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Binding of Atropine and Muscarone to Rat Brain Fractions and its Relation to the Acetylcholine Receptor

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SUMMARY

FARROW, J. T., AND O'BRIEN, R. D.: Binding of atropine and muscarone to rat brain fractions and its relation to the acetylcholine receptor. *Mol. Pharmacol.* **9**, 33–40 (1973). The binding of atropine and muscarone to subcellular fractions from whole rat brain was measured by equilibrium dialysis. At 10 nM, muscarone and atropine, but not decamethonium, dimethyltubocurarine, or nicotine, bound to a crude mitochondrial fraction from brain, and the binding was reversible. Atropine and muscarone at 10 nM did not bind to homogenates of kidney or lung. Atropine, but not muscarone, bound to liver homogenates and liver mitochondria, probably to atropinesterase. Atropine at 10 nM bound to a greater extent, on a protein basis, to fractions enriched in synaptosomes, pre- and postsynaptic membranes, and membrane of uncertain origin, than to other fractions. Muscarone showed a similar binding profile but also bound to purified mitochondrial fractions. Atropine bound to the synaptic membrane fraction at two sites present at 0.089 ± 0.018 and 0.91 ± 0.20 nmole/g of tissue (fresh weight), with binding constants of 0.6 ± 0.01 nM and 0.9 ± 0.18 μM, respectively. Only scopolamine, of the sixteen drugs tested (at 50 nM), interfered with atropine binding at 5 nM to the same fraction. These findings are interpreted as showing that the higher-affinity site for atropine binding is the site more likely to be related to the physiological acetylcholine receptor.

INTRODUCTION

Recent studies in our laboratory (1–9) have demonstrated the binding of cholinergic ligands *in vitro* to fractions from various species. This binding has many of the characteristics one would expect of binding to the acetylcholine receptor. Phospholipoproteins in particulate fractions from *Torpedo* and *Electrophorus* electroplax bound muscarone and nicotine reversibly at either a single site (*Electrophorus*) or at two sites (*Torpedo*), probably to the acetylcholine receptor. Dimethyltubocurarine bound at two sites in *Torpedo* and three in *Electrophorus*; decamethonium bound to these fractions at three and four sites, respectively. Muscarone, nicotine, decamethonium, and atropine bound reversibly to a protein from housefly brain. Cholinergic agonists and antagonists interfered with the binding whereas the majority of noncholinergic drugs did not. Acetylcholine bound reversibly to two high-affinity sites present in phospholipoproteins from *Torpedo* electroplax. The binding was blocked by nicotinic drugs.

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To assure that the bindings of the type described occur to the physiological acetylcholine receptor, one would like to show that the binding occurred with excitable tissues and not with inexcitable ones, and that the binding was located in postsynaptic membranes rather than (for instance) to presynaptic vesicles. The present study explores this aspect with brain tissue, for which a standardized subcellular fractionation technique exists.

This study was undertaken to determine whether high-affinity binding of cholinergic ligands to subcellular fractions can be observed in rat brain and not in non-nervous tissues; if so, to determine whether such binding is reversible, is present in appropriate amounts, shows appropriate interaction with cholinergic and noncholinergic drugs, and has an appropriate subcellular localization.

METHODS

Whole brains were quickly removed from decapitated female, 3–6 month-old Holtzman rats. The brains were homogenized in 0.32 M sucrose (10%, w/v) in a Teflon-glass Potter-Elvehjem homogenizer with a clearance of 0.25 mm and rotating at 840 rpm. Following centrifugation at 1000 × *g* for 11 min (3400 rpm, Spinco 30 rotor) the supernatant fluid was spun at 10,000 × *g* for 20 min (10,000 rpm, Spinco 30 rotor) to give a crude mitochondrial pellet. The supernatant fraction was separated into soluble and microsomal fractions by centriguation at 100,000 × *g* for 60 min (30,000 rpm, Spinco 30 rotor). The crude mitochondrial pellet was subdivided to give fraction A (myelin), fraction B (synaptosomes), and fraction C (mitochondria) by resuspension of the pellet in 0.32 M sucrose, which was then layered on top of a discontinuous density gradient (4.5 ml of 1.2 M sucrose, 4.5 ml of 0.8 M sucrose, and 4.5 ml of sample in 0.32 M sucrose) and centrifuged at 284,000 × *g* for 55 min (40,000 rpm, Spinco SW 40 rotor) (10).

