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EFFECTS OF COPPER ON THE BINDING OF AGONISTS AND ANTAGONISTS TO MUSCARINIC RECEPTORS IN RAT BRAIN

JUDITH R. FARRAR* and WAYNE HOSS†

Center for Brain Research, University of Rochester, School of Medicine and Dentistry, Rochester, NY 14642, U.S.A.

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Abstract—Studies were performed to assess the effects of copper treatment *in vitro* on muscarinic binding parameters in rat brain homogenates. Brainstem, an area low in copper, was found to be insensitive to copper treatment as compared to forebrain, a region of relatively high copper content. Inclusion of 3 μM copper in forebrain homogenates decreased the number of sites seen by [^{3}H]-*l*-quinuclidinyl benzilate (QNB) by 40–50%. Copper-enhanced displacement of bound QNB was noted for agonists and antagonists. Both ligands showed maximal effects at 6 μM copper, although quantitative differences could be determined at any copper level. At levels of maximal effect, the increase in QNB displacement was greater than or less than 50% for agonists and antagonists respectively. Two-site analyses of carbamylcholine (CCH) binding showed that the addition of 1 μM copper to forebrain homogenates increased the percentage of high affinity sites (α) from 42 to 70%. The IC_{50} decreased from 3.1 to 1.7 μM, but the dissociation constants for the high and low affinity sites were not changed. The effect of added copper on CCH binding to muscarinic receptors was reversible with the addition of the copper-chelating agent triethylene tetramine.

Metal ions *in vitro* can function as allosteric effectors modifying binding parameters in a variety of hormone and neurotransmitter systems including anterior and posterior pituitary preparations [1–4], peptide hormone [5] and adrenergic [6–8] receptor mechanisms, and assays of opiate [9, 10], histaminergic [11], dopaminergic [12, 13], muscarinic [14], and progesterone [15] binding sites. A potential role for copper in the normal function of brain muscarinic receptors is suggested by binding alterations noted in the presence and absence of copper and by receptor localization in areas of relatively high copper content.

A number of workers have attempted to define the copper content of specific brain regions [16–20]. These studies have generally indicated high copper areas overlapping sites of muscarinic innervation despite different methods and/or units of measurement. The hippocampus and striatum have the highest copper content.

Copper localization, in areas of muscarinic innervation, may not be enzyme-related, reflecting, perhaps, a more basic involvement in receptor mechanisms. Work examining the effects of heavy metal ions on muscarinic binding found Cu^{2+} to be specially potent, exerting effects on agonist and antagonist binding at micromolar concentrations [14].

An *in vitro* model of muscarinic function based on work performed in this laboratory incorporates membrane thiol groups as determinants of binding properties [14]. Speculation as to the existence of any comparable *in vivo* system must take into account copper-related changes in muscarinic binding and the distributional overlap of brain copper and muscarinic receptors. In addition, the level of brain copper can be manipulated *in vivo*. Experiments were, therefore, designed to characterize further the action of copper on brain muscarinic receptors. This paper describes the effects of added copper *in vitro* on the binding of muscarinic antagonists and agonists in regional homogenates of rat brain.

MATERIALS AND METHODS

Materials. Male albino rats of the Sprague–Dawley strain were purchased from the Charles River Breeding Laboratories. Carbamylcholine (CCH) chloride, gallamine triethiodide and cupric chloride were obtained from the Aldrich Chemical Co., K & K Laboratories, and Mallinckrodt, Inc., respectively. Scintanalyzed grade toluene was acquired from the Fisher Chemical Co. The nitric and perchloric acids used for the preparation of atomic absorption samples were ACS grade (Baker-analyzed) from VWR. The copper standard solution for atomic absorption spectrophotometry was purchased from Ventron (Alfa division). [^{3}H]-Quinuclidinyl benzilate (QNB)-*l*-isomer, sp. act. 32–44 Ci/mmole, was obtained from Amersham. Radiolabeled samples were counted with a Searle model 300 liquid scintillation counter at an efficiency of 30%.

Brain metal content. Metal-ion content was deter-

* Present address: Department of Ophthalmology, University of Rochester, School of Medicine and Dentistry, Rochester, NY 14642.

