of modified Krebs buffer, 200 μM DFP, and the indicated concentrations of [^{3}H]AcCh. After 1 hr of incubation with gentle shaking at 25°, 3 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 3 ml of buffer within less than 3 sec. Filters were counted for tritium using a scintillation cocktail (Hydroluma) and a scintillation spectrometer (LKB-1218) at 48% efficiency. The above conditions were chosen in order to ensure that equilibrium has been achieved and to eliminate the loss of bound [^{3}H]AcCh during filtration. At 25°, $t_{1/2}$ for association of 40 nM [^{3}H]AcCh was 10–20 sec in all regions, and the half-time dissociation was 10–40 sec. Nevertheless, equilibrium was reached only after 20–30 min at 25°, possibly due to the onset of a slower phase of binding after the rapid early phase (13). Thus, an incubation period of 1 hr was chosen, since after this period even the lowest concentration of [^{3}H]AcCh employed (4 nM) has reached equilibrium. Ice-cold buffer was used for washes to prevent dissociation of [^{3}H]AcCh from the receptors, since at 0° the dissociation half-time was relatively slow (90–250 sec).

Specific binding was determined by calculating the differences between the total binding and the nonspecific binding, the latter in the presence of 10 μM atropine. The same values for nonspecific binding were obtained when 10 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° for 10 min.

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 washing of the membrane pellet three times with 1 ml of ice-cold buffer and determination of radioactivity in the pellets.

All determinations were carried out in quadruplicate and varied by no more than 15%.

[^{3}H]4-NMPB binding. Aliquots (20 μl) of the membrane preparation were incubated for 60 min at 25° with a saturating concentration (25 nM) of [^{3}H]4-NMPB 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. These assays were routinely carried out in parallel with [^{3}H]AcCh binding assays in the same preparations.

Data analysis. Results of binding experiments are presented as mean values or means ± 1 SD. Values for maximal binding capacity (B_{max}) and dissociation constants (K_d) were obtained by linear regression analysis of binding isotherms. The competition curves were analyzed by a nonlinear least square curve-fitting procedure using a model for either one or two binding sites (4). Theoretical competition curves were fitted to the experimental data points using the nonlinear least squares regression computer program BMDPAR (November 1978 revision), developed at the Health Science Computing Facility (University of California, Los Angeles, CA).

RESULTS

Considerations related to the binding assay. The binding at equilibrium of [^{3}H]AcCh at 25° was studied in rat tissue homogenates obtained from the following regions: cerebral cortex, hippocampus, striatum, hypothalamus, brainstem, cerebellum, olfactory bulb, and heart atrium. In order to avoid the isotopic dilution of [^{3}H]AcCh by endogenous acetylcholine, the membranes were subjected to hypotonic washing prior to binding assay (see Experimental Procedures). Moreover, since [^{3}H]AcCh is susceptible to acetylcholinesterase, an inhibitor of the enzyme was added prior to the addition of [^{3}H]AcCh. We examined the effects of a few inhibitors on the binding of [^{3}H]AcCh to homogenates of atrium, brainstem, and cerebral cortex. Binding of the tritiated agonist was critically dependent on both the concentration and the nature of the inhibitor. In the absence of cholinesterase inhibitor, specific binding of [^{3}H]AcCh could not be detected. Of the three cholinesterase inhibitors tested, namely DFP, physostigmine, and neostigmine, the first seems to be the most suitable, since it did not induce any loss of [^{3}H]AcCh-binding sites in homogenates prepared from atrium, brainstem, and cerebral cortex over a wide concentration range (0.1–1.0 mM). On the other hand, the application of physostigmine and neostigmine at concentrations effective in inhibiting acetylcholinesterase (10–50 μM) resulted in reduced binding of tritiated agonist. For example, specific binding at 34 nM [^{3}H]AcCh obtained in the presence of 200 μM DFP in homogenates of atrium, brainstem, and cerebral cortex were 140, 110, and 296 fmol/mg protein, respectively, as compared to the corresponding values of 121, 101, and 253 fmol/mg protein in the presence of 10 μM neostigmine. All subsequent binding experiments were, therefore, performed in the presence of 200 μM DFP. The inhibitor did not affect the binding of the antagonist [^{3}H]4-NMPB in any of the tissues under investigation.

Since [^{3}H]AcCh can also bind to nicotinic receptor sites (21), a series of binding experiments was performed in the presence and in the absence of 10 μM nicotine or cytisin, potent nicotinic agonists. The specific binding of [^{3}H]AcCh to homogenates of the various regions was not affected by the nicotinic ligands but was completely blocked by atropine, oxotremorine, or arecoline (10 μM) and, therefore, represents binding to muscarinic receptors. Thus, under our experimental conditions, essentially all the [^{3}H]AcCh sites measured were muscarinic, and there was no requirement for the addition of unlabeled nicotinic ligands (16). This is most likely due to