In other experiments the crude mitochondrial pellet was resuspended in water (2 ml/g of tissue, fresh weight), layered on a discontinuous density gradient consisting of 1.8 ml each of 1.2, 1.0, 0.8, 0.6, and 0.4 M sucrose, and then centrifuged at 284,000 × *g* for 55 min (40,000 rpm, Spinco SW 40 rotor). This procedure yielded fractions D (synaptic vesicles), E and F (membranes of uncertain origin), G (synaptosome ghosts), H (intact synaptosomes), and I (mitochondria) (11). All isolated fractions were diluted with water to 0.32 M sucrose and centrifuged at 100,000 × *g* for 45 min (30,000 rpm, Spinco 30 rotor). The pellets were resuspended in small volumes of Krebs-Ringer solution. This modification of Whittaker's centrifugation technique yields the desired fractions (12).

The binding of radioactive cholinergic ligands to the various subcellular fractions was measured by equilibrium dialysis at 4°, using the methods, chemicals, and expressions of results described by Eldefrawi, Eldefrawi, and O'Brien (5). After dialysis against radioactive muscarone, quadruplicate 0.1-ml samples from bag and bath were heated with 0.2 ml of 30% H_2O_2 at 100° for 20 min in sealed counting vials. After atropine dialysis, digestion was unnecessary, as digested and undigested samples yielded equivalent counts. Radioactivity was measured as described by Eldefrawi *et al.* (5), except that twice the concentration of Beckman BBS-3 solubilizer (80 ml/liter) was used in the counting solution. Protein was determined by the method of Lowry *et al.* (13).

Binding constants were determined by a computerized iterative procedure designed to yield the theoretical curve for multicomponent binding expressed as a Scatchard plot that best fit the data.

The reversibility of muscarone and atropine binding was examined by first dialyzing seven identical bags containing the crude mitochondrial fraction against 10 nm radioactive ligand and then subjecting the bags to a second 24-hr dialysis against 100 volumes of Ringer's solution containing 0.01, 0.1, 1, or 10 μM nonradioactive ligand or, in the case of the control, 0.01 μM radioactive ligand. The radioactivity of bath and bag contents was then determined as usual.

Acetylcholinesterase activity was measured in a pH-stat at pH 7 and 25°, using 3

mM acetylcholine as substrate and 6.25 mM NaOH as titrating agent.

Thin-layer chromatography was carried out as described by Farrow and O'Brien (12), using chloroform–diethylamine (90:10) as solvent system for the separation of atropine, tropane, and tropic acid.

RESULTS

We first attempted to observe, by equilibrium dialysis, the binding of cholinergic ligands to a crude mitochondrial fraction containing synaptosomes, myelin, and mitochondria. For muscarone at 1 μM the radioactivity in a 0.10-ml sample from the dialysis bag was only slightly in excess of the bath radioactivity, but muscarone and atropine, both active at muscarinic receptors, showed considerable binding at 10 nm ligand (Table 1). By contrast, the nicotinic ligands decamethonium, curare, and nicotine bound minimally at 10 nM (Table 1).

We then examined the reversibility of muscarone and atropine binding at 10 nm (see METHODS). Muscarone binding was fully reversible when redialyzed against Ringer's solution. Atropine binding was not fully reversible when redialyzed against 100 volumes of Ringer's solution (Fig. 1), but 94% of the bound radioactive atropine was displaced by increasing concentrations of unlabeled atropine. Because atropine is an ester it was necessary to rule out the possibility that the "reversible" atropine binding we observed by this method was actually irreversible binding exhibiting a relatively rapid rate of hydrolysis of the bound ligand, i.e., a half-life of less than 6 hr. Therefore we assayed, by thin-layer chromatography, the amounts of atropine, tropane, and tropic acid in the supernatant fraction following centrifugation (100,000 × *g* for 45 min) of the contents of a sac after dialysis once against radioactive atropine at 10 nM. In duplicate experiments only 6 and 8% of the total radioactivity was not atropine. These findings are consistent with very high-affinity reversible binding. Indeed, subsequent experiments (see below) revealed an atropine binding constant of 0.6 nM.

TABLE 1

Binding of radioactive cholinergic ligands to a crude mitochondrial fraction (P_2) from rat brain

Fraction P_2, the crude mitochondrial fraction, was dialyzed overnight at 4° against a number of tritiated cholinergic ligands. The difference in disintegrations per minute between 0.10-ml samples from the bath and the dialysis bag contents is referred to as "radioactivity bound." Where standard deviations are not indicated the data are mean values from two experiments.

