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COPPER IONS AND DIAMIDE INDUCE A HIGH AFFINITY GUANINE-NUCLEOTIDE-INSENSITIVE STATE FOR MUSCARINIC AGONISTS

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The binding capacity of [^{3}H]-acetylcholine for muscarinic receptors of rat cerebral cortex membranes is increased in the presence of Cu^{2+} ions from 690 to 1320 fmol/mg protein with no significant change in affinity. Membranes treated with 50 μM Cu^{2+} and washed retain the increased binding capacity. Agonist binding in copper-treated membranes is insensitive to guanylylimidodiphosphate even at high concentrations (>200 μM). Similar results were obtained when the sulfhydryl oxidizing agent, diamide (2 mM) was substituted for Cu^{2+} in the treatment of membranes. These data suggest the involvement of inter- or intra-molecular SH / S-S transitions in the interaction between the muscarinic receptor and a guanine nucleotide binding regulatory protein.

The interaction of agonists with the muscarinic cholinergic receptor complex is thought to involve multiple binding sites of differing affinities for the agonist (for recent reviews see 1,2). Transition metals (3-5), and guanine nucleotides (6) exert strong effects on muscarinic agonist affinity, which have been interpreted as resulting from modifications in the proportions of the different agonist binding states within the heterogeneous site population (3-5). The conversion induced by transition metals can be reversed by micromolar concentrations of guanine nucleotides suggesting a common site of action (3).

Copper is a transition metal of ubiquitous distribution in mammalian tissues including the central nervous system (7-9), and is thus potentially capable of influencing neuronal function. Addition of copper has been shown to increase the apparent affinity of muscarinic agonists (4) by increasing the proportion of high affinity sites (5). Recently we suggested that the action of this metal at cholinergic receptor sites may contribute to certain types of hypersensitivity reactions to copper (10). In this communication we explore the effect of cupric ions on the muscarinic receptor by direct measurement of agonist occupancy with [^{3}H]-acetylcholine, ([^{3}H]-ACh) (11). A possible mechanism for copper's action on the muscarinic receptor involves its known affinity for sulfhydryl residues. Sulfhydryl modification has been shown to result is marked changes in muscarinic agonist affinity (4, 12-16). Therefore, in addition we compare the effects of cupric ions with those of the sulfhydryl oxidizing agent, diamide, which has been previously used to modify opiate binding sites (17). The results illustrate the importance of sulfhydryl groups as regulators of the interaction between the neurotransmitter, acetylcholine, and the muscarinic receptor.

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METHODS

[^{3}H]-acetylcholine of high specific radioactivity (86 Ci/mmol, 97% purity) was purchased from Amersham Corporation. The labeled muscarinic antagonist [^{3}H]-N-methyl-4-piperidyl benzilate, ([^{3}H]-4NMPB), (69 Ci/mmol) was the same as previously described (18). Metals (in the form of chloride salts) were from Merck. Nucleotides, diamide, and other reagents were from Sigma Chemical Company.

Hypotonically washed rat (male, C-D strain) cerebral cortex or medulla-pons membranes were prepared as described elsewhere (11). Essentially, two preincubations in 50 mM Tris-HCl (pH 7.4, 25°C, 30 min.) preceded all experimentation described. The final pellet was resuspended in modified Krebs buffer (118 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, 1.9 mM $CaCl_2$ and 25 mM Tris-HCl pH 7.4 at 25°C) and then used for binding assays or submitted to the pretreatments indicated. A fresh solution of diisopropyl-fluorophosphate (DFP) in water was prepared and an appropriate amount added to acheive a 200 µM final concentration. After a further 30 min. preincubation, 20 µl aliquots were assayed in a volume of 40 µl containing 200 µM DFP and the indicated concentrations of ligands and reagents. Following a one hour incubation at 25°C, 2 ml of ice-cold buffer was added and the contents were filtered under high pressure through GF/C filters (Whatman). The filters were immediately washed with an additional 2 ml of buffer; filtration time was ~2 sec. Filters were counted for tritium using a scintillation cocktail (Hydroluma) and a scintillation spectrometer (TriCarb 300, Packard). Specific binding was taken as the difference between the total binding to control membranes and the measured non-specific binding, i.e., binding to membranes after adding 20 µM atropine during the last 10 min. of the preincubation step. Binding of the antagonist [^{3}H]-4NMPB was carried out similarly, except that the filters were washed with 4x3ml of buffer. Competition assays were performed as previously described (3) employing 2 nM [^{3}H]-4NMPB and various concentrations of carbamylcholine. All determinations were carried out in quadurplicate, each varying by <15%. Protein concentration was determined by the procedure of Markwell et al (19) using BSA as a standard.