† Author to whom all correspondence should be addressed.

mined in six specific brain regions (cerebellum, brainstem, midbrain, cortex, hippocampus, and striatum) as well as forebrain. Brain tissue was prepared for analysis using the wet digestion technique of Harrison *et al.* [17]. Briefly, this involved drying the tissue at 110° for 12 hr followed by digestion in 1:1 concentrated HNO_3:$HClO_4$ at 200°. Digestion was allowed to proceed until the white fumes of $HClO_4$ were evolved, and the samples were water clear. Copper content was determined by atomic absorption spectrophotometry using a Perkin Elmer model 372 Atomic Absorption Spectrophotometer at a wavelength of 324.8 nm and a slit width of 0.7 nm. Acetylene and air were used as fuel and oxidizer respectively. Digested samples were diluted as necessary with deionized water and measured against a reagent blank. Copper recovery, as measured by the addition of two different amounts of copper to tissue digests, was virtually 100% for all brain areas.

Neural membrane preparation. Crude membrane preparations from rat hippocampus, striatum and cortex, as well as from forebrain and brainstem, were used in the various binding assays. The tissue was homogenized in 10 vol. of 40 mM sodium–potassium phosphate buffer, pH 7.4, and centrifuged at 3000 *g* for 10 min. The supernatant fraction was then centrifuged at 60,000 *g* for 30 min. The resulting pellet was suspended in 40 mM sodium–potassium phosphate buffer, pH 7.4. Protein content was determined by a modification of the method of Lowry *et al.* [21]. Aliquots of the suspension were frozen rapidly in liquid nitrogen and stored at −20°. Binding studies showed these samples to remain stable and comparable to fresh homogenates for at least 4 weeks.

Binding assays. The muscarinic binding assays employed a modification of the filtration assay developed by Yamamura and Snyder [22] which utilizes the binding of QNB, a specific and potent muscarinic antagonist. Suspensions containing 60 μg protein/ml for forebrain and 30 μg protein/ml for brainstem, 40 mM Na^+–K^+ phosphate buffer, pH 7.4, and various concentrations of [^{3}H]-QNB were incubated at room temperature for 1 hr. Control samples contained a 1000-fold excess of unlabeled QNB. Samples were filtered with suction through Whatman GF/B glass fiber filters, and washed twice with 5 ml of cold phosphate buffer. The filters were placed in Nalge scintillation bags with 4 ml of Triton–toluene scintillation fluid. The bags were then placed in scintillation vials and held for at least 12 hr before counting. Specific binding was defined as the difference between the values obtained in the absence and presence of excess unlabeled QNB.

Indirect binding was determined by the ability of the ligand to compete with 50 pM [^{3}H]-*l*-QNB for the receptor. Experimental conditions were carefully determined and strictly maintained. Each sample contained approximately 10 pM receptors (4.0 μg/ml protein for forebrain and 5.4 μg/ml protein for brainstem) in a final volume of 20 ml. This receptor concentration had no significant effect on the ligand-induced inhibition of [^{3}H]-QNB binding. Addition of receptor homogenate to individual sample tubes was staggered so that the incubation time for each tube was exactly 90 min.

The effect of copper on muscarinic binding properties *in vitro* was determined by including 3 μM $CuCl_2$ in the incubation medium for direct binding assays and 1 μM $CuCl_2$ in the medium for indirect studies. The concentration of Cu^{2+} was unbuffered and thus represents total added Cu^{2+}.

The effect of the copper chelators, penicillamine (PA) and triethylene tetramine (TETA), on muscarinic receptors were determined by incubating membrane aliquots with 1 mM reagent for 20 min at room temperature. The modifying reagents were removed by centrifugation at 30,000 *g* for 20 min. The pellets were rinsed with cold phosphate buffer, pH 7.4, resuspended, and recentrifuged prior to use. Preincubation with 50 μM copper was performed in a similar manner. In this case, the incubation and washing procedures were then repeated for the other modifying agents.