Ligand	Ratio of radioactivity bound to that in bath	Amount bound
	dpm:dpm	*nmoles/g protein*
Atropine, 10 nM	364:1,005	0.98
Muscarone, 1 μM	502 ± 330:19,560 ± 360	3.1
Muscarone, 10 nM	39 ± 9:204 ± 22	0.36
Decamethonium, 10 nM	10:419	0.06
Dimethyltubocurarine, 10 nM	−5:244	−0.04
Nicotine, 10 nM	21:826	0.07

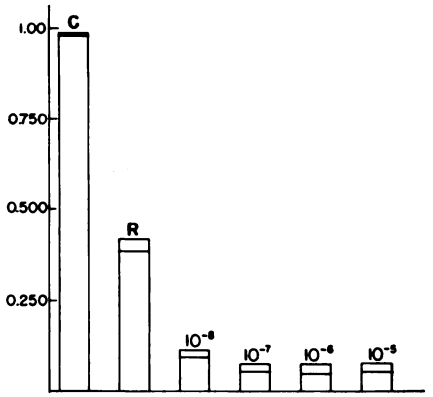

FIG. 1. *Reversibility of atropine binding*

Seven identical dialysis bags containing the crude mitochondrial fraction were first dialyzed against 10 nM [^{3}H]atropine (generally labeled) and then subjected to a second 24-hr dialysis against 100 volumes of Ringer's solution (R), 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M unlabeled atropine, or, in the case of the control (C), 10 nM [^{3}H]atropine. The radioactivity of bath and bag contents was then determined as usual. The atropine bound after the second dialysis step is expressed as a percentage of the atropine bound after the first dialysis step. Data are from duplicate experiments.

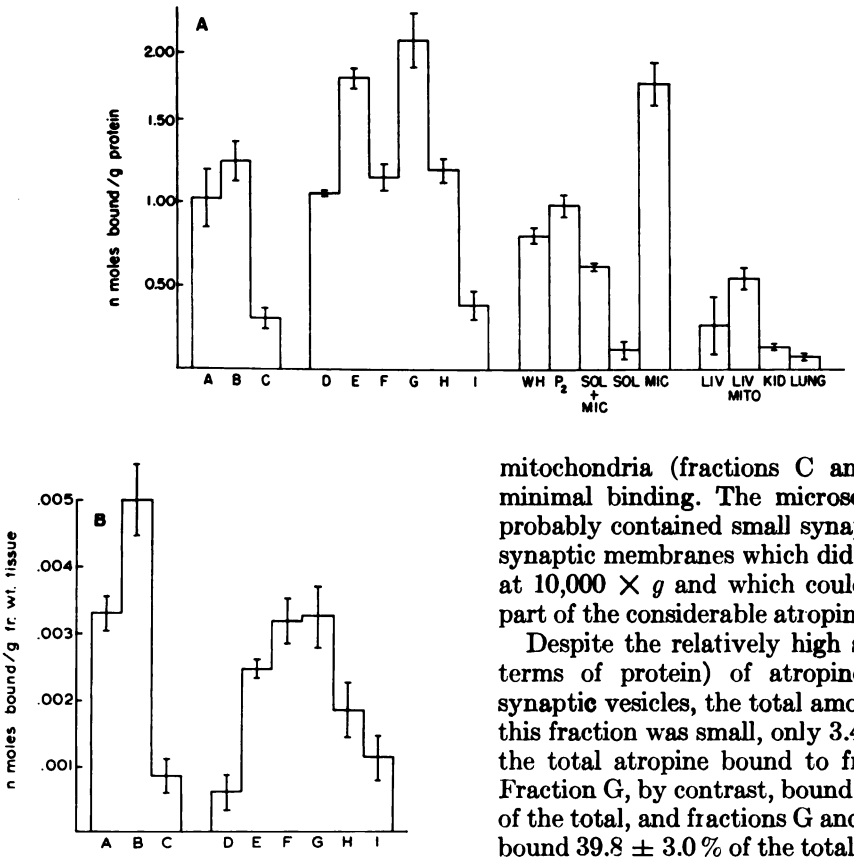

FIG. 2. *Binding of 10 nM atropine to subcellular fractions from brain, expressed as amount bound per gram of protein (A) or as amount bound per gram of tissue, fresh weight (B)*

Fraction A = myelin, B = synaptosomes, and C = mitochondria. D = synaptic vesicles, E and F = membranes of uncertain origin, G = pre- and postsynaptic membranes, H = intact synaptosomes, and I = mitochondria. WH = whole homogenate. P_2 = crude mitochondria. Sol = soluble. Mic = microsomal. Liv = liver. Liv mito = liver nitochondria. Kid = kidney.