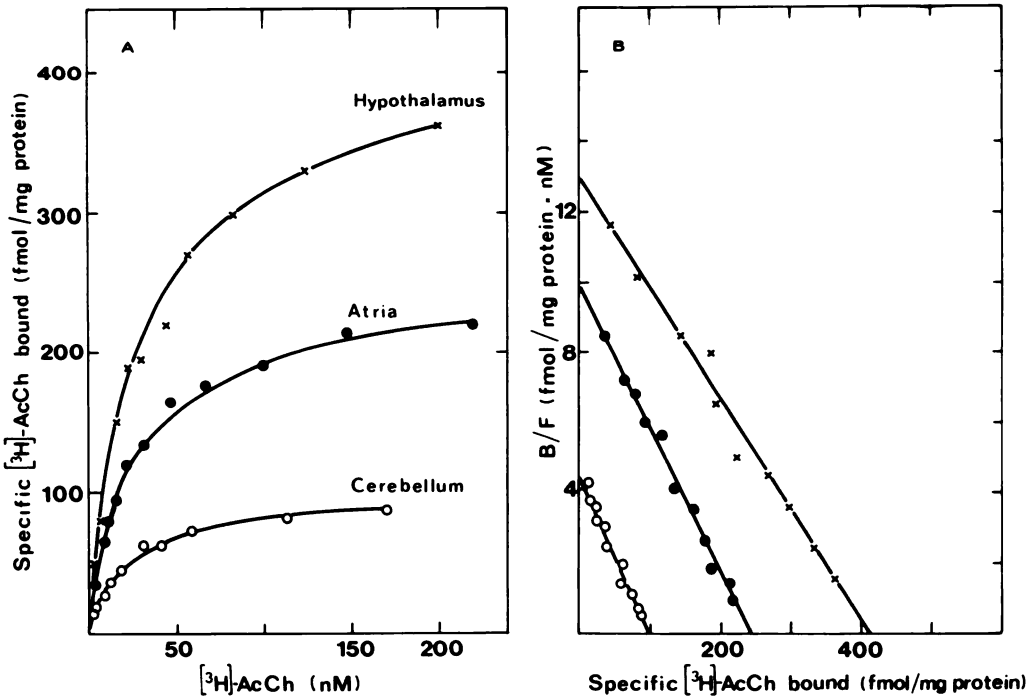

FIG. 1. *A. Specific binding at equilibrium of [³H]AcCh to membranes prepared from rat cerebellum, hypothalamus, and atrium (A) and Scatchard plots of the same data (B)*

Freshly prepared tissue was pooled from 4 (cerebellum and atrium) or 8 (hypothalamus) rats. Membranes were prepared and assayed for specific [³H]AcCh binding to muscarinic receptors as detailed in the text. Data are shown for a single experiment for each tissue, each carried out in quadruplicate with variations of <15%. Linear regression analysis yielded the following parameters: B_{max} = 98, 245, and 415 fmol/mg protein and K_d = 22, 25, and 32 nM for the cerebellum, atrium, and hypothalamus preparations, respectively. Specific binding of 25 nM [³H]4-NMPB in the same preparations was 193, 336, and 1298 fmol/mg of protein for the cerebellum, atrium, and hypothalamus, respectively.

the much higher concentration of muscarinic relative to nicotinic receptors in the tissues under study (21) and due to the definition of all the [³H]AcCh binding observed in the presence of atropine (which includes nicotinic sites) as nonspecific.

Binding of [³H]AcCh to membranes prepared from brainstem, atrium, hippocampus, and cerebral cortex could be detected by using either the centrifugation or the rapid filtration technique. In each case the bound ligand comprised two components, one that could be inhibited by 10 μM atropine or oxotremorine (specific binding) and one that could not be inhibited by these muscarinic ligands (nonspecific binding). Both techniques yielded similar values for specific binding of [³H] AcCh, indicating that there was no significant loss of specifically bound ligand during the filtration process. In binding experiments performed by the filtration technique the filters were always washed with ice-cold buffer; filtration was terminated within 2–3 sec. Nonspecific binding, however, was much higher when measured by the centrifugation technique than by filtration. Consequently, all further experiments were carried out by the filtration method in order to obtain more accurate determinations of the specific binding. Yet, relatively high nonspecific binding was observed in several regions even when filtration techniques were used; for example, in brainstem and cerebellum preparations, specific binding of 40 nM [³H]AcCh accounted for about 50% of the total binding, while in the atrium and cortex it accounted for 75 and 65% of the total binding, respectively.

The concentration dependence of specific binding of [³H]AcCh at equilibrium is shown in Fig. 1 for three tissue preparations. As reported previously for the binding of this ligand to rat cerebral cortex (12–14), the specific binding for the tissues shown in Fig. 1(as well as in the other tissues studied) is saturable in the ligand concentration range of 4–200 nM. The specific binding of [³H]AcCh was found to be a linear function of protein concentration (0.05–0.5 mg/assay) in each of the eight regions studied. Tissue concentrations of 0.08–0.2 mg of protein/assay were, therefore, used for all experiments.

Binding in all regions reached equilibrium within about 20–30 min and remained unchanged even after 2 hr of incubation at 25°; binding studies at equilibrium were, therefore, conducted for 60 min at 25°.

Characterization and regional distribution of [³H]AcCh binding. Specific binding of [³H]AcCh in the eight regions studied was saturable over a concentration range of 4–200 nM. Representative binding isotherms are depicted in Fig. 1A. In all tissues, Scatchard plots of the binding data were linear (Fig. 1B) and Hill coefficients of the saturation isotherms were close to unity, indicating that over this concentration range the labeled acetylcholine binds to a single class of sites with no evidence of cooperativity. Binding characteristics obtained from these equilibrium studies are given in Table 1. The mean dissociation constants for [³H]AcCh are 19–39 nM in the brainstem, cerebellum, atrium, hypothalamus, cerebral cortex, and olfactory bulb, and somewhat higher (55–73 nM) in the striatum and hippocampus. The density of

TABLE 1

Specific binding at equilibrium of [^{3}H]AcCh and [^{3}H]4-NMPB to muscarinic receptors in various rat brain regions and in cardiac atrium

Mean and SD values are shown for the indicated number of experiments for each preparation. [^{3}H]AcCh binding was determined as described in the text, using at least 11 concentrations in each curve (4–200 nM). The B_{max} and K_d, values were calculated from Scatchard analysis. Specific binding of the antagonist [^{3}H]4-NMPB was determined in incubations carried out at the same conditions (except that the reaction volume was increased to 1 ml) with 25 nM ligand, which saturates 98–99% of muscarinic receptors in all tissues studied. Protein was determined by the Lowry method using bovine serum albumin as a standard.

