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High-affinity Binding of [^{3}H]Acetylcholine to Muscarinic Cholinergic Receptors[1]

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Abstract

High-affinity binding of [^{3}H]acetylcholine to muscarinic cholinergic sites in rat CNS and peripheral tissues was measured in the presence of cytisin, which occupies nicotinic cholinergic receptors. The muscarinic sites were characterized with regard to binding kinetics, pharmacology, anatomical distribution, and regulation by guanyl nucleotides. These binding sites have characteristics of high-affinity muscarinic cholinergic receptors with a K_d of approximately 30 nM. Most of the muscarinic agonist and antagonist drugs tested have high affinity for the [^{3}H]acetylcholine binding site, but pirenzepine, an antagonist which is selective for M-1 receptors, has relatively low affinity. The ratio of high-affinity [^{3}H]acetylcholine binding sites to total muscarinic binding sites labeled by [^{3}H]quinuclidinyl benzilate varies from 9 to 90% in different tissues, with the highest ratios in the pons, medulla, and heart atrium. In the presence of guanyl nucleotides, [^{3}H]acetylcholine binding is decreased, but the extent of decrease varies from 40 to 90% in different tissues, with the largest decreases being found in the pons, medulla, cerebellum, and heart atrium. The results indicate that [^{3}H]acetylcholine binds to high-affinity M-1 and M-2 muscarinic receptors, and they suggest that most M-2 sites have high affinity for acetylcholine but that only a small fraction of M-1 sites have such high affinity.

Muscarinic cholinergic receptors in brain and peripheral tissues have been studied extensively using radiolabeled antagonist ligands such as [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB), [^{3}H]N-methylscopolamine, and [^{3}H]propylbenzilylcholine (Yamamura and Snyder, 1974; Birdsall et al., 1978; Hulme et al., 1978). Muscarinic antagonists bind to receptors with Hill coefficients (n_H) close to 1; however, muscarinic agonists compete for [^{3}H]antagonist binding sites with shallow competition slopes ($n_H < 1$) which deviate from simple mass action predictions for a single class of sites (Birdsall et al., 1978). One reasonable explanation for this is that agonists bind to multiple sites or states of muscarinic receptors with varying affinities, while most antagonists bind to the different sites or states with equal affinities (Birdsall et al., 1978). Evidence for this was obtained using the agonist ligand [^{3}H]oxotremorine-M (Birdsall et al., 1978).

Studies of the actions of muscarinic agonists and detailed analyses of binding competition curves between muscarinic agonists and [^{3}H]antagonists have led to the concept of muscarinic receptor subtypes (Rattan and Goyal, 1974; Goyal and Rattan, 1978; Birdsall et al., 1978). This concept was reinforced by the discovery of the selective actions and binding properties of the antagonist pirenzepine (Hammer et al., 1980; Hammer and Giachetti, 1982; Watson et al., 1983; Luthin and Wolfe, 1984). An evolving classification scheme for these muscarinic receptors divides them into M-1 and M-2 subtypes (Goyal and Rattan, 1978; for reviews, see Hirschowitz et al., 1984). Although details of the distinctions between M-1 and M-2 receptor subtypes are still emerging, this classification offers a convenient framework in which to compare the binding properties of ligands.

The M-1 subtype has high affinity for pirenzepine and appears to be the prevalent muscarinic receptor in brain areas such as cerebral cortex, corpus striatum, and hippocampus, as well as in sympathetic ganglia. The M-2 subtype has relatively much lower affinity for pirenzepine and is found in high proportions in brain areas such as pons, medulla, and cerebellum, as well as in heart atrium and the ileum of the small intestine. In addition, guanyl nucleotides affect the binding of agonists at muscarinic receptors, and this effect appears to be greatest in tissues with a high proportion of M-2 sites. This is consistent with evidence that some muscarinic receptors are coupled to adenylate cyclase and inhibit the formation of cAMP (Murad et al., 1962; Brown, 1979). In fact, the differences between M-1 and M-2 receptor subtypes could derive largely from the effector mechanisms to which they are coupled.

Attempts to measure muscarinic cholinergic receptor binding sites with [^{3}H]acetylcholine ([^{3}H]ACh) have generally met with very limited success, due to high nonspecific binding, low specific binding, and low specific radioactivity of the available ligand.[4] We have circumvented these technical difficulties by utilizing [^{3}H]ACh of high specific radioactivity and an assay procedure which minimizes nonspecific binding. Since in brain [^{3}H]ACh also binds to nicotinic cholinergic receptor sites (Schwartz et al., 1982), we have carried out the assay for muscarinic sites in the presence of a saturating concentration of cytisin, a nicotinic agonist. Under conditions of the assay, [^{3}H]ACh binds to sites with the characteristics of muscarinic cholinergic receptor agonist recognition sites in brain and peripheral tissues. The purpose of this study was to characterize the kinetics of binding, pharmacology, anatomical distribution, and regulation of these sites.