We used two fractionation schemes to determine the subcellular localization of the muscarone and atropine binding at 10 nM (see METHODS). Atropine (Fig. 2A and B) bound more extensively, on either a protein or a tissue weight basis, to synaptosomes (fraction B), pre- and postsynaptic membranes (fraction G), and membranes of uncertain origin (fraction E and F) than to other fractions. Synaptic vesicles (fraction D) exhibited moderate binding whereas mitochondria (fractions C and I) showed minimal binding. The microsomal fraction probably contained small synaptosomes and synaptic membranes which did not sediment at 10,000 × *g* and which could account for part of the considerable atropine binding.

Despite the relatively high specificity (in terms of protein) of atropine binding to synaptic vesicles, the total amount bound to this fraction was small, only 3.4 ± 0.87% of the total atropine bound to fractions D–I. Fraction G, by contrast, bound 26.0 ± 0.2% of the total, and fractions G and H combined bound 39.8 ± 3.0% of the total.

The large variability of muscarone binding to subcellular fractions (Fig. 3A and B) resulted from low counts at 10 nM. Nevertheless it is clear that synaptosomes, synaptic membranes, and fraction E bound muscarone with higher specific activity (on a protein or tissue weight basis) than did myelin (fraction A) or synaptic vesicles. Unlike the atropine case, however, mitochondria exhibited binding activity. Of the total binding in fractions D–I, 21.6 ± 9.2% was to fraction I as compared to 23.4 ± 3.5% to fraction G and 38.5 ± 6.9% to fractions G and H combined.

To establish the tissue specificity of this binding we examined three non-neural tissues (Figs. 2A and 3A). Muscarone did not bind to whole 10% homogenates of liver, kidney, or lung or to liver mitochondria. Atropine did not bind to kidney or lung. The apparent binding of atropine to liver and liver mitochondria is probably due to the high concentration of atropines-

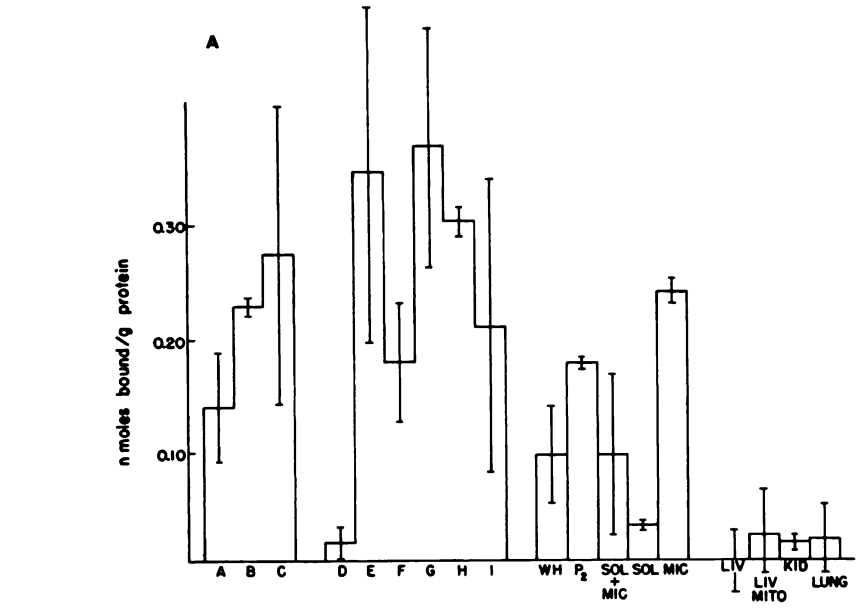

FIG. 3. *Binding of 10 nM muscarone to subcellular fractions from brain, expressed as amovnt bound per gram of protein (A) or as amount bound per gram of tissue, fresh weight (B)*

Fraction A = myelin, B = synaptosomes, and C = mitochondria. D = synaptic vesicles, E and F = membranes of uncertain origin, G = pre- and postsynaptic membranes, H = intact synaptosomes, and I = mitochondria. WH = whole homogenate. P_2 = crude mitochondria. Sol = soluble. Mic = microsomal. Liv = liver. Liv mito = liver mitochondria. Kid = kidney.

terase (atropine acylhydrolase, EC 3.1.1.10) in this tissue, in contrast to its absence in brain (14).