RESULTS AND DISCUSSION

Binding of the unlabeled agonist, carbamylcholine, was measured over the full range of receptor occupancy by its competition for [^{3}H]-4NMPB binding sites on cerebral cortical membranes in the presence and in the absence of cupric ions. Analysis of the data according to a two-site model indicates that Cu^{2+} (100 µM) increased the proportion of high affinity agonist binding sites from 29% to 55% without substantially affecting the affinity constants for the binding reaction. We also utilized the binding of [^{3}H]-ACh as a tool to directly investigate the binding of the neurotransmitter to these sites. Under the conditions employed in this study the labeled neurotransmitter apparently interacts specifically with the high affinity binding sites (11). Equilibrium binding of [^{3}H]-ACh to cortical membranes exhibited an apparently homogeneous receptor population (Kd = 34 nM) saturating at 690 fmol/mg protein (Fig. 1), which amounts to ~25% of the sites available for antagonist binding in the same preparation. In the presence of 100 µM cupric ions the value of Bmax for [^{3}H]-ACh was increased by 90% (1320 fmol/mg) with only slight changes in its binding affinity (42 nM) (Fig. 1). The increases in high affinity sites observed with carbamylcholine and [^{3}H]-ACh are very similar and demonstrate that [^{3}H]-ACh is a sensitive probe for measurement of these changes. Thus in the presence of Cu^{2+},

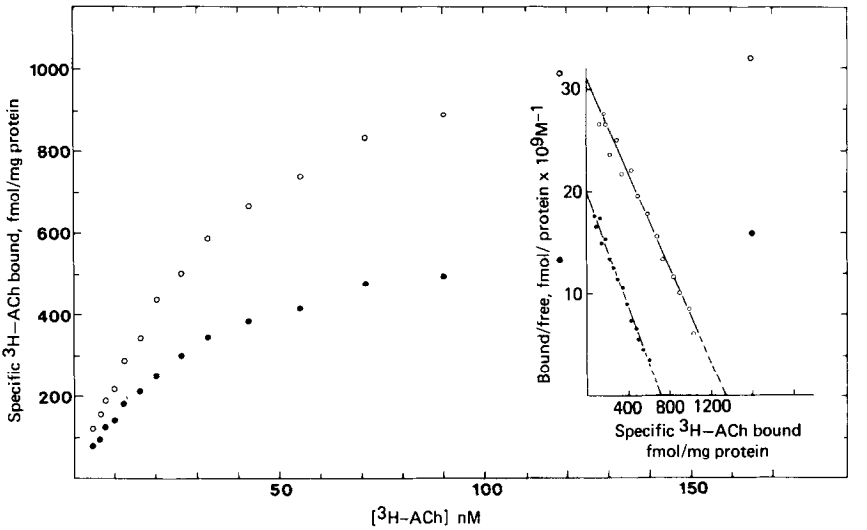

FIGURE 1 Equilibrium specific binding isotherms of [^{3}H]-ACh to rat cerebral cortex membranes in the absence (●) and presence (○) of 100 μM Cu^{2+}. Membranes (0.125 mg protein per 40 μl assay) were incubated with the indicated concentrations of [^{3}H]-ACh as described under Methods. Non-specific binding was determined using 10 μM atropine, and was linear over the concentration range studied. Results shown are from a typical experiment, carried out in quadruplicate and agreeing within 15%. Specific binding of [^{3}H]-4NMPB in the same preparation was 2940 fmol/mg protein (control) and 2750 fmol/mg protein (100 μM Cu^{2+}). Insert: Scatchard plot of the same data. Linear regression yielded K_d = 34 nM and 42 nM, B_{max} = 690 fmol/mg protein and 1320 fmol/mg protein, in the absence (●) and presence (○) of 100 μM Cu^{2+}.