Tissue	No. of experiments	Specific binding of 25 nM [^{3}H]4-NMPB	Specific binding of[^{3}H]AcCh		[^{3}H]AcCh / [^{3}H]4-NMPB
			B_{max}	K_d	
		fmol/mg protein	*fmol/mg protein*	*nM*	
Olfactory bulb	4	2860 ± 590	750 ± 185	32 ± 8	0.26 ± 0.04
Cerebral cortex	6	3020 ± 570	626 ± 138	34 ± 13	0.22 ± 0.03
Hippocampus	4	2845 ± 570	421 ± 88	73 ± 21	0.15 ± 0.02
Striatum	4	3244 ± 67	529 ± 127	55 ± 20	0.16 ± 0.04
Hypothalamus	3	1238 ± 290	402 ± 95	39 ± 14	0.33 ± 0.01
Brain stem	10	398 ± 112	176 ± 42	26 ± 8	0.48 ± 0.07
Cerebellum	4	196 ± 46	100 ± 29	19 ± 4	0.51 ± 0.06
Atrium	6	323 ± 90	209 ± 58	23 ± 7	0.71 ± 0.11

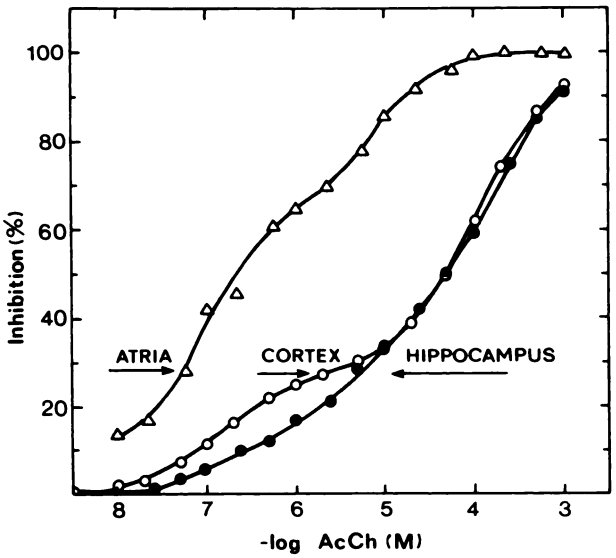

FIG. 2. *Inhibition by AcCh of specific [^{3}H]4-NMPB binding to membranes prepared from rat cortex (○), hippocampus (●), and atrium (△)*

Membranes were prepared and assayed as detailed in Experimental Procedures. Data are shown for a single experiment for each tissue, each carried out in quadruplicate with variations of <15%. The [^{3}H]4-NMPB concentrations were 2.0, 1.8, and 2.1 nM in the cortex, hipocampus, and atrium, respectively. The dissociation constants for [^{3}H]4-NMPB in these tissues are, respectively: 0.4, 0.4, and 1.0 nM. Data were analyzed according to a two-sites model by the use of a nonlinear least squares regression computer program and the indicated concentrations and K_d values for [^{3}H]4-NMPB.

[^{3}H]AcCh sites was 15–26% of that of [^{3}H]-labeled antagonist sites in the cerebral cortex, hippocampus, striatum, and olfactory bulb, 33% in the hypothalamus, and 45–71% in the brainstem, cerebellum, and atrium. It should be noted that decreased B_{max} values for [^{3}H]AcCh were obtained when frozen tissues (kept at −70°) were used (about 75–85% of control values obtained with fresh preparations). Our results, therefore, relate only to experiments carried out with freshly dissected tissues.

The data obtained in direct binding studies with [^{3}H]AcCh were compared with competition experiments using [^{3}H]4-NMPB and unlabeled AcCh. The results of such experiments performed on homogenates of atria, cortex, and hippocampus are depicted in Fig. 2. Analysis of the curves according to a two-sites model for agonist binding (4) yielded K_d values of 23, 24, and 136 nM for the high-affinity sites of atria, cortex, and hippocampus, respectively. The K_d values for AcCh binding to the low-affinity sites in these regions were 2.1, 19, and 33 μM, and the fractions of high-affinity sites were 0.65, 0.28, and 0.23 for atria, cortex, and hippocampus. It, therefore, appears that the binding characteristics observed in the direct binding studies with [^{3}H]AcCh represent the binding of AcCh to the sites defined as high affinity sites in the competition experiments.