To examine whether atropine was bound by acetylcholinesterase, we measured the activity of this enzyme and atropine binding at a concentration of 10 nM on the same brain fractions (Table 2). The ratio of

TABLE 2

Hydrolysis of acetylcholine by brain fractions and its relationship to atropine binding

In duplicate experiments, binding of 10 nM atropine and hydrolysis of 3 mM acetylcholine were measured on the same samples. Acetylcholine hydrolysis was measured at pH 7.4 and 25° by titration with 6.25 mM NaOH, as described in (4). Fractions E and F are membranes of uncertain origin, fraction G is synaptic membranes, fraction H is intact synaptosomes, and fraction I is mitochondria.

Fraction	Atropine bound (*a*)	Acetylcholine hydrolyzed (*b*)	*a*:*b*
	pmoles/ml	*moles/ml/10 min*	
E	4.97	2.28	2.18
	6.29	3.12	2.01
F	7.48	3.48	2.14
	7.92	4.05	1.96
G	6.59	3.13	2.10
	6.41	2.85	2.25
H	1.92	0.813	2.36
	1.91	0.563	3.39
I	3.27	1.17	2.79
	3.12	1.11	2.81

atropine bound to acetylcholine hydrolyzed is similar in fractions E, F, and G but is markedly different in fraction I, which indicates that atropine binding cannot be ac-

counted for by binding to acetylcholinesterase. The nearly constant ratio in fractions E, F, and G, however, suggests a fixed relationship between the concentrations of the enzyme in these fractions.

Because fraction G (pre- and postsynaptic membranes) is both the most interesting fraction with regard to receptor activity and the most specific site of atropine binding (Fig. 2), we confined subsequent experiments to this fraction.

Binding to fraction G of atropine was measured and is expressed as a Scatchard plot in Fig. 4. Two modes of binding were detected, with binding constants of 0.6 ± 0.01 nM and 0.9 ± 0.18 μM and amounts of 6.6 ± 0.4 and 86 ± 27 pmoles/g of brain, wet weight, from which it was derived. We performed a similar experiment, employing a whole homogenate of rat brain. The two binding sites were present at 89 ± 18 and 910 ± 200 pmoles/g of tissue, fresh weight, respectively. Consequently the ratio of amounts of low- and high-affinity binding was similar in whole brain to that in fraction G; the respective ratios were 13 and 10. However, fraction G as isolated contained only about 8% of the atropine-binding activity of whole brain. We cannot specify the extent to which the other 92% represents synaptic membranes not recovered in fraction G, or atropine-binding activity in other structures.

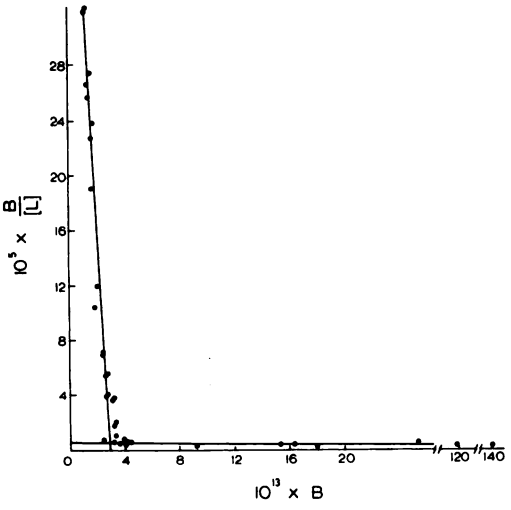

FIG. 4. *Binding of atropine from 40 pM to 5 μM to fraction G (synaptic membranes), expressed as a Scatchard plot*

B = moles of atropine bound per 100 μg of protein. *L* = final free atropine concentration (molar). The points are from two experiments. The lines result from computerized best-fit calculations.

TABLE 3

Effects of cholinergic and noncholinergic drugs on atropine binding to fraction G (synaptic membranes)

The atropine concentration was 5 nM and the drug concentration was 50 nM. The percentages are mean values from two experiments.