Materials and Methods

Synthesis of [^{3}H]acetylcholine. [^{3}H]ACh (80 Ci/mmol) was synthesized by esterification of [^{3}H]choline (80 Ci/mmol; New England Nuclear) as

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[4] During the preparation of this paper, Gurwitz, Kloog, and Sokolovsky (1984) reported the successful use of high specific radioactivity [^{3}H]ACh to label muscarinic sites in rat brain.

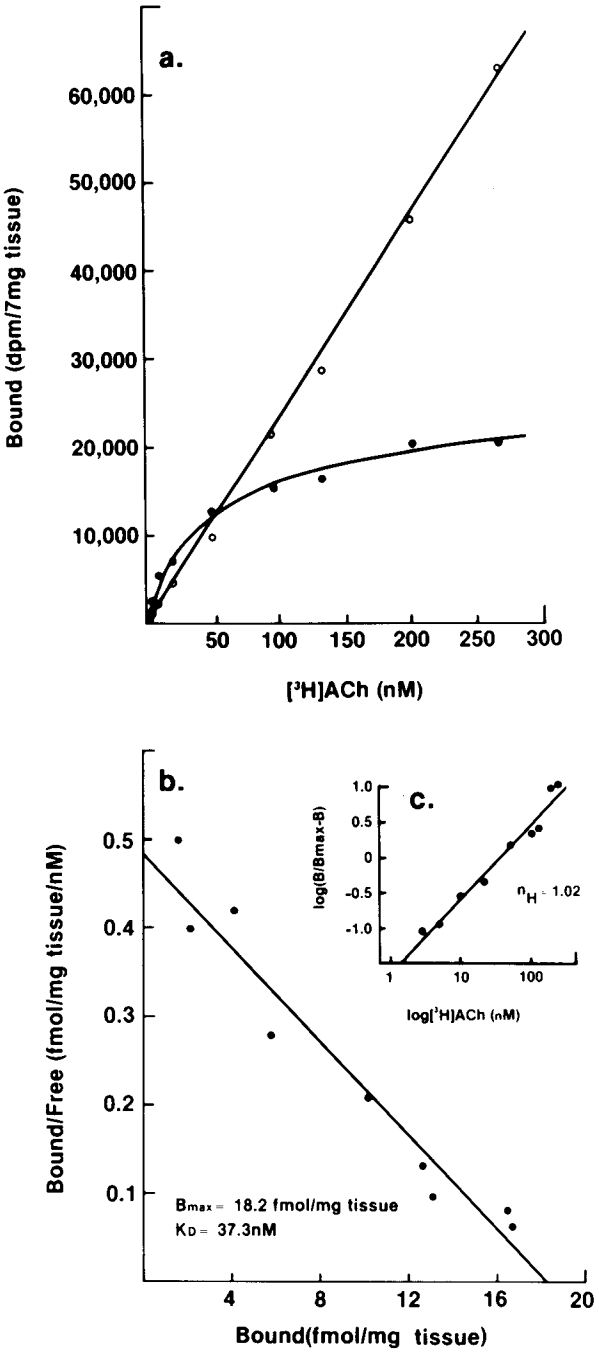

Figure 1. [³H]Acetylcholine binding in rat brain. *a*, Saturation analysis of [³H]ACh binding in rat cerebral cortex. Homogenates of cortex were incubated in buffer containing 1 μM cytisin and different concentrations of [³H]ACh (2 to 300 nM) for 60 min at 25°. Nonspecific binding (○) was determined in the presence of 1.5 μM atropine. Specific binding (●) is the difference between total binding and nonspecific binding. *b*, Scatchard plot of specific binding of [³H]ACh shown in *a*. The K_d and B_{max} were determined by linear regression analysis. *c*, Hill plot of specific binding shown in *a*. The Hill coefficient (n_H) was determined by linear regression analysis. This experiment is representative of 10 separate experiments.