The characteristics of [^{3}H]AcCh-binding sites were further determined by measuring the inhibition of binding by various drugs. Representative competition curves for the atrium are given in Fig. 3. Muscarinic drugs were potent inhibitors of specific [^{3}H]AcCh binding, whereas neither nicotinic drugs (nicotine, cytisin, succinylcholine, *d*-tubocurarine, and α-bungarotoxin) nor noncholinergic drugs (norepinephrine, dopamine, serotonin, morphine, and glycine) could inhibit the binding of [^{3}H]AcCh at a concentration of 10 μM. Muscarinic antagonists were more potent than agonists as inhibitors. Table 2 shows the following order of potency, which was similar for all regions under study: (−)-QNB ≥ *N*-methylscopolamine > atropine > oxotremorine > oxotremorine-M > AcCh ≥ (+)-QNB > carbamylcholine > arecoline > pilocarpine. The K_d values obtained in three of the regions studied (cerebral cortex, brainstem, and atria) are summarized in Table 2. Note the high degree of stereospecificity of the sites. (−)-QNB, for example, is more potent than (+)-QNB; the apparent inhibition constants in competition binding experiments with [^{3}H]AcCh are 1.8 and 2.0 nM with (−)-QNB and 37 and 54 nM with (+)-QNB for brainstem and atrial preparations, respectively. Thus, the affinity ratio of the enantiomers, as measured by the ratio of their K_d values, holds both for

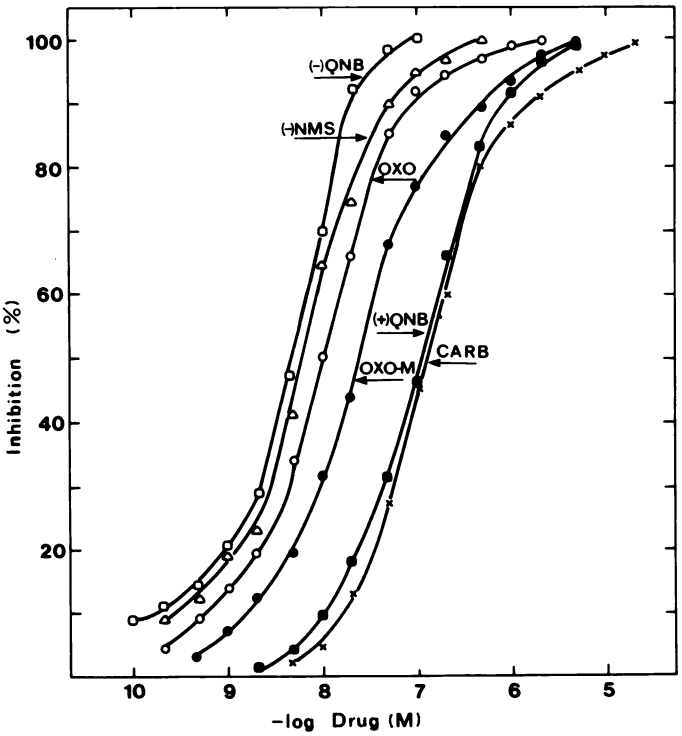

FIG. 3. *Inhibition of specific [³H]AcCh binding to rat atrium membranes by muscarinic ligands*

Membranes (0.08 mg of protein) were incubated for 60 min at 25° with 24 nM [³H]AcCh and the indicated concentrations of the drugs. Results shown are means of 2–3 experiments with each drug; for clarity, standard deviations (which were <10%) are not shown. *NMS*, *N*-methylscopolamine; *CARB*, carbamylcholine.

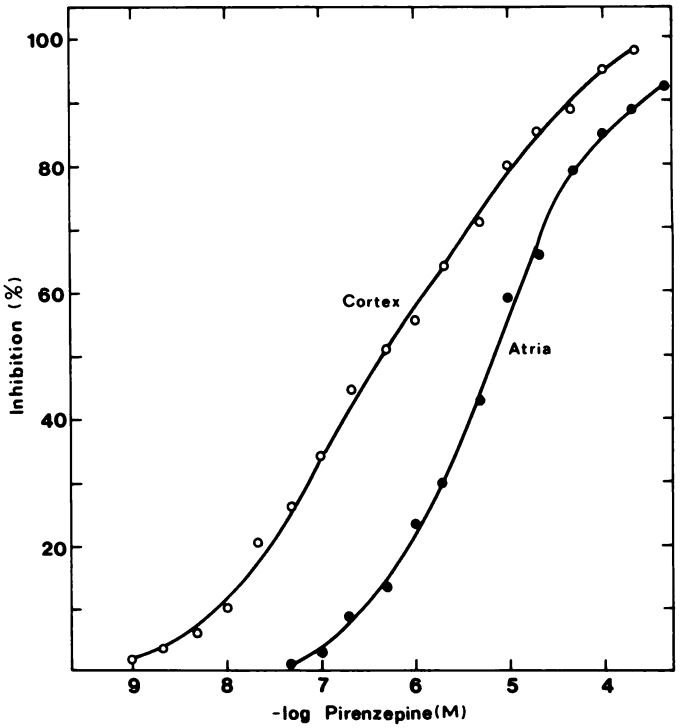

FIG. 4. *Inhibition by pirenzepine of specific [³H]AcCh binding to rat cerebral cortex and atrium membranes*

See legend to Fig. 3 and text for details.

TABLE 2

K_d of muscarinic ligands as determined by competition with [³H]AcCh

The experiments were performed under the conditions described in Fig. 3. The results are mean ± SD of 3 separate experiments, each performed in triplicate. The binding curves yielded linear Scatchard plots (Hill slopes of 0.85–1.1) and were thus analyzed according to binding to a single site.