Drug	[Atropine binding (drug present)/ atropine binding (no drug)] × 100
Pilocarpine	102
Carbamylcholine chloride	88
Scopolamine	8
Nicotine	100
Succinylcholine chloride	104
Dimethyltubocurarine	96
Decamethonium bromide	102
Hexamethonium chloride	91
Tetraethylammonium iodide	98
DL-Norepinephrine	107
Epinephrine	102
Serotonin	102
γ-Aminobutyric acid	91
Glutamate	94
Iproniazid	101
Picrotoxin	97

Finally, we examined the interference of drugs at 50 nM with atropine binding at 5 nM to fraction G (Table 3). Scopolamine, the only muscarinic antagonist tested, was also the only drug that largely blocked atropine binding. Muscarinic agonists, nitotinic agonists and antagonists, and noncholinergic transmitters and drugs had little or no effect on atropine binding.

DISCUSSION

Our early studies of muscarone binding to rat brain employed 1 μM ligand (1) and failed to demonstrate binding. It is now apparent that this failure was due in part to the use of too high a muscarone concentration, in part to the very low amount of binding in brain, in comparison with the *Torpedo* electroplax

then under study, and in part to the use of the relatively insensitive centrifugal technique.

The crucial question is: What is the evidence that the bindings reported herein are to the physiological acetylcholine receptor?

As in previous studies, we have been able to show suitable reversibility, affinity, and sensitivity to drug blockade (although we were surprised by the specificity of that blockade, with only scopolamine giving a marked effect at the low drug concentration tested). It is noteworthy that no binding of the nicotinic type was found; that is, nicotinic ligands (nicotine, decamethonium, dimethyltubocurarine) did not bind, nor did they block atropine binding. This finding contrasts with that in fly brain (3) where nicotinic and muscarinic characteristics were noted in the binding. Data from the microelectrophoretic application of drugs to single neurons in mammalian brains strongly suggest that both types of receptor are present (15), but do not indicate the relative amounts. To account for the absence of nicotinic binding, one must assume either that the muscarinic agents have higher affinity for their binding sites or that these sites are present at several times the concentration of the nicotinic sites.

The principal new features of this study are that the bindings observed, for both muscarone and atropine, were found essentially only in nervous tissue, not in nonnervous; and the binding showed highest specific activity to the subfraction from disrupted synaptosomes which contained postsynaptic membranes.

Two binding modes for atropine were found in the synaptic membrane fraction, with K_1 = 0.6 nM and K_2 = 0.9 μM. Paton and Rang (16) found two binding modes for atropine in guinea pig intestine, with K values remarkably similar to ours; that is, K_1 = 1.11 nM and K_2 = 0.45 μM. In their case, only the high-affinity binding could be demonstrated in studies of the physiological interference of atropine with the stimulatory action of acetylcholine, for which K = 1.11 nM. This suggests that the high-affinity binding was to the physiological receptor, and the low-affinity binding was to some other inactive binding site. The same may be true in rat brain.

Because the low-affinity site had a binding constant of about 800 nM, the data of Figs. 1 and 2, measured with 10 nM atropine, would show a minimal contribution from the low-affinity binding site (only 1% of which binds at 10 nM) despite the existence of about 10 times more low-affinity than high-affinity binding sites in whole brain and in fraction G. By contrast, at 10 nM atropine the high-affinity binding sites are 94% saturated and so represent most of the observed binding. The same is true of the data on drugs in Table 3, obtained at 5 nM atropine. It follows that the data on distrbution and drug response refer primarily to high-affinity binding. The low-affinity binding is not characterized in this paper.

Pilocarpine and carbamylcholine, muscarinic agonists, did not block atropine binding, but scopolamine (like atropine, a muscarinic antagonist) did. Either the atropine-binding site has a higher affinity for the antagonists than for the agonists or it is distinct from the agonist sites. More detailed study will decide this point.

The binding constant of atropine for butyrylcholinesterase is in the region of 0.1 mM, while for acetylcholinesterase the affinity is even less (17). The data reported in Table 2 suggest that atropine binding is not to acetylcholinesterase. These findings agree with the extensive data reported for *Torpedo* electroplax, showing that cholinergic ligand binding and acetylcholinesterase activity are associated with different macromolecules (6).

In conclusion, these observations are consistent with the hypothesis that the observed binding of atropine is to a physiological site of atropine action, which is either identical with or associated with a set of muscarinic receptors in brain. High-affinity, reversible muscarone binding is similarly concentrated in synaptosomes and synaptic membranes and absent from kidney, liver, and lung. The finding supports the view that in previous studies with labeled muscarone we were indeed measuring binding to the acetylcholine receptor in electroplax and housefly brain.

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