RESULTS

Antagonist binding. Studies comparing the effects of increasing total added Cu^{2+} concentration on QNB binding in forebrain and brainstem are shown in Fig. 1. Forebrain was more sensitive than brainstem to increasing medium Cu^{2+}. Receptor occupancy in the presence of 60 μM Cu^{2+} declined by approximately 56% in the forebrain and 17% in the brainstem. A

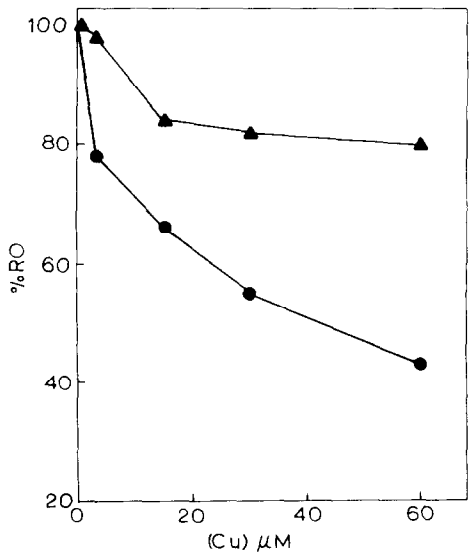

Fig. 1. Effect of increasing medium copper on the specific binding of 0.1 nM [^{3}H]-QNB in homogenates prepared from brainstem (▲), a low copper region, and forebrain (●), an area of relatively high copper content. The data show the means of three experiments each performed in triplicate. The ordinate represents percent receptor occupancy.

level of 3 μM Cu^{2+} was sufficient to reduce the number of forebrain sites seen by QNB by more than 20% without significantly altering receptor occupancy in the brainstem.

Specific effects of Cu^{2+} on antagonist binding were examined using homogenates prepared from three forebrain regions (hippocampus, striatum and cortex) as well as whole forebrain. A representative QNB binding curve for a striatal preparation in the presence and absence of 3 μM Cu^{2+} is shown in Fig. 2.

Table 1. Effects of copper on the binding of *l*-QNB to rat brain regions

	Apparent number of binding sites (pmoles/mg protein)			Dissociation constant for QNB binding ($K_d \times 10^{-10}$ M)		
	N*	No added copper	+3 μM copper	N	No added copper	+3 μM copper
Hippocampus	4	2.70 ± 0.01†	1.40 ± 0.02‡	4	0.53 ± 0.04	0.44 ± 0.06§
Striatum	4	3.60 ± 0.03	2.40 ± 0.01‡	4	0.53 ± 0.08	0.43 ± 0.05§
Cortex	4	2.10 ± 0.02	1.05 ± 0.01‡	4	0.49 ± 0.07	0.45 ± 0.07§
Forebrain	4	2.45 ± 0.01	1.30 ± 0.01‡	4	0.59 ± 0.08	0.37 ± 0.03§
Brainstem¶	2	1.85	1.45	2	0.44	0.46

* Number of experiments.
† S.E.M.
‡ $P < 0.001$.
§ $0.01 < P < 0.025$.
¶ Values are the means of two experiments each performed in triplicate and differing by less than 10%.

Cu^{2+}-induced alterations are evident. Copper treatment decreased the number of binding sites seen by QNB and enhanced receptor affinity for the drug. Similar trends were noted in all brain regions examined. Values for receptor occupancy (B_{max}) and dissociation constants (K_d) are shown in Table 1. Correlations between copper treatment and the decrease in the values of B_{max} and K_d were significant ($P < 0.001$ and $0.010 < P < 0.025$ respectively).

Agonist binding. Figure 3 shows the effect of increasing medium Cu^{2+} on the binding of the muscarinic agonist, carbamylcholine (CCH), in forebrain and brainstem. As was the case for QNB binding, the forebrain showed a greater sensitivity to the inclusion of Cu^{2+} in the incubation medium. Displacement of bound QNB by 10^{-6} M CCH in the brainstem was not affected significantly by any level of medium Cu^{2+}. However, copper levels as low as 3 μM increased CCH binding by more than 40% in the forebrain. A copper level of 1 μM enhanced the binding of CCH in the forebrain by 20% without any effect on direct antagonist binding.

