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AGONIST-SPECIFIC REVERSE REGULATION OF MUSCARINIC RECEPTORS BY TRANSITION METAL IONS AND GUANINE NUCLEOTIDES

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Transition metal ions, e.g. Mn^{2+}, Ni^{2+} and Co^{2+} enhance in vitro agonist binding to muscarinic receptors in mouse cortex or hippocampus. This effect arises mainly from the conversion of low to high affinity binding sites. Binding properties of antagonists in these brain areas, as well as those of both agonists and antagonists of medulla-pons muscarinic receptors, are insensitive to these ions. The induced interconversion can be reversed by either of the following procedures: (i) removal of the ions; (ii) thermal exposure; (iii) addition of micromolar concentrations of guanine nucleotides.

Previous in vitro binding studies to muscarinic receptors have shown that agonist binding sites are more sensitive than antagonist sites to environmental restraints - they are more hydrophobic for low affinity sites and more hydrophilic for high affinity sites - and that interconversion between high and low affinity states can be induced (1 and ref. therein). Since transition metals might exert an effect on the water structure and thus affect the environmental restraints at the binding sites, we examined the effect of Mn^{2+}, Ni^{2+} and Co^{2+} ions on the binding of muscarinic agonists and antagonists to membrane-bound receptors obtained from mouse brain. The recently described GTP effect in the regulation of muscarinic receptors, which is known to be Mg^{2+}-dependent (2-4), was investigated further in the presence and absence of these divalent cations. We report here that guanine nucleotides, in the presence of Mn^{2+}, Ni^{2+} or Co^{2+} exerted a specific effect on agonist binding in brain regions where they had only minimal effect under standard assay conditions, i.e. in the cortex and the hippocampus (3). Thus, transition metal ions and guanine nucleotides exert antagonistic effects on muscarinic agonist binding, similar to those observed in several other neurotransmitter systems, e.g. α-adrenergic (5) and opiate receptors (6,7).

MATERIALS AND METHODS

The potent muscarinic antagonist(^{3}H)-N-methyl-4-piperidyl benzilate (4NMPB) (33 Ci/mmole) and unlabeled muscarinic ligands have been previously described (8). Metals (in the form of chloride salts) were from Merck. Nucleotides were from Sigma.

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Adult male ICR mice (20-25 gr) were decapitated, their brains rapidly removed and the brain regions dissected and homogenized in 0.32 M sucrose as previously described (8).

For the binding assays, 50 µl aliquots of homogenate were incubated at 37°, for the time periods indicated, in buffer (2 ml) containing 118 mM NaCl, 5 mM KCl, 10 mM glucose, 25 mM Tris-HCl (pH = 7.4 at 25°) and 1 mM of the divalent cation indicated. For antagonist binding studies various concentrations of (^{3}H)-4NMPB were included. Agonist binding was studied in competition experiments with 2 nM (^{3}H)-4NMPB. The reaction was terminated by filtration as described (8). Assays were carried out in triplicate and specific binding was defined as the total minus the non-specific, i.e. binding in the presence of 1 µM unlabeled atropine. Protein was determined by the Lowry method using bovine serum albumin as a standard. Data analysis was carried out as described in detail previously (9).

RESULTS

Substitution of the divalent cation Mg^{2+} by Mn^{2+} (as chloride salts, 1mM) had no effect on the equilibrium binding of(^{3}H)-4NMPB to cortical homogenate (Fig. 1A). However, the ability of the agonist carbamylcholine to inhibit (^{3}H)-4NMPB binding was increased, the shift to the left in the displacement curve indicating a 4-fold decrease in I_{50} value (Fig. 1B). Similar results were obtained in hippocampal preparations, but in medulla-pons homogenates such replacement had only a negligible effect (i.e. a 1.5-fold shift to the left). Substituting Ni^{2+} (1mM) or Co^{2+} (1mM) instead of Mn^{2+} (1mM) for magnesium ions had similar effects in these instances, and indeed in all the experimentation reported in the present work. For the sake of simplicity, we restrict our reporting to Mn^{2+}, but it should be noted that all observations apply equally to Ni^{2+} and to Co^{2+}. These phenomena were unaltered by washing the membranes twice (20,000 g x 20 min, in assay buffer excluding divalent cations), prior to assay with Mg^{2+} or Mn^{2+}, or if the divalent cations tested were included as 1 mM excess over 1 mM EDTA. Thus participation of endogenous divalent cations is unlikely. It should be noted that addition of 1 mM Ca^{2+} to the assay buffer had a negligible effect on the phenomena described. The effect of manganese on agonist binding was completely reversible after preincubation for 10 or 30 min at 37°, upon withdrawl of the cation by double-washing the membranes and subsequent assay with 1 mM Mg^{2+}. Alternatively, excess (3 mM) EDTA was added to the assay tubes after 10 min incubation with muscarinic ligands at 37° and the reaction allowed to proceed for a further 5 min to reach a new equilibrium prior to filtration. Under these experimental conditions the curve of carbamylcholine/(^{3}H)-4NMPB competition was indistinguishable from that of the control experiment (in which EDTA was in excess of manganese for the entire incubation period).