[^{3}H]-ACh could saturate about half of the muscarinic receptors. Therefore further experimentation was performed to more fully characterize the effect of copper using the labeled neurotransmitter (39-42 nM). It should be noted that at these concentrations non-specific binding represents about 20-30% of the total [^{3}H]-ACh binding, and its level is unaffected by the various ions and sulfhydryl reagents employed.

A comparison of the increases in specific [^{3}H]-ACh binding brought about by 2 mM Ni^{2+} (11) with those observed with 100 μM Cu^{2+} (Table 1) shows that they are similar in magnitude and are not additive; however, they differ in their sensitivity to the stable GTP analog, guanylylimidodiphosphate (GppNHp), the latter being insensitive. The effect of copper ions predominates, so that even when nickel ions are also present in the assay buffer the decrease in [^{3}H]-ACh binding induced by GppNHp is minimal (18% in the presence of Cu^{2+} vs. 54% in its absence, Table 1). A copper concentration above 10 μM was required to overcome the effect of the guanine nucleotide. We have previously reported that the increases induced by millimolar concentrations of Ni^{2+}, Co^{2+}, or Mn^{2+} ions are readily reversible by washing, chelation, or addition of micromolar concentrations of guanine nucleotides (3,11). The effects displayed by copper ions were more persistent. The increased specific [^{3}H]-ACh binding and the insensitivity

TABLE 1. EFFECTS OF Ni^{2+}, Cu^{2+}, GppNHp AND THEIR COMBINATION ON THE SPECIFIC BINDING OF [^{3}H]-ACh TO RAT CEREBRAL CORTEX MEMBRANES AT EQUILIBRIUM[a]

Transition metal ions added to assay buffer	Specific binding of 39 nM [^{3}H]-ACh (fmoles/mg protein assayed with the additions:[b]	
	none	200 µM GppNHp
none	277	183
2 mM Ni^{2+}	476	220[c]
100 µM Cu^{2+}	465	392[c]
2 mM Ni^{2+} + 100 µM Cu^{2+}	470	385[c]

[a]Values are means of 4 experiments (each carried out in quadruplicate); standard deviations were within 15% of given values.
[b]Specific binding was determined as described in legend to Fig. 1.
[c]Both ions and GppNHp were added to the assay buffer prior to addition of membranes.

to GppNHp were apparent even after removal of the ions by extensive washing of the membranes (Table 2), indicating a more permanent modification of the receptor complex consistent with a possible SH / S-S transition. Therefore we selected the sulfhydryl oxidizing agent, diamide, to further explore this hypothesis.

Indeed, similar phenomona were also observed following treatment of the membranes with diamide, which increased specific [^{3}H]-ACh binding by 50% (Table 2). Subsequent treatment of the membranes with a sulfhydryl reducing agent (10 mM DTT at pH 7.4), which by itself did not change [^{3}H]-ACh binding, decreased agonist binding to control levels, and restored its sensitivity to guanine nucleotides (Table 2).