A comparison of Figs. 1 and 3 indicates differences in the effects of medium Cu^{2+} on agonist and antagonist behavior. As *in vitro* copper levels increased, QNB binding measured directly decreased, whereas CCH binding indirectly measured by the displacement of QNB increased. The question arises as to whether these trends represent a real difference in the copper-induced binding behavior of agonists and antagonists, or whether they merely reflect the direct versus indirect nature of the binding studies performed. Figure 4 shows the effect of *in vitro* copper on the displacement of bound QNB by 5×10^{-10} M *d,l*-QNB and by 5×10^{-6} M CCH in normal forebrain. Both show increased levels of displacement

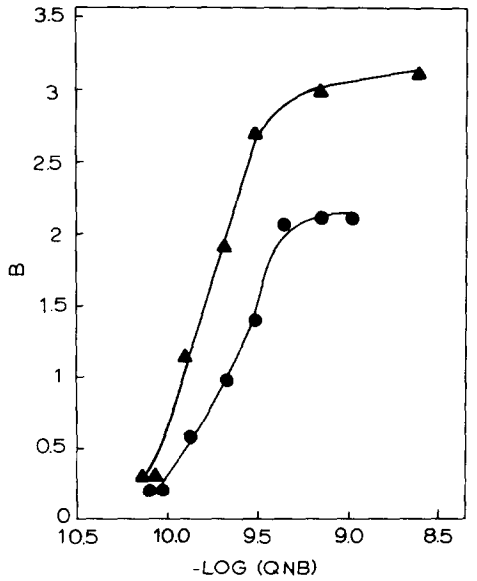

Fig. 2. Log-dose curves for a typical experiment depicting the specific binding of [^{3}H]-QNB in crude synaptosomal membranes from normal striatum in the presence (▲) and absence (■) of 3 μM Cu^{2+} in the incubation medium. Binding (B) is expressed as picomoles per mg protein. The curves represent a single example of four experiments each performed in triplicate.

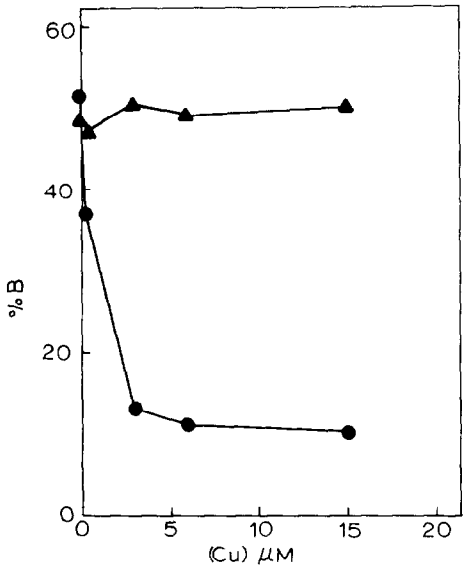

Fig. 3. Effect of increasing medium Cu^{2+} on the displacement of 50 pM [^{3}H]-QNB by 5 μM and 1 μM carbamylcholine in forebrain (●) and brainstem (▲) preparations respectively. The reduction in % QNB bound (% B) indicates increased carbamylcholine binding. The data represent the means of three experiments each performed in triplicate.

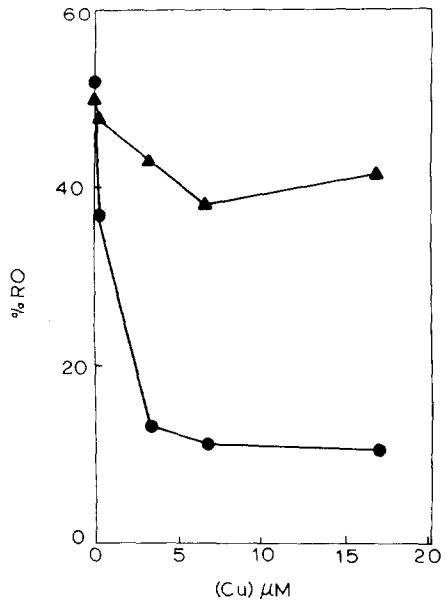

Fig. 4. Effect of increasing medium Cu^{2+} on the displacement of bound [^{3}H]-QNB induced by 0.5 nM *d,l*-QNB (▲) and 5 μM carbamylcholine (●) in forebrain. The binding of [^{3}H]-QNB is shown as percent receptor occupancy (% RO). Decreased receptor occupancy is indicative of increased binding by the unlabeled ligand. The data represent the composite of two experiments each performed in triplicate and differing by less than 10%.

with increased medium copper. However, the effect of copper on *d,l*-QNB was so slight as to be insignificant at any level of medium copper. The effect of copper treatment on the displacement of bound QNB by CCH was dramatic and significant ($P < 0.001$) in comparison.