The manganese effect occurs rapidly, with maximal shift in agonist binding already seen after 5 min incubation at 37°, the minimum period required to reach equilibrium. No further effect on agonist binding was seen after further

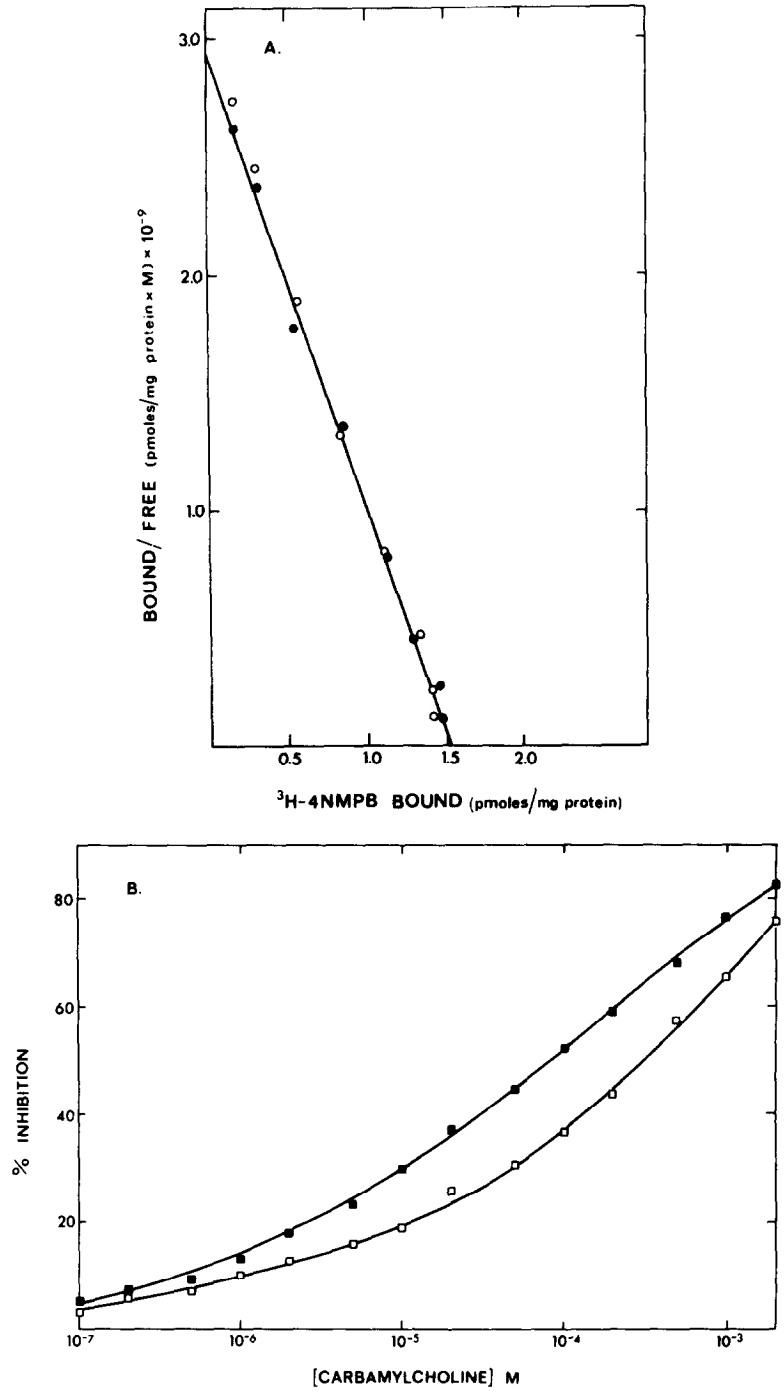

Fig. 1. The binding of (^{3}H)-4NMPB (A) and of carbamylcholine (B) to cortex homogenate in the presence of 1 mM Mg^{2+} (open symbols) and 1 mM Mn^{2+} (closed symbols). Samples (0.05 ml) were assayed at 37° for 10 min in 2 ml buffer containing various concentrations of (^{3}H)-4NMPB (A), or 2 nM (^{3}H)-4NMPB and the concentrations of carbamylcholine indicated (B). Concentrations of (^{3}H)-4NMPB binding sites were in all cases < 0.1 nM.

incubation (up to 60 min). A similar shift was observed when homogenates were preincubated for various time periods at 37° in the same buffer solution including Mn^{2+} but excluding muscarinic ligands, and reaction was then terminated 5 min after the ligands were added. The manganese effect was only slightly potentiated by increasing its concentration above 1 mM (e.g. at 5 mM the shift in the carbamylcholine curve was 5-fold); however, at concentrations below 1 mM the effect was diminished.We thus chose one millimolar as the concentration for further experiments, which conveniently allowed us to employ the physiological control conditions of 1 mM Mg^{2+}.