	K_d		
	Cerebral cortex	Brainstem	Atria
	nM	*nM*	*nM*
(−)-QNB	3.8 ± 0.8	1.8 ± 0.4	2.0 ± 0.6
(−)-NMS[a]	2.6 ± 0.7	1.7 ± 0.7	3.7 ± 0.7
Atropine	3.5 ± 0.9	2.1 ± 0.5	1.9 ± 0.3
(+)-QNB	36.4 ± 9.1	37.7 ± 6.3	54.5 ± 7.2
Oxotremorine	15.6 ± 6.7	12.0 ± 4.0	7.5 ± 0.4
OXO-M	28.0 ± 7.0	15.0 ± 3.0	26.0 ± 4.0
AcCh	41.0 ± 6.0	29.0 ± 5.0	27.0 ± 4.0
Carbamylcholine	132.0 ± 25.0	122.0 ± 30.0	83.0 ± 17.0
Arecoline	155.0 ± 32.0	150.0 ± 40.0	100.0 ± 15.0
Pilocarpine	590.0 ± 70.0	610.0 ± 20.0	450.0 ± 60.0

[a] *N*-Methylscopolamine.

the binding experiments and for the bioassay in the intact animal (19).

Competition binding experiments between [³H]AcCh and the nonclassical muscarinic antagonist pirenzepine were conducted using heart atrium and cerebral cortex membranes (Fig. 4). This antagonist was postulated to have different affinities toward two subclasses (M_1, with high affinity toward pirenzepine, and M_2, which exhibits low affinity to this drug) of the muscarinic receptors (5, 22–27). Analysis of the data using a computerized non-linear least-square regression program (see Experimental Procedures) indicated that the competition data in atrial preparations are best fitted by a one-site model (K_d = 3.7 μM) while those from the cerebral cortex are best fitted by a two-site model in which the K_d value is 31 nM for 48% of the sites and 3.9 μM for 52% of the sites. Thus, labeling the binding sites with [³H]AcCh demonstrates that pirenzepine binding sites in the atrium appear to have characteristics of low affinity sites, while in the cortex high and low affinity sites can be detected.

Modulation of [³H]AcCh binding. Binding of agonists to muscarinic receptors was previously shown to be modulated by guanine nucleotides (28–35) and transition metal ions (7, 13, 36, 37). We, therefore, examined the effects of these modulators on the high affinity [³H]AcCh binding in the various tissues under study. Incubation with GTP or its stable analog Gpp(NH)p decreased the binding for [³H]AcCh in all tissues examined. This is shown, for example, for membrane preparations from cerebral cortex, brainstem, and atrium (Fig. 5). It was found that 50% inhibition of displaceable [³H]AcCh binding (at 40 nM) occurs at 1–6 μM Gpp(NH)p in brain preparations and at 0.3 μM Gpp(NH)p in the atrial preparation. The maximal decrease in [³H]AcCh binding was obtained at 1 mM Gpp(NH)p; no further decrease occurred at higher Gpp(NH)p concentrations. As shown in Fig. 6, Gpp(NH)p did not affect the affinity of [³H]AcCh but rather brought about a reduction in its binding. Clear differences between the tissues were found in the proportion of sites displaying sensitivity to Gpp(NH)p.

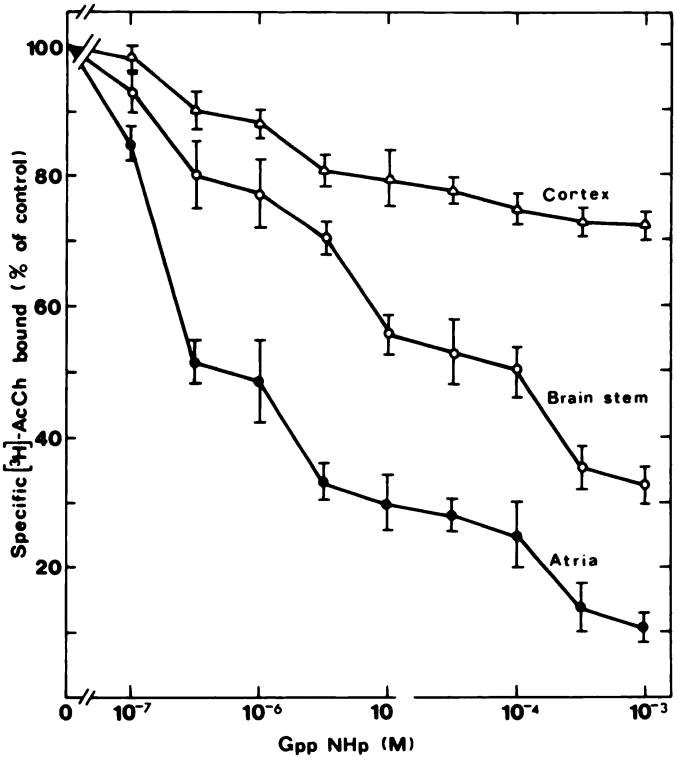

FIG. 5. *Inhibition of specific [^{3}H]AcCh binding by Gpp(NH)p*

Membranes prepared from rat cerebral cortex, brainstem, and atrium were assayed for specific binding of [^{3}H]AcCh (40 nM) in the presence of the indicated concentrations of Gpp(NH)p. The data shown are means ± SD of two representative experiments for each tissue. Specific binding in control preparation (in the absence of nucleotide) was 342 ± 46, 98 ± 15, and 138 ± 30 fmol/mg protein for the cerebral cortex, brainstem, and atrial preparations, respectively. Nonspecific binding (determined with 10 μM atropine) was not affected by 1 mM Gpp(NH)p (not shown).