Studies were performed to determine whether the effects of copper on the displacement of QNB *d,l*-QNB and CCH were specific to these ligands or could be generalized to all muscarinic antagonists and agonists. Figures 5 and 6 show the effects of increasing medium copper on the displacement of bound QNB by a number of muscarinic antagonists and agonists respectively. In all cases, the effect on agonist binding was considerably greater than that on antagonist binding. Both types of ligands attained maximal effects at 6 μM copper. However, quantitative differences between the copper-induced binding behavior of agonists and antagonists could be determined at any level. Inclusion of as little as 1 μM copper in the incubation medium increased displacement of bound QNB by both groups. However, the increase was always less than 12% for antagonists and greater than 12% for agonists. Likewise, at levels of maximal effect (≥ 6 μM copper), the increase in QNB displacement was less than 50% or greater than 50% for antagonists or agonists respectively. This quantitative difference in copper-induced binding can be used to distinguish ligands of 'questionable' agonist–antagonist behavior. For example, in Fig. 5, gallamine, an antagonist which sees affinity subpopulations similar to those seen by CCH [23], displays

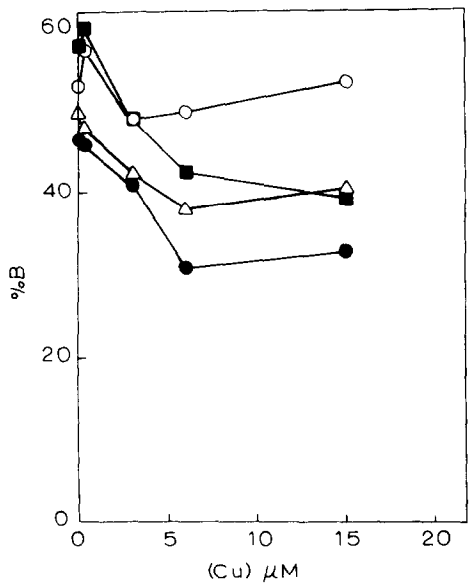

Fig. 5. Antagonist-induced displacement of bound [^{3}H]-QNB in the presence of increasing medium Cu^{2+}. Displacement was determined in forebrain homogenates using the following antagonists at concentrations approximating the appropriate dissociation constants: 0.5 nM *d,l*-QNB (△), 0.5 nM scopolamine (○), 1 nM atropine (■), and 1 μM gallamine (●). The decrease in percent QNB bound (% B) indicates increased binding by the unlabeled ligand. Data shown represent the means of two experiments each performed in triplicate and differing by less than 10%.

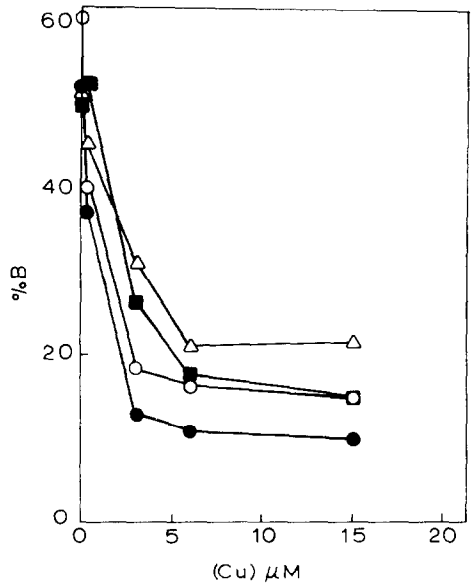

Fig. 6. Agonist-induced displacement of bound [^{3}H]-QNB in the presence of increasing medium Cu^{2+}. Displacement was determined in forebrain homogenates using the following agonists at concentrations approximating reported IC_{50} values: 5 μM carbamylcholine (●), 5 μM arecoline (△), 0.5 μM oxotremorine (■), and 1 μM pilocarpine (○). The reduction in percent QNB bound (% B) represents increased agonist binding. The data show the means of two experiments each performed in triplicate and differing by less than 10%.

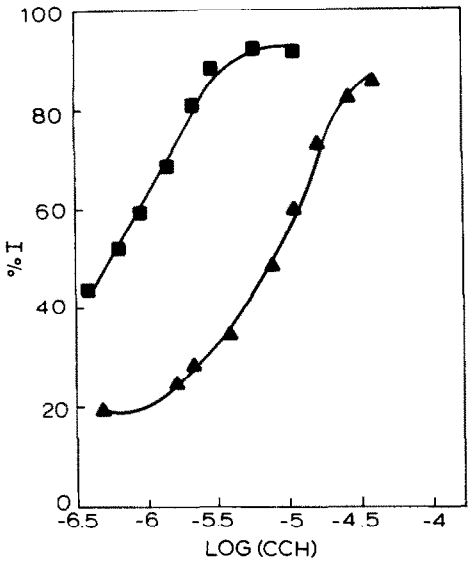

Fig. 7. Plots of percent inhibition (% I) by carbamylcholine (CCH) of the binding of 50 pM [^{3}H]-QNB in a normal forebrain homogenate in the presence (■) and absence (▲) of 1 μM Cu^{2+}. The curves represent a single example of four experiments each performed in triplicate.

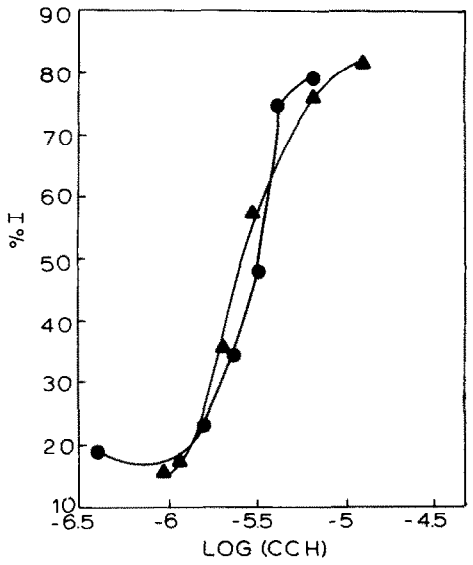

Fig. 8. Inhibition of the binding of 50 pM [^{3}H]-QNB by carbamylcholine (CCH) in a brainstem preparation in the presence (●) and absence (▲) of 1 μM Cu^{2+}. The curves represent a single example of two experiments each performed in triplicate and differing by less than 10%.

copper-induced binding changes that identify it as an antagonist (e.g. 50% increase in binding at 6 μM copper). On the other hand, in Fig. 6 pilocarpine, a partial agonist, clearly shows agonistic behavior with respect to the inclusion of copper in the incubation medium.

Binding assays were performed to substantiate further the effect of 1 μM Cu^{2+} on agonist (CCH) binding in brainstem and forebrain. Representative inhibition curves for the two regions in the presence

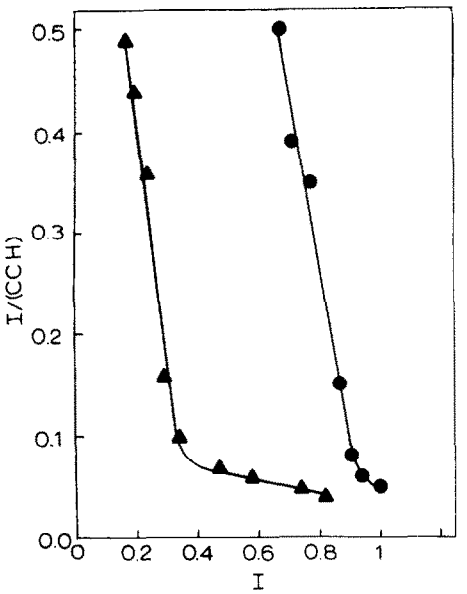

Fig. 9. Scatchard plots for a typical assay of the binding of carbamylcholine (CCH) to crude synaptosomal membranes from rat forebrain in the presence (●) and absence (▲) of 1 μM Cu^{2+}. The abscissa represents inhibition (I) of [^{3}H]-*l*-QNB binding. The curves represent a single example of four experiments each performed in triplicate.

and absence of Cu^{2+} are presented in Figs. 7 and 8. An increase in the affinity of the forebrain binding sites for CCH in the presence of Cu^{2+} is suggested by the displacement of the curve to the left. Copper had little effect on the binding of CCH in brainstem as indicated by the overlap of the 0 copper and + copper curves for this region. Cu^{2+}-enhanced affinity of forebrain sites for CCH is also evident in the shift of the Scatchard plot to the right (Fig. 9). Scatchard analysis using the method of Rubinow [24] indicated that the inclusion of 1 μM Cu^{2+} in forebrain but not the brainstem homogenates resulted in a significant drop in the value of IC_{50} and a significant increase in the proportion of high affinity sites (α). Values for the agonist binding parameters, α, IC_{50}, K_H, K_L are presented in Table 2. Cu^{2+} had no significant effects on any of the values determined for brainstem sites.