The data obtained for the (^{3}H)-antagonist/agonist inhibition curves were analyzed according to the two-site and three-site models (10). The best fit observed was for the two-site model and since no statistically significant improvement was noted on adding a third state, data from inhibition curves were subsequently fitted by a two-site model specifying a high (H) and a low (L) affinity state. The binding data for carbamylcholine are given in Table I. As shown in this table, substitution of Mg^{2+} by Mn^{2+}, Ni^{2+} or Co^{2+} resulted in an increased proportion (α) of high affinity sites with a concomitant small decrease in K_H and essentially no change in K_L.

High concentrations (up to 100 μM) of GTP have a negligible effect on agonist binding in cortical preparations under our standard assay conditions,

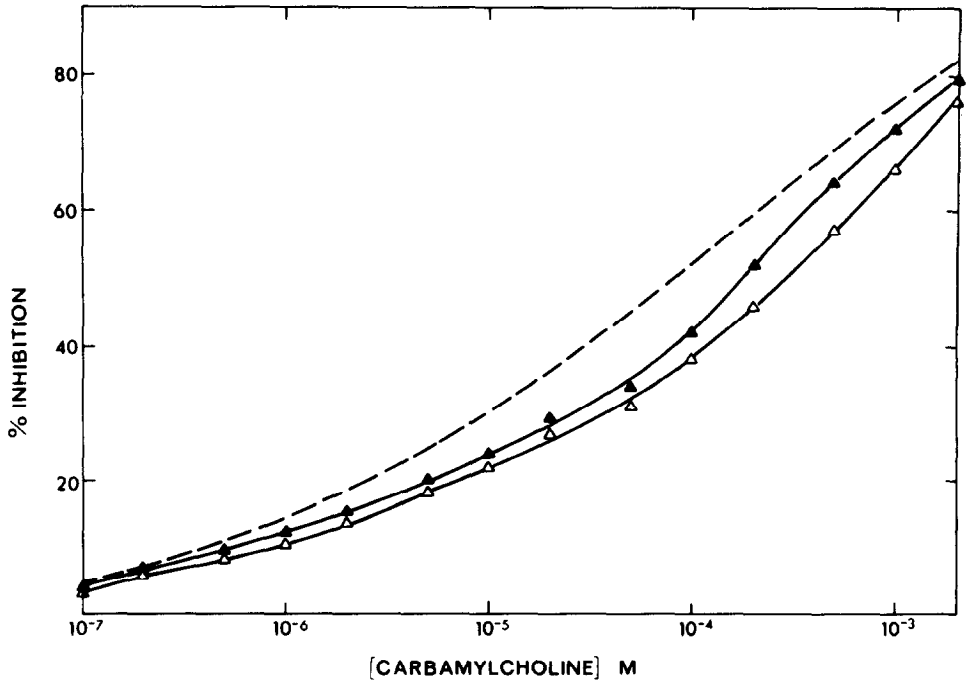

Fig. 2. Effect of GTP on the binding of carbamylcholine to cortex homogenate in the presence of 1 mM Mn^{2+}. Assays were carried out as described in legend to Fig. 1B. GTP concentrations were 5 μM (open triangles), 0.5 μM (closed triangles) or 0 (dashed line, data from Fig. 1B).