The maximal Gpp(NH)p-induced decrease in [^{3}H]AcCh binding was 32, 67, and 90% for cerebral cortex, brainstem, and atria (Fig. 6). In other tissues (hippocampus, striatum, and cerebellum) the maximal decrease was 56, 50, and 73%, respectively. Note that in all regions except atrium a certain fraction of high affinity [^{3}H]AcCh sites remains insensitive to guanyl nucleotides even at 1 mM.

As shown in Fig. 6, Ni^{2+} (as well as other transition metal ions such as Co^{2+} and Mn^{2+}, not shown), at a concentration of 2 mM, doubled the capacity for [^{3}H]AcCh binding to hippocampus and striatum preparations, without substantially affecting its affinity. We have previously reported a similar finding for cerebral cortex (13). A similar increase in B_{max} for [^{3}H]AcCh was detected in the olfactory bulb preparaton (not shown). However, these transition metal ions increased the B_{max} for [^{3}H]AcCh only minimally (10–15%) in the hypothalamus, brainstem, and cerebellum, and not at all in the atrium. Equilibrium binding of the antagonist [^{3}H]4-NMPB was not affected by the presence of 2 mM Ni^{2+} in any of the preparations investigated.

The induced increase in [^{3}H]AcCh-binding capacity was completely inhibited in the presence of Gpp(NH)p (not shown), as observed previously for the cerebral cortex (13). The potency of Gpp(NH)p for inhibiting the Ni^{2+}-induced increase in specific [^{3}H]AcCh binding was about equal to its potency in reducing [^{3}H]AcCh binding in the absence of Ni^{2+}, *i.e.*, 50% inhibition at ~2 μM. The increase in [^{3}H]AcCh binding induced by the transition metal ions was readily reversible, *i.e.*, it was diminished within 90–120 sec following the addition of 200 μM Gpp(NH)p (or 10 mM EDTA) to membranes preincubated for 60 min with [^{3}H]AcCh and 2 mM Ni^{2+} (not shown).

DISCUSSION

High affinity binding of [^{3}H]AcCh to muscarinic receptors. The binding of [^{3}H]AcCh to various tissue homogenates shows the typical characteristics of specific interaction with high affinity between the ligand and muscarinic receptors. In all preparations examined, [^{3}H]AcCh binding is saturable over the concentration range of 4–200 nM; the binding is reversible, and the labeled ligand can be displaced by muscarinic agonists and antagonists with the expected rank order of potency and stereospecificity; nicotinic or noncholinergic drugs have no effect. Binding studies carried out under the experimental conditions employed in this work indicate that the interaction of the labeled ligand is with an apparently homogeneous population of high affinity binding sites within each preparation.

It should be noted that the B_{max} values obtained for direct binding of [^{3}H]AcCh are significantly lower than those observed for antagonist binding to the same preparations (Table 1). In fact, the B_{max} values for the direct binding of the tritiated agonist are similar to the concentration of high affinity agonist binding sites observed in competition experiments between unlabeled agonist and labeled antagonist (1–5) (Table 3). Comparison between the B_{max} values and the dissociation constants for direct [^{3}H]AcCh binding and competition experiments employing [^{3}H]4-NMPB (described under Results) clearly indicates that the [^{3}H]AcCh sites measured in the direct binding experiments represent the population of high affinity agonist binding sites detected in the competition experiments. The distribution of [^{3}H]AcCh binding sites in the various tissues resembles that of the total muscarinic sites measured by [^{3}H]4-NMPB binding (Table 1, Refs. 4 and 18). However, the fraction of muscarinic receptors to which [^{3}H]AcCh binding is measured (B_{max} of [^{3}H]AcCh/B_{max} of [^{3}H]4-NMPB) is not constant and reaches higher values at the tissues with *lower* muscarinic receptor density (Table 1).

Mode of interaction of agonists and antagonists with the muscarinic receptors. Comparison of our data on [^{3}H]AcCh binding to muscarinic receptors with the binding of synthetic labeled muscarinic agonists reveals both similar and different features. The regional distribution of agonist binding sites is clearly similar. Thus, tissues or brain regions rich in [^{3}H]AcCh binding sites (Table 1) also display a high density of [^{3}H]OXO-M (3, 8, 32) and of [^{3}H]CD (10, 11) sites. However, the latter two drugs differ from the labeled neurotransmitter, [^{3}H]AcCh, in two features. (a) In most tissues, curvilinear Scatchard plots are obtained for [^{3}H]OXO-M and [^{3}H]CD binding in the nanomolar range (3, 8, 10, 11, 28, 32),