Brain copper levels. To determine whether the concentration of Cu^{2+} required to produce effects on muscarinic receptors *in vitro* was in the range of Cu^{2+} levels *in vivo*, brain copper was determined by atomic absorption spectrophotometry. The levels of copper in several brain regions are shown in Table 3. Assuming that brain tissue is 80% water, the overall concentration of copper in the tissue ranged from 44 to 108 μM. Although most of the copper is expected to be bound in various compartments, including membranes, the amount of copper required for the effects on the binding of ligands to muscarinic receptors represents only a small fraction of the total copper.

Reversibility of the copper effect. To address the reversibility of Cu^{2+}-induced effects on muscarinic receptors *in vitro*, a membrane preparation from rat forebrain was preincubated with 50 μM Cu^{2+} and washed by centrifugation. This produces approxi-

Table 2. Effect of copper on the binding of carbamylcholine to rat brain muscarinic receptors

	N*	IC_{50} (μM)	α†	K_H (μM)	K_L (μM)
No added copper					
Forebrain	4	5.69 ± 0.21‡	0.42 ± 0.08	0.26 ± 0.02	40.14 ± 10.24
Brainstem§	2	1.60	0.54	0.03	1.91
+1 μM copper					
Forebrain	4	1.00 ± 0.14‖	0.70 ± 0.06¶	0.28 ± 0.05	34.42 ± 5.82
Brainstem§	2	1.48	0.52	0.03	2.26

* Number of experiments.
† Proportion of high affinity receptors.
‡ S.E.M.
§ Values are the means of two experiments each performed in triplicate and differing by less than 10%.
‖ $P < 0.01$.
¶ $P < 0.05$.

Table 3. Relative distribution of copper in the rat brain

Region	Copper (μg/g dry wt)
Cerebellum	13.3 ± 0.57*
Midbrain	15.5 ± 1.10
Brainstem	14.1 ± 0.46
Hippocampus	34.0 ± 1.84
Striatum	34.5 ± 1.11
Cortex	16.5 ± 0.99
Forebrain	32.4

* S.E.M. of four experiments each performed in quadruplicate. The forebrain value is the mean of two experiments each performed in quadruplicate.

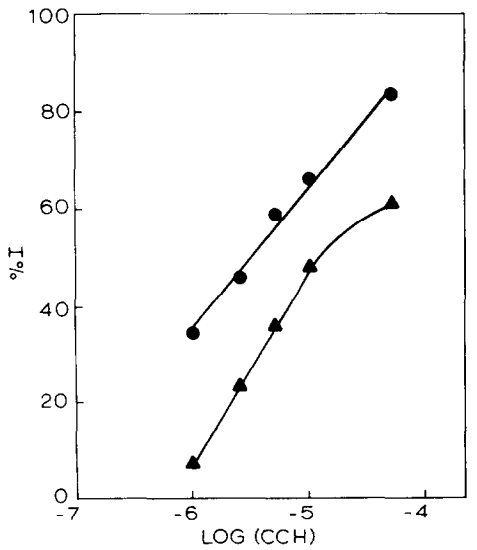

Fig. 10. Effect of the treatment of forebrain preparations with 1 mM triethylenetetramine (▲) on the carbamylcholine-induced displacement of [^{3}H]-QNB (50 pM) binding after preincubation with 50 μM Cu^{2+}. The inhibition curve for the buffer-washed control (●) is shown for comparison. Data shown represent the means of two experiments each performed in triplicate and differing by less than 10%. The ordinate represents percent inhibition (% I).

mately the same effects on the binding parameters as observed with the inclusion of 1 μM Cu^{2+} during the binding assay. Subsequent incubation with 1 mM of the copper-chelating agent TETA shifted the CCH inhibition curve to the right (Fig. 10) as expected for removal of copper. On the other hand, TETA had virtually no effect on a washed control membrane preparation without pretreatment with Cu^{2+}.

DISCUSSION

Low concentrations of copper *in vitro* altered the binding of both agonists and antagonists to muscarinic cholinergic receptors in rat brain. The major effects were a decrease in apparent receptor number, an increase in affinity for QNB, and a dramatic decline in agonist affinity. Using a two-site model, the data are compatible with a Cu^{2+}-induced shift in receptor subtype towards higher affinity receptors. The concentration of copper required to produce these effects was a small fraction of the total copper normally present in brain.

In general, tissues prepared from brain regions of high copper content are the most sensitive to copper treatment. The cortex is exceptional, having a relatively low level of copper but displaying a robust response to Cu^{2+} *in vitro*. Lack of a strict correlation is not surprising since most brain copper is associated with enzymes such as cytochrome oxidase present in large amounts. In addition, muscarinic cholinergic receptors represent only a very small fraction of 1% of the total protein in brain.

The copper chelator, TETA, which together with PA is used clinically to deplete excess copper in Wilson's Disease and industrial copper poisoning [25], reversed the effects of Cu^{2+} treatment. The binding alterations are, thus, not owing to any Cu^{2+}-related oxidative or catalytic reaction. In contrast to TETA, pencillamine itself produced effects similar to copper. This was probably due to the formation of a disulfide bond between the chelator and protein sulfhydryl moieties. It is well known that sulfhydryl modifying reagents such as *N*-ethylmaleimide can influence muscarinic binding parameters.

There is a fundamental difference between the binding of antagonists and agonists to muscarinic receptors distinguishable by response to copper treatment. The interaction of phenyl, hydroxyl, and

other antagonist substituents with accessory regions near the receptive site may contribute to this effect.

Copper treatment also differentiates brainstem and forebrain receptors. The disparity is particularly striking for agonist binding. This, together with the dissimilarity of forebrain and brainstem in the binding of the weak antagonists pirenzepine [26] and gallamine [23], raises the possibility of qualitative differences between brainstem and forebrain receptors.

Differences between brainstem and forebrain have been reported for the distribution of agonist affinity binding sites and the effects of various agents, including sulfhydryl ligands [14, 27–29] and ions [14, 30–32] on these sites. The response of cyclic nucleotide levels to muscarinic agents is also dependent on brain region [33–35]. Although brainstem contains fewer total receptors, they have a relatively higher affinity for agonists. Guanine nucleotides in the presence of Mg^{2+} modulate the binding of agonists in brainstem but not forebrain receptors [36].

Variations in the sensitivity of forebrain and brainstem muscarinic receptors to copper treatment could result from the presence of specific copper binding proteins in the brainstem, reducing the amount of copper available for interaction with receptors. The occurrence of abnormal metal-binding proteins in Wilson's Disease and Menke's Syndrome, human copper pathologies with well-documented neurologies [37, 38], supports involvement of copper-binding proteins other than cuproenzymes in brain function.

Whether or not copper serves as a modulator of muscarinic function in brain remains a question; however, certain observations are relevant. It has been established that relatively minor alterations in membrane fluidity, redox state, or protein constituents can alter significantly muscarinic binding properties. Copper, like other trace metals, can influence biomembrane stability by all of these mechanisms. The cupric ion is a strong sulfhydryl ligand and has a propensity for forming extremely stable square planar complexes. These properties make this ion a likely candidate for membrane–protein interactions such as have been suggested to regulate agonist affinity properties. The amount of Cu^{+} versus Cu^{2+} resulting from the addition of Cu^{2+} to the incubation medium containing the tissue is unknown. Nevertheless, the addition of low concentrations of Cu^{2+} produces consistent changes in muscarinic receptor properties. Regulation of receptor function by a Cu^{2+} protein *in vivo* has been demonstrated for opiate binding [39].

In conclusion, a role for copper in normal muscarinic function is suggested by (1) the distributional overlap between brain copper and muscarinic receptors, (2) the effects of copper treatment on muscarinic binding in brain regions differing in copper content, and (3) the chemical properties of copper and its protein complexes. The functional significance of copper distribution and muscarinic heterogeneity in brain warrants further investigation. A future communication will describe modulation by copper of muscarinic receptor function in brain *in vivo* using a nutritionally controlled animal model.

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