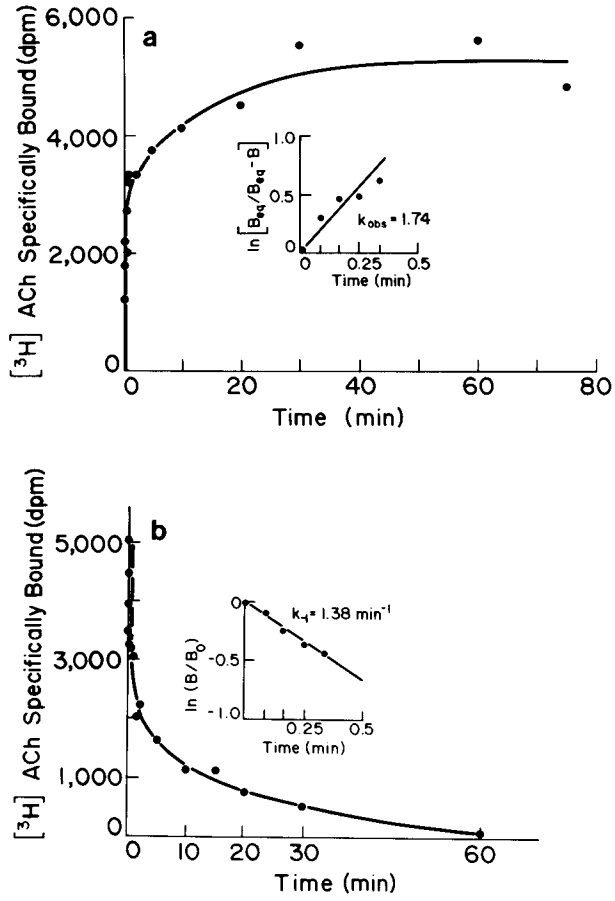

Figure 2. Rate of association and dissociation of [³H]ACh binding to cerebral cortex. *a*, The association rate was determined by incubating the tissue as described in the text with 6 nM [³H]ACh for various times before filtration. Specific binding is plotted and is representative of four experiments. *Inset*, pseudo-first order kinetic plot of [³H]ACh specific binding. The association rate constant, k_1, was determined by the equation $k_1 = (k_{obs} - k_{-1})/$ [³H]ACh concentration. *b*, The rate of dissociation was determined by initially incubating the tissue with 6 nM [³H]ACh for 60 min and subsequently adding 1.5 μM atropine. The reaction mixture was filtered at the times shown. Specific binding is plotted and is representative of four experiments. *Inset*, first order kinetic plot of the dissociation of [³H]ACh specific binding. The dissociation rate constant, k_{-1}, is given by the slope of the *line*.

described previously (Schwartz et al., 1982). The procedure yields [³H]ACh of high purity (>98%) that is conveniently and economically repurified or resynthesized weekly (Schwartz et al., 1982). Analysis of [³H]ACh after incubation with tissue for 1 hr in the presence of a cholinesterase inhibitor showed that less than 2% of the material had been hydrolyzed.

Tissues. Sprague-Dawley rats (male, 250 to 350 gm) were decapitated, and the brains were dissected into various regions, frozen on dry ice, and kept at −80°C for up to 1 week before assaying. Spinal cord and peripheral tissues were similarly frozen.

Binding assays. Tissues were homogenized with a Brinkmann Polytron in 50 mM Tris-HCl buffer containing 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$ (pH 7.4 at 25°C). The tissue homogenates were washed twice by centrifugation at 48,000 × *g* for 10 min with intermediate homogenization in fresh buffer. The final tissue pellet was resuspended in buffer containing 100 μM diisopropyl fluorophosphate (DFP) to inhibit cholinesterases and 1.5 μM cytisin to occupy nicotinic receptors. Routinely, the binding assays were initiated by the addition of tissue homogenate (5 to 7 mg of original weight = 250 to 450 μg of protein) to tubes containing buffer and [³H]ACh. In pharmacological studies, drugs were dissolved in buffer and added to the tubes before the tissue. Total volume of the reaction mixture was 500 μl, and all assays were run in triplicate. Nonspecific binding was measured in parallel in the presence of 1.5 μM atropine. The reaction mixtures were incubated at 25°C for 60 min and were then filtered under reduced pressure through Whatman GF/C filters which had been wet with buffer containing 0.05% polyethyleneimine to eliminate displaceable binding to the filters (Schwartz et al., 1982). The filters were washed 3 times with 4-ml aliquots of cold buffer, placed in vials to which Liquiscint scintillation fluid was added, and counted by liquid scintillation spectrometry at a counting efficiency of 37%. Specific binding was defined as the difference between total binding and nonspecific binding. In some experiments, the time, temperature, or volume of incubation was varied to determine the effects of these parameters on binding.

Binding of [³H](−)QNB (38 Ci/mmol; Amersham Corp.) was carried out under conditions similar to those for [³H]ACh, except that the amount of

TABLE I

Subcellular distribution of [^{3}H]ACh and [^{3}H]QNB binding in rat cerebral cortex

The specific binding of 30 nM [^{3}H]ACh and 170 pM [^{3}H]QNB was measured in the total homogenate and in the myelin, synaptosomal, and mitochondrial fractions, which were separated by sucrose density centrifugation as described in the text. The data are reported as means from four animals.

Fraction	[^{3}H]ACh Binding		[^{3}H]QNB Binding	
	fmol/mg of Protein	Percentage of Total Homogenate	fmol/mg of Protein	Percentage of Total Homogenate
Total homogenate	76 ± 2.1[a]		547.8 ± 46.4	
Myelin	37.6 ± 3.1	49	258.3 ± 23.6	47
Synaptosomal	109.9 ± 3.1	145	763.6 ± 67.8	139
Mitochondrial	72.4 ± 0.8	95	465.8 ± 57.0	85

[a] Mean ± SE.

TABLE II

Competition by drugs for [^{3}H]ACh binding sites in rat cerebral cortex

Tissue homogenates were added to tubes containing 6 nM [^{3}H]ACh and 8 to 10 concentrations of drug. The IC_{50} values were determined graphically and are the means of 3 to 5 determinations. The [^{3}H]ACh concentration used was well below the K_d; therefore, where the Hill coefficient is close to 1, the IC_{50} value closely approximates the inhibition constant, K_i.

Drug[a]	IC_{50} (nM)	n_H
Muscarinic agonists		
Oxotremorine	17.5 ± 3.1[b]	1.12 ± 0.08
Acetylcholine	25.7 ± 2.8	0.91 ± 0.06
Arecoline	140.2 ± 20.2	0.93 ± 0.03
Methacholine	154.9 ± 6.4	0.86 ± 0.12
Carbachol	172.2 ± 17.8	0.98 ± 0.11
Muscarine	360.9 ± 145.7	0.83 ± 0.12
Pilocarpine	397.2 ± 14.2	0.80 ± 0.05
Bethanechol	1309 ± 419	0.73 ± 0.09
Muscarinic antagonists		
(−)QNB	1.4 ± 0.2	0.92 ± 0.08
Atropine	4.6 ± 0.7	0.97 ± 0.07
Dexetimide	5.8 ± 1.6	0.68 ± 0.03
Scopolamine	6.4 ± 0.9	0.78 ± 0.08
Pirenzepine	846 ± 47.1	0.64 ± 0.06
Levetimide	9513 ± 2112	0.87 ± 0.20

[a] The following drugs decreased binding by less than 50% at a concentration of 30 μM: Choline, Cytisin, Nicotine, Dihydro-β-erythroidine, Mecamylamine, hemicholinium-3, and Physostigmine.

[b] Mean ± SE.

tissue assayed was decreased to 1 mg (approximately 50 μg protein) per tube, and the volume of the assay was increased to 3 ml.

Subcellular fractions of cerebral cortex were isolated by discontinuous sucrose gradient centrifugation according to the method of Whittaker and Barker (1972). The myelin, synaptosomal, and mitochondrial fractions and a portion of the initial homogenate were then diluted in buffer and prepared for the binding assays as described above. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Drugs. Drugs were obtained from commercial sources with the exceptions of dexetimide and levetimide, which were generous gifts from Dr. John J. O'Neill (Temple University) and Dr. Barry Wolfe (University of Pennsylvania), pirenzepine, which was a generous gift from Dr. Henry Yamamura (University of Arizona), and mecamylamine and dihydro-β-erythroidine, which were kindly provided by Dr. Clement A. Stone (Merck Sharp and Dohme Research Laboratories).

Results

Kinetics of [^{3}H]ACh binding. Specific binding of [^{3}H]ACh in the cerebral cortex was saturable over a concentration range of 2 to 300 nM. The binding capacity (B_{max}) was 18.2 ± 1.0 fmol/mg tissue (318 ± 17 fmol/mg protein), and the equilibrium dissociation constant (K_d) at 25°C was 35.2 ± 3.5 nM (Fig. 1, *a* and *b*). The Hill coefficient (n_H) of the saturation isotherm was 1.01 ± 0.01 (Fig. 1c), indicating an absence of cooperativity and that, over this concentration range, [^{3}H]ACh was binding to a single class of sites.

At a [^{3}H]ACh concentration of 30 nM, specific binding to 7 mg of cerebral cortex (400 μg protein) was approximately 10,000 dpm and represented 65 to 70% of total binding (Fig. 1*a*). Specific binding was linear between 2 and 20 mg of cortex tissue, and no specific binding was detected in tissue which had been placed in a boiling water bath for 5 min prior to assay. In the absence of a cholinesterase inhibitor, specific binding of [^{3}H]ACh could not be detected. In the presence of 100 μM DFP, nonspecific binding was decreased, and specific binding was increased. Concentrations of DFP as high as 1 mM did not interfere with the assay.

The rates of association and dissociation were rapid. At 25°C, the half-time ($t_{1/2}$) for association was approximately 40 sec, and the binding reached equilibrium within 30 min (Fig. 2*a*). The $t_{1/2}$ for dissociation was approximately 30 sec, and the dissociation was essentially complete within 60 min (Fig. 2*b*). The rate constants for association (k_1) and dissociation (k_{-1}) were determined graphically to be 0.034 $nM^{-1} \cdot min^{-1}$ and 1.04 min^{-1}, respectively (Fig. 2, *insets*). The kinetic dissociation constant determined by the ratio k_{-1}/k_1 was 31 nM, in close agreement with the K_d determined by equilibrium studies. At 0°C, the $t_{1/2}$ for dissociation was 2 min and, consistent with this slower dissociation, the equilibrium K_d for [^{3}H]ACh binding in cortex at 0° was 16.3 ± 1.0 nM (data not shown).

Subcellular location. In subcellular distribution studies, binding was measured in the myelin, synaptosomal, and mitochondrial fractions of cerebral cortex and compared with the binding in the total homogenate. These studies indicated that [^{3}H]ACh binding sites were located primarily within the synaptosomal and mitochondrial fractions (Table I). This subcellular distribution of [^{3}H]ACh binding sites was similar to that of [^{3}H]QNB binding sites (Table I).

Pharmacological characteristics. The pharmacological characteristics of the [^{3}H]ACh binding site in cerebral cortex were determined by measuring the inhibition of binding by drugs (Table II and Fig. 3). Muscarinic cholinergic drugs were potent inhibitors of binding, whereas neither nicotinic drugs, cholinesterase inhibitors, nor hemicholinium-3 competed effectively for the site (Table II and Fig. 3). In particular, cytisin, which routinely was present in the assay at a concentration of 1.5 μM, did not decrease binding at concentrations up to 100 μM.

In general, muscarinic antagonists were more potent than agonists. All of the agonists and most of the antagonists competed for the [^{3}H]ACh binding sites with Hill coefficients close to 1 (Table II). Oxotremorine was the most potent agonist tested, while bethanechol was 50 to 100 times less potent, and choline was practically inactive (Table II). The antagonists (−)QNB, atropine, scopolamine, and dexetimide had IC_{50} values between 1 and 7 nM (Table II). Levetimide, the pharmacologically inactive enantiomer of dexetimide, was nearly 2000 times less potent in competing for [^{3}H]ACh binding sites (Table II and Fig. 2), indicating a high degree of stereospecificity of the site. Interestingly, pirenzepine, a muscarinic antagonist that is relatively selective for the M-1 subtype of muscarinic receptor (Hammer and Giachetti, 1982; Watson et al., 1983), competed for the [^{3}H]ACh site in brain with a shallow Hill slope and relatively low potency (Table II and Fig. 3). In fact, pirenzepine was approximately 12 times more potent in competing for [^{3}H]QNB binding sites than for [^{3}H]ACh binding sites in cortex (Fig. 4).

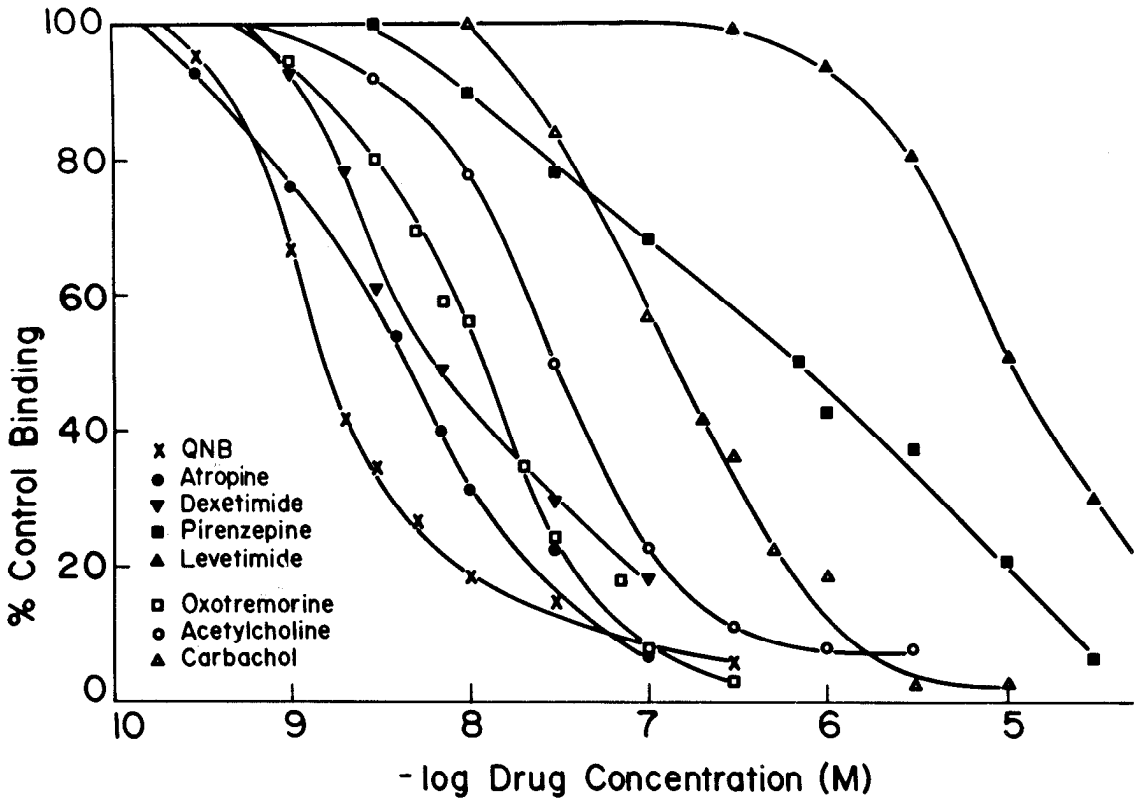

Figure 3. Competition for [^{3}H]ACh binding sites in cerebral cortex by drugs. Cortical homogenates were added to tubes containing 6 nM [^{3}H]ACh and the competing drug at various concentrations. The mixtures were incubated for 60 min at 25° and filtered as described in the text. Specific binding is expressed as the percentage of binding in the absence of competing drug. The curves are representative of at least three separate experiments.

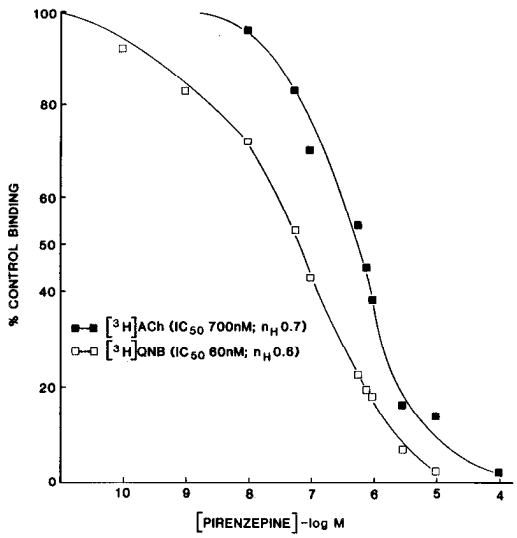

Figure 4. Pirenzepine competition for [^{3}H]ACh and [^{3}H]QNB binding sites in cerebral cortex. Cortical homogenates were added to tubes containing various concentrations of pirnzepine and either [^{3}H]ACh (6 nM) or [^{3}H] QNB (50 pM). The mixtures were incubated for 60 min at 25° and then filtered as described in the text. Specific binding is expressed as the percentage of binding in the absence of pirenzepine. The curves are representative of three experiments.

Muscarinic agonists were much more potent competing for [^{3}H] ACh binding sites than for muscarinic sites labeled by [^{3}H]antagonists (cf. Yamamura and Snyder, 1974; Birdsall et al., 1978). This is consistent with previous reports of high potencies of agonists in competing for muscarinic sites labeled by the agonists [^{3}H]oxotremorine-M (Birdsall et al., 1978) and [^{3}H]*cis*-methyldioxolane (Ehlert et al., 1980). In contrast, the potencies of most of the antagonists in competing for [^{3}H]ACh binding sites were comparable to their potencies at sites labeled by [^{3}H]antagonists.

Distribution of [^{3}H]ACh binding sites in CNS and periphery. The [^{3}H]ACh binding site is widely distributed in the CNS and is also found in certain peripheral tissues (Table III). Within the CNS, the binding was highest in the cerebral cortex, pons, medulla, and thalamus and lowest in the cerebellum, olfactory tubercle, and spinal cord. Among the peripheral tissues examined, specific binding was found in the heart atria and ventricles, pituitary gland, ileum of the small intestine, bronchial tissue, submandibular gland, and parotid gland (Table III). Very little specific binding was detected in liver, kidney, or testes.

Saturation studies of [^{3}H]ACh binding in several regions of brain and in heart atrium indicated that the K_d in most of the tissues was between 25 and 35 nM, but the K_d values in the hippocampus and striatum were consistently higher (Table IV). The Hill coefficients in all of the tissues examined were close to 1 (Table IV). The density (B_{max}) of the [^{3}H]ACh binding site in different brain regions and in the atrium was compared to the density of [^{3}H]QNB binding sites in these tissues. The number of [^{3}H]ACh sites was 10 to 20% of the number of [^{3}H]QNB sites in the cortex, hippocampus, and striatum, 30 to 50% of the [^{3}H]QNB sites in the hypothalamus, thalamus, and cerebellum, and 70 to 90% of the number of [^{3}H]QNB sites in the pons and medulla. In the heart atrium, the number of [^{3}H]ACh sites was approximately 60% of the [^{3}H]QNB sites (Table IV).

Sensitivity to guanyl nucleotides. Incubation with GTP or its stable analogue 5′-guanylylimidodiphosphate [Gpp(NH)p] decreased binding of [^{3}H]ACh in all tissues examined. The binding was decreased to a much greater extent in the medulla, pons, cerebellum, and atrium than in the cerebral cortex, striatum, or hippocampus (Fig 5. and Table V); but in all tissues, the fraction of sites that was affected by the nucleotide displayed similar high sensitivity to Gpp(NH)p, with a half-maximal effect at 0.2 to 0.7 μM.

TABLE III

Relative distribution of [^{3}H]ACh binding in CNS and peripheral tissues

The specific binding of [^{3}H]ACh (30 nM) to CNS areas and peripheral tissues was determined as described in the text. Values are means of at least three separate determinations.

Tissue	Specific Binding (fmol/mg of tissue)
CNS areas	
Cortex	8.0 ± 0.2[a]
Pons	10.0 ± 0.3
Medulla	11.0 ± 0.3
Thalamus	8.1 ± 0.8
Hippocampus	4.6 ± 0.2
Striatum	4.7 ± 0.2
Hypothalamus	7.8 ± 0.1
Cerebellum	3.8 ± 0.1
Olfactory bulb	5.0 ± 0.6
Olfactory tubercles	3.6 ± 0.7
Spinal cord	3.9 ± 0.7
Colliculi	6.4 ± 0.4
Tegmentum	5.8 ± 0.1
Septum	4.7 ± 0.6
Peripheral areas	
Heart atrium	4.0 ± 0.2
Heart ventricle	1.5 ± 0.1
Bronchi	1.3 ± 0.1
Ileum	1.8 ± 0.1
Pituitary gland	0.5 ± 0.1
Submandibular gland	1.1 ± 0.2
Parotid gland	0.3 ± 0.1
Liver[b]	0.4 ± 0.1
Kidney[b]	0.2 ± 0.1
Testis[b]	0.3 ± 0.2

[a] Mean ± SE.

[b] Specific binding was barely detectable in these tissues.

Discussion

The studies presented here indicate that [^{3}H]ACh, at concentrations between 2 and 300 nM, specifically labels and saturates a muscarinic cholinergic recognition site in brain and peripheral tissues. In contrast to the complex kinetics derived from studies of agonist competition for [^{3}H]antagonist binding sites, the kinetics of [^{3}H]ACh binding appear to be much simpler and to follow mass action predictions for a single site. This difference is probably related to the use of [^{3}H]ACh, which presumably labels the agonist recognition site of the muscarinic receptor molecule. Although muscarinic antagonists clearly label the same receptor molecule and are potent inhibitors of [^{3}H]ACh binding (see Table II), the antagonist binding site(s) may not be identical to the agonist recognition site. This could result in complex kinetics in experiments in which competition between agonists and [^{3}H]antagonists is examined, because each could bind to both sites with different affinities.

A subtle difference between [^{3}H]ACh and [^{3}H]QNB binding sites is suggested by the competition experiments in which the affinity of ACh for [^{3}H]ACh binding sites is approximately 100 times greater than its apparent potency in competing for [^{3}H]antagonist binding sites (Table II; see also, Yamamura and Snyder, 1974; Birdsall et al., 1978). In addition, ACh and most of the other agonists tested compete for [^{3}H]ACh binding sites with Hill coefficients close to 1, whereas most of these agonists compete for [^{3}H]antagonist binding sites with Hill coefficients significantly less than 1 (Birdsall et al., 1978). These differences are unlikely to be due to agonist-induced conversion of the site to a desensitized state with high affinity for ACh, because the IC_{50} of ACh in competing for [^{3}H]antagonist binding sites in rat brain is 2 to 5 μM (Yamamura and Snyder, 1974; Birdsall et al., 1978), indicating that even in the presence of high concentrations of ACh, the antagonist binding site does not appear to convert to a state with high affinity for agonists.

The M-1 selective antagonist pirenzepine is more than 100 times less potent than the other antagonists tested in competing for [^{3}H]ACh binding sites. This indicates that within the M-1 and M-2 classification scheme, the [^{3}H]ACh site has a key characteristic of an M-2 subtype of receptor.

The distribution of [^{3}H]ACh binding sites in rat brain is markedly different from that of [^{3}H]QNB sites. Binding of [^{3}H]ACh is highest in the medulla, pons, thalamus, and cerebral cortex; whereas, binding of [^{3}H]QNB is highest in the striatum, hippocampus, and cerebral cortex. This too indicates that, under the conditions of this assay, [^{3}H]ACh labels a subtype of muscarinic receptor. The ratio of [^{3}H]ACh sites to [^{3}H]QNB sites is highest in the medulla, pons, and cerebellum, brain areas which are thought to contain a high proportion of the M-2 subtype of muscarinic receptor (Watson et al., 1983; Luber-Narod and Potter, 1982). Similarly, in the heart atrium, a tissue in which the M-2 subtype of receptor predominates (Watson et al., 1983), the number of [^{3}H]ACh binding sites represents a large proportion of the number of [^{3}H]QNB sites. Thus, consistent with the low affinity of pirenzepine, the distribution of the [^{3}H]ACh binding site is similar to that described for M-2 receptors.

The reduction of [^{3}H]ACh binding by guanyl nucleotides is most pronounced (80 to 90%) in the medulla, pons, cerebellum, and atrium, tissues rich in M-2 receptors. This is consistent with previous studies which found that guanyl nucleotides reduced the potency of agonists in competing for [^{3}H]antagonist binding sites in these tissues (Berrie et al., 1979; Rosenberger et al., 1980; Sokolovsky et al., 1980). In contrast, in the cerebral cortex, hippocampus, and striatum, the maximum reduction of [^{3}H]ACh binding by Gpp(NH)p was approximately 50%, indicating a more obvious heterogeneity

TABLE IV

Binding constants for [^{3}H]acetylcholine in rat brain areas and heart atrium

Tissue homogenates were incubated with 2 to 300 nM [^{3}H]ACh. For comparison, the B_{max} of [^{3}H]QNB binding sites in the same tissues is shown. Values are the means for 3 to 7 experiments.

Tissue	[^{3}H]ACh			[^{3}H]QNB: B_{max} (fmol/mg of tissue)	[^{3}H]ACh Sites/[^{3}H]QNB Sites
	K_d (nM)	n_H	B_{max} (fmol/mg of tissue)		
Cerebral cortex	34.6 ± 4.1[a]	1.01 ± 0.01	18.4 ± 1.5	120.6 ± 3.0	15%
Pons	33.8 ± 4.9	1.00 ± 0.03	20.3 ± 0.9	22.3 ± 1.1	91%
Medulla	24.5 ± 0.5	0.96 ± 0.02	19.3 ± 0.02	26.6 ± 1.9	72%
Thalamus	38.2 ± 3.5	1.04 ± 0.03	17.5 ± 1.4	59.9 ± 0.8	29%
Hippocampus	65.4 ± 4.0	0.99 ± 0.01	14.3 ± 0.5	108.6 ± 1.3	13%
Striatum	61.9 ± 4.8	0.97 ± 0.01	13.2 ± 0.9	149.0 ± 14.2	9%
Hypothalamus	25.9 ± 1.0	1.00 ± 0.01	12.8 ± 0.2	36.5 ± 1.4	35%
Cerebellum	24.0 ± 0.7	0.98 ± 0.08	6.5 ± 0.2	12.8 ± 1.2	51%
Atrium	34.4 ± 8.8	1.02 ± 0.06	12.7 ± 1.3	20.6 ± 1.8	62%

[a] Mean ± SE.

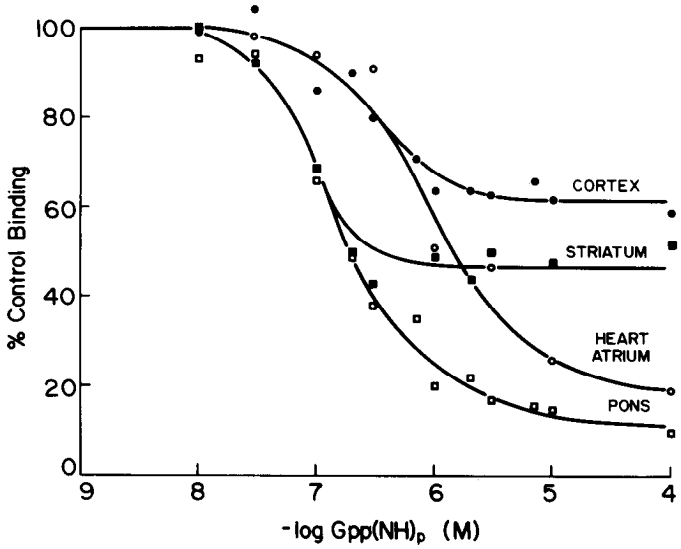

Figure 5. The effect of Gpp(NH)p on [^{3}H]ACh binding in different tissues. Tissue homogenates were incubated for 60 min at 25° with 6 nM [^{3}H]ACh and various concentrations of Gpp(NH)p. Specific binding is expressed as the percent of binding in the absence of added Gpp(NH)p. The *curves* are representative of at least four experiments.

TABLE V

Effects of Gpp(NH)p on [^{3}H]ACh binding in brain areas and heart atrium

Tissues were incubated with 6 nM [^{3}H]ACh and various concentrations of Gpp(NH)p (0.01 to 100 μM). The maximum inhibition and the concentration of Gpp(NH)p which reduced binding by one-half the maximum extent (IC_{50e}) were determined graphically. Values are the means of 4 to 6 experiments.

Tissue	Maximum Inhibition (%)	IC_{50e} (μM)
Cerebral cortex	43.2 ± 4.5[a]	0.42 ± 0.11
Striatum	46.0 ± 2.9	0.20 ± 0.07
Hippocampus	58.0 ± 1.6	0.52 ± 0.12
Pons	85.3 ± 3.7	0.15 ± 0.03
Medulla	85.0 ± 3.0	0.17 ± 0.03
Cerebellum	85.3 ± 3.2	0.43 ± 0.24
Heart atrium	86.0 ± 5.0	0.75 ± 0.25

[a] Mean ± SE.

among the binding sites in these tissues. The difference between the guanyl nucleotide-sensitive and -insensitive sites is important. In the simplest case, the reduction in binding could reflect high-affinity M-2 receptors that are coupled to a guanyl nucleotide-sensitive effector mechanism, possibly as a ternary complex formed by the agonist, the receptor, and a nucleotide regulatory protein. These may be the receptors that are linked to adenylate cyclase. The guanyl nucleotide-insensitive sites could then be high-affinity M-2 receptors that do not form such a complex and presumably are not linked to adenylate cyclase. However, it is also possible that the guanyl nucleotide sensitivity distinguishes between two different receptor subtypes, possibly M-1 and M-2, with the same high affinity for [^{3}H]ACh. In fact, the low Hill coefficient of pirenzepine in competition experiments (see Table II) favors such an interpretation. If this is the case, based on the ratio of [^{3}H]ACh to [^{3}H]QNB binding sites and the extent of reduction of [^{3}H]ACh binding by Gpp(NH)p in the different tissues, most of the receptors designated as the M-2 type appear to have high affinity for [^{3}H]ACh, while only a small fraction (5 to 10%) of the M-1 type appear to have such high affinity.

In conclusion, the kinetics, tissue distribution, pharmacology, and regulation by guanyl nucleotides are consistent with the [^{3}H]ACh binding sites being the endogenous ligand recognition sites of high-affinity muscarinic receptors. Within the context of the M-1 and M-2 classification scheme, most (if not all) of the M-2 receptors appear to have high affinity for [^{3}H]ACh, but only a small fraction of the M-1 sites appear to have such high affinity. Consistent with this, the overall potency of ACh in competing for [^{3}H]QNB binding sites is 10 to 30 times greater in M-2-rich tissues, such as the medulla, pons, and atrium, than in M-1-rich tissues, such as the cerebral cortex, striatum, and hippocampus (K. J. Kellar, and A. M. Martino, unpublished observations).

The selective labeling of these high-affinity muscarinic receptors with the endogenous neurotransmitter ligand should prove useful in furthering progress toward understanding agonist actions at and regulation of muscarinic receptors.

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