However, treatment with a reducing agent alone could not restore agonist binding to control levels following exposure of the membranes to Cu^{2+} (Table 2). Attempts to reverse the copper-induced effects by exposing the membranes to high concentrations of chelators (10 mM D(-)penicillamine or EDTA) were unsuccessful (not shown). Only a combination of DTT and D(-)penicillamine reversed the copper-induced effects (Table 2) and this was accompanied by a return of the [^{3}H]-ACh / [^{3}H]-4NMPB binding ratio to its control level (Table 2). This may suggest that copper ions, like diamide, also exert their effect through modification of sulfhydryls; however, unlike diamide, copper ions most likely remain complexed at their site of interaction, which would explain why DTT alone proved insufficient. It should be noted that pretreatment of the membrane preparation with 10 mM DTT followed by washing, prior to exposure to copper ions, did not modify any of the effects of copper treatment on control membranes (not shown). Hence, it seems that the sulfhydryls involved in the copper-induced modifications of muscarinic agonist binding and insensitivity to guanine nucleotides are already in the reduced state in the native membrane. Interestingly, treatment of cerebral cortex membranes with DTT in combination with D(-)penicillamine at pH 8.4 resulted in a tendency to increase the specific binding of both [^{3}H]-ACh

TABLE 2. EQUILIBRIUM SPECIFIC BINDING OF [^{3}H]-ACh AND [^{3}H]-4NMPB TO RAT CEREBRAL CORTEX MEMBRANES FOLLOWING VARIOUS PRETREATMENT PROCEDURES[a]

Additions during pretreatments[b]		Specific binding of 42 nM [^{3}H]-ACh (fmoles/mg protein), with the additions:[c]			Specific binding of 25 nM [^{3}H]-4NMPB (fmoles/mg protein)	Specific Binding Ratio[d]	
I	II	none	GppNHp	Ni^{2+}			
none	none	282	192	515	3105	0.090	100%
Cu^{2+}	none	396	344	410	2730	0.145	160%
Cu^{2+}	DTT	364	305	425	2660	0.137	152%
Cu^{2+}	DTT, penicil-amine	305	204	---	3160	0.096	107%
none	DTT, penicil-amine	336	212	---	3470	0.097	108%
diamide	none	420	340	510	3360	0.125	139%
diamide	DTT	270	220	440	3220	0.084	93%

a Values are means of 3-4 experiments except for control and copper treatment values which are means of 9 experiments. Standard deviation was within 12% of given values for [^{3}H]-ACh and within 8% for [^{3}H]-4NMPB.

b Pretreatments (I and II) were carried out in Krebs buffer (pH 7.4, except those employing penicillamine in which pH was 8.4) at protein concentration of 1.5-2.5 mg/ml, each followed by centrifugation in 20 volumes of Krebs buffer (15 min., 30,000 g). Copper concentration was 50 µM, DTT and penicillamine were both 10 mM, and diamide was 2 mM.

c Specific binding was determined as described in Legend to Fig. 1. Nickel concentration was 2 mM and GppNHp was 200 µM.

d Specific binding ratio is given both as the ratio of specifically bound [^{3}H]-ACh / [^{3}H]-4NMPB and as a percentage of the control ratio. Note that since the [^{3}H]-ACh concentration used was too low to saturate its binding sites (K_d about 34 nM), while [^{3}H]-4NMPB binding is 99% saturated at 25 nM, this ratio has no absolute meaning and is only included for comparison.

(19%) and [^{3}H]-4NMPB (12%; Table 2), possibly indicating that endogenous metal ions might be interacting with the native muscarinic receptor complex.

Competition experiments using muscarinic receptors obtained from the medulla-pons (Fig. 2) also confirm the appearance of a high affinity guanine-nucleotide-insensitive state in the presence of copper. The metal elevated the percentage of high affinity sites from 60% in control membranes to 72% in the presence of 100 µM copper. Furthermore, while GTP reduced the percentage of sites exhibiting high affinity to 42% in control membranes this transition was completely blocked in the presence of copper. Specific binding of [^{3}H]-ACh in this preparation was also highly sensitive to GppNHp (52% decrease at 200 µM nucleotide). This sensitivity was completely lost following pretreatment of the membranes with 50 µM copper or with 2 mM diamide (not shown).