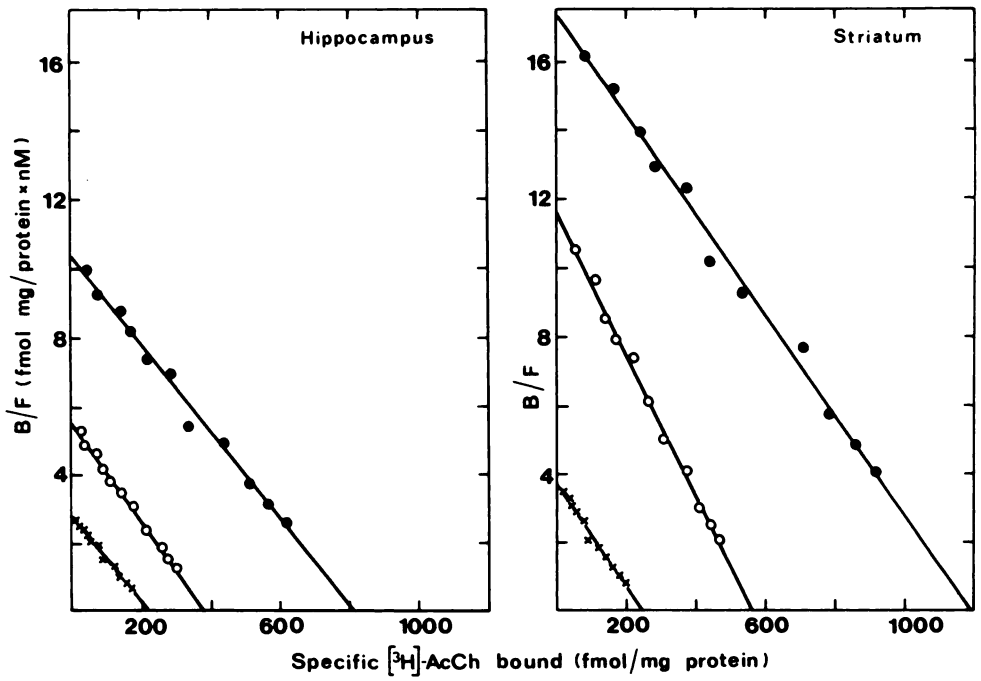

FIG. 6. *Effect of Ni^{2+} and Gpp(NH)p on specific binding at equilibrium of [³H]AcCh to membranes from rat hippocampus and striatum*

Specific [³H]AcCh binding was assayed as detailed in the text, in the absence (○) and in the presence of 2 mM Ni^{2+} (●) or 200 μM Gpp(NH)p (+). These additions did not affect nonspecific binding of [³H]AcCh (determined with 10 μM atropine, not shown). Scatchard plots of data from a single experiment for each tissue are shown. Linear regression analysis yielded the following parameters: K_d = 69, 78, and 82 nM (hippocampus) and 49, 69, and 66 nM (striatum); B_{max} = 380, 810, and 226 fmol/mg protein (hippocampus) and 564, 1190, and 242 fmol/mg protein (striatum) for control buffer and buffer containing 2 mM Ni^{2+} or 200 μM Gpp(NH)p, respectively. Specific binding of 25 mM [³H]4-NMPB in the same preparations was 2450 and 3272 fmol/mg protein in the hippocampus and striatum, respectively, and was the same in the absence or presence of 2 mM Ni^{2+} or 200 μM (Gpp(NH)p (not shown).

TABLE 3

High affinity AcCh-binding sites, determined in direct binding with [³H]AcCh and in competition experiments with [³H]4-NMPB, and their modulation by Gpp(NH)p and Ni^{2+}

The proportion of high affinity AcCh-binding sites (α) was determined in competition binding experiments (2 experiments) as described in Fig. 2. The effects of 1 mM Gpp(NH)p and 2 mM Ni^{2+} were determined with 40 nM [³H]AcCh. Data presented are mean of 2 experiments each performed in quadruplicate.

Tissue	α in competition with [³H]4-NMPB	[³H]AcCh / [³H]4-NMPB	Decrease by 1 mM Gpp(NH)p	Increase by 2 mM Ni^{2+}
	%	%	%	%
Cerebral cortex	28	22	32	174
Hippocampus	23	15	56	220
Striatum	25	16	50	212
Olfactory bulb		26	46	192
Hypothalamus		33	64	116
Brainstem	61	48	67	112
Cerebellum	64	51	73	114
Atrium	65	71	91	None

while [³H]AcCh yields linear Scatchard plots at the wide concentration range employed (4–200 nM; see Fig. 1). (b) The B_{max} values derived from the direct binding experiments with labeled agonists are higher for [³H]AcCh than for the two synthetic agonists (Table I; Refs. 3, 7, 10, 11, 32, 37). The nonlinear Scatchard plots of [³H]OXO-M and [³H]CD could result from their binding to both superhigh and high affinity sites (2, 3, 10, 11), while [³H]AcCh binds to all high affinity sites (both superhigh and high) with a similar affinity. Indeed, binding of [³H]AcCh to a homogeneous class of high affinity muscarinic sites was recently reported by Kellar *et al.* (15–17). Since the B_{max} values for [³H]OXO-M and [³H]CD are determined mostly by the fraction of superhigh affinity sites (10, 11, 23), this could as well explain the lower B_{max} values observed for these ligands relative to [³H]AcCh. As for the origin of the superhigh and high affinity sites observed with [³H]OXO-M and [³H]CD, there are two possibilities. First, there could be pre-existing subpopulations of sites (superhigh and high) which are recognized differently by certain agonists, but not by the neurotransmitter itself. A second explanation is that the superhigh affinity sites reflect a receptor conformation induced by the binding of OXO-M or CD, but not by AcCh. The latter possibility suggests that muscarinic agonists may be capable of inducing different conformational changes in the receptor. Such a phenomenon was demonstrated earlier for the binding of muscarinic antagonists (18, 38). The induction of different receptor conformations by different agonists is in line with recent observations on differences in the modes of binding of oxotremorine and AcCh, as revealed by their binding kinetics (39) and by the different sensitivities of their binding to modulation by batrachotoxin (40). A further support for this notion is provided by the earlier demonstration that oxotremorine differs from AcCh and carbamylcholine in the effects

on cAMP formation and phosphatidylinositol hydrolysis (41, 42, 47). These observations might be correlated with the above suggestions.

It is interesting to note that the K_d values determined for the muscarinic antagonists atropine and (−)-*N*-methylscopolamine by competition with [^{3}H]AcCh (Table 2) are slightly higher (2–3-fold) than those obtained for these ligands by direct binding or by competition against tritiated antagonists (1, 3, 4, 18). In the case of (−)-QNB, a much higher difference between these K_d values was observed. Thus, competition of (−)-QNB with [^{3}H]AcCh yielded K_d of 2–4 nM (Table 2), whereas direct binding of [^{3}H](−)-QNB yields K_d values of 0.1 nM or lower (Ref. 1 and references therein). A high K_d value for (−)-QNB in competition against [^{3}H]AcCh was also reported by Kellar *et al.* (15). The reason for the lower affinities of muscarinic antagonists as determined by competition with [^{3}H]AcCh is yet unknown. A possible explanation for this phenomenon is the existence of site-site interactions among muscarinic receptors, which differ for the pairs agonist-antagonist and antagonist-antagonist. The differences may extend also to the nature of the antagonists (38, 43) or agonist molecules themselves.

Relation to subtypes of muscarinic receptors. The existence of two subclasses (M_1 and M_2) of muscarinic receptors, which bind the nonclassical muscarinic antagonist pirenzepine with different affinities, was proposed previously (5, 22–27). In order to explore whether the high affinity [^{3}H]AcCh-binding sites correlate with one of the postulated pirenzepine receptor subclasses, we have conducted competition experiments with [^{3}H]AcCh and pirenzepine, employing tissues enriched in either low or high affinity pirenzepine-binding sites (Fig. 4). Interestingly, similar distribution patterns of high and low pirenzepine-binding sites are observed when pirenzepine competes against [^{3}H]AcCh and against tritiated antagonists (Fig. 4, Refs, 5, 26, 27). Since the measured [^{3}H]AcCh binding is to a fraction of the antagonist binding sites, these findings indicate that the [^{3}H]AcCh-binding sites detected in the direct binding studies do not correlate with either of the postulated M_1 or M_2 pirenzepine receptor subclasses. This conclusion is in accord with the suggestion (27) that high affinity pirenzepine binding sites are *not* similar to the low affinity agonist-binding sites.

Sensitivity to guanyl nucleotides. The sensitivity of [^{3}H]AcCh binding to guanyl nucleotides (Figs. 5 and 6) is most pronounced in those preparations enriched in agonist high affinity binding sites, *i.e.* brainstem, cerebellum, and atrium (Table 3). The main effect of the guanyl nucleotides is reduction in the binding of [^{3}H]AcCh with minor changes in the corresponding K_d values. This is entirely in agreement with previous findings derived from curves representing [^{3}H]antagonist/agonist competition binding in the presence and absence of guanyl nucleotides (28–33) and with a recent report employing [^{3}H]OXO-M (7). In all brain tissues under investigation, the conversion of high affinity to low affinity sites induced by guanyl nucleotides is only partial, unlike in other receptors where a complete conversion has been observed (35, 44). Partial conversion of muscarinic receptors has also been reported by other laboratories, using [^{3}H]OXO-M (7) or [^{3}H]CD (34). Thus, under the experimental conditions employed, the muscarinic site detected by [^{3}H]AcCh binding can be divided into GTP-sensitive and GTP-insensitive sites. The former are most probably those sites that are reversibly coupled to nucleotide-binding proteins (44, 45). We have recently shown that the conversion of low to high affinity [^{3}H]AcCh sites which are GTP insensitive may be induced by treatment of cerebral cortex or brainstem preparations with Cu^{2+} ions, probably via sulfhydryl groups on the receptor or on the guanyl nucleotide-binding protein (14). The reason for the presence or the induction of high affinity [^{3}H]AcCh sites which are insensitive to GTP is unknown, although preliminary experimentation has indicated the possible role of SH/S-S transformation in such processes (14). Finally, it should be noted that atrial and brain preparations reveal different sensitivities to GTP or Gpp(NH)p (I_{50} of the Gpp(NH)p effect is 0.3 μM *versus* 1–6 μM in atria and in various brain regions, respectively). These different sensitivities could stem from different coupling of heart and brain muscarinic receptors with guanyl nucleotide-binding proteins. Alternatively, they could indicate that the guanyl nucleotide protein designated Ni which transduces the inhibitory coupling of heart muscarinic receptors to adenylate cyclase (46) differs from the yet unknown guanyl nucleotide which is coupled to brain muscarinic receptors.

Low affinity agonist sites can be converted into a GTP-sensitive high affinity state by co-incubation with transition metal ions (Fig. 6). In brainstem, cerebellum, and atrium, 2 mM Ni^{2+} had a negligible effect on [^{3}H]AcCh binding, while in preparations from the hippocampus, striatum, olfactory bulb, and cerebral cortex, the B_{max} for [^{3}H]AcCh was increased by about 2-fold (Table 3). This is in agreement with our previous findings (13, 36) and with recent reports on [^{3}H]OXO-M binding to rat cerebral cortex (7) and for [^{3}H]CD binding to porcine striatum (37).

Mechanisms leading to reversible transitions of high to low affinity states and vice versa might involve conformational changes induced directly by modulators or modification induced directly via activation or inhibition of enzymes (as discussed in detail in Ref. 1 and references therein). Studies aimed at investigating these mechanisms using the endogenous neurotransmitter should prove useful in elucidating the interaction of the muscarinic receptor with effector systems and its regulation.

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