as reported previously (3) and confirmed here (Table I). However, the "new" agonist binding properties revealed in the presence of Mn^{2+} are strikingly sensitive to GTP. At concentrations as low as 5 μM, GTP completely restores the curve to its original position, with half maximal effect achieved by 0.5 μM GTP (Fig. 2). Concentrations in the range of 5-100 μM shifted the curve only slightly further. No effect was seen on antagonist binding (not shown). Under the conditions employed here, the effect of GTP could already be observed at concentrations as low as 0.1 μM. Analysis of the binding curves for the Mn^{2+}/ GTP combinations (Table I) indicates that the nucleotide restores the"original" binding properties in cortical preparations. GDP and Gpp(NH)p were equipotent with GTP in reversing of the Mn^{2+} induced changes in agonist binding parameters (not shown). ITP could mimic this reversal but was less potent (i.e. exerting half-maximal effect at 10-20 μM). ATP, cAMP and cGMP in concentrations up to 100 μM were ineffective.

The effect of submicromolar concentrations of GTP on the Mn^{2+} induced agonist binding properties was already evident after 5 min incubation at 37°, regardless of whether the nucleotide was added after preincubation with Mn^{2+} or was present throughout the incubation period. This was only slightly weakened by prolonging incubation with Mn^{2+} + GTP to 30 min. Agonist binding in the presence of Mn^{2+} and 0.5 μM GTP was unaltered by co-incubation with 50 μM ATP.

The antagonistic effects of Mn^{2+} and GTP on muscarinic agonist binding to cortical homogenate could also be demonstrated for the agonists acetylcholine

Table I: Effects of divalent cations and GTP on parameters of carbamylcholine binding to mouse cortex homogenates.

divalent cation (1 mM)	GTP(μM)	I_{50} (μM)	α(%)	$K_H x10^6 (M^{-1})$	$K_L x10^3 (M^{-1})$
Mg^{2+}	-	300	25	3.6	6.5
Mg^{2+}	50	320	25	3.1	6.2
Mn^{2+}	-	80	39	2.6	8.6
Mn^{2+}	5	320	26	3.1	8.0
Mn^{2+}	0.5	180	30	3.1	8.3
Ni^{2+}	-	50	46	2.1	8.0
Co^{2+}	-	40	47	2.4	8.2

I_{50} is the carbamylcholine concentration inhibiting 50% of 2 nM (^{3}H)-4NMPB binding. α is the proportion of high affinity sites. K_H and K_L are the affinity constants for high and low affinity sites respectively. α, K_H and K_L were calculated by computer fit. Values are the means of 4-6 experiments for each combination, each analysed separately by computer. Standard deviations are < ±0.08 of mean values for I_{50} and α,and < ±0.25 of mean values for K_H and K_L.

(in the presence of 10 μM physostigmine), acetoxyquinuclidine and oxotremorine, which were used in similar competition experiments with (^{3}H)-4NMPB. It should be noted that the shift in I_{50} values induced by 1 mM Mn^{2+} was only two-fold for acetoxyquinuclidine and for oxotremorine.

By including Mn^{2+} in the receptor assay we were able to demonstrate a second phenomenon which we previously reported to be absent in mouse cortex, namely, sensitivity of agonist binding properties to thermal exposure (1). In homogenates of mouse medulla-pons and cerebellum the thermal effect on agonist binding reflects a conversion of high to low affinity binding sites (1). Since manganese ions increase the population of high affinity binding sites for agonists, we investigated whether the ligand binding properties of the cortical preparation would now be sensitive to thermal exposure. Treatment of the cortical membranes at 50