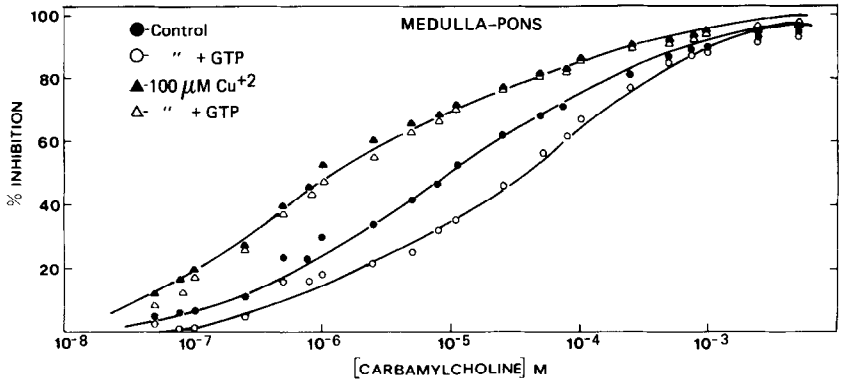

FIGURE 2 Effect of copper on carbamylcholine binding to rat medulla pons: insensitivity to GTP. Membranes (0.075 mg protein per 2 ml assay) were incubated with 2 nM [^{3}H]-4NMPB and various concentrations of carbamylcholine in the absence (circles) or presence (triangles) of 100 μM Cu^{2+} for 30 minutes at 25°C. The additional presence of GTP (250 μM) is indicated by open symbols and its absence by closed symbols. Non-specific binding is that ocurring in the presence of 5 μM atropine. Reaction was initiated by addition of the membranes to tubes containing all reagents in Krebs buffer.

Aronstam and Eldefrawi (12) have previously demonstrated dependence of agonist binding on the sulfhydryl redox state. Treatment with potassium ferricyanide or DTNB increased agonist affinity in competition assays with [^{3}H]-QNB; this was reversible by treatment with reducing agents. Matsumoto et al. observed that when DTNB and GppNHp were employed in combination, the net effect was similar to that produced by the oxidant alone (20). The latter report is in accord with our findings that sulfhydryl oxidation abolishes nucleotide effects in cerebral cortical and medulla-pons preparations. However, copper treatment is unique in that its effect cannot be reversed by treatment with a reducing agent alone. The finding that neither the reducing agent by itself nor the chelator by itself is able to fully reverse the effects of copper on muscarinic binding suggests that the copper-complexed sulfhydryl residues tend to become oxidized in the process of copper removal by the chelator.

Our data, combined with results reported by other workers for alterations in muscarinic agonist binding by sulfhydryl modification (4, 12-16), lead us to suggest that copper and diamide treatments increase agonist binding to the muscarinic receptor and remove guanine nucleotide sensitivity through a common mechanism involving sulfhydryl residues. Two possible mechanisms might explain the data, involving either an intermolecular or an intramolecular reaction with formation of a copper-complexed or a diamide-induced disulfide bridge via sulfhydryls. The sensitive groups may be on the receptor protein (R), on a guanine nucleotide binding protein (N), or on another, as yet unidentified subunit of the receptor oligomer (1,2). We postulate that the modification of these sulfhydryl residues either favors the formation of R-N oligomers which exhibit high

affinity for [^{3}H]-ACh binding, or acts directly at the N protein, blocking the dissociation to the low affinity agonist binding conformer by guanine nucleotides (1,2,15). Further experimentation will be needed to elucidate the model more precisely. It should be noted that SH / S-S transformations have been linked to changes in the functional state of other receptors such as the nicotinic (21) and the CNS opiate receptor (22).

Finally, we would like to point out that the Cu^{2+} concentrations employed in this study are well within the range observed in the rat (7,8) as well as in the human (9) cerebral cortex. Although most of the copper ions in brain tissue are probably complexed to proteins, they might play a role in the modification of normal CNS activity as well as in the neurological deficits related to the cholinergic system found in certain pathological conditions associated with abnormal levels of copper (5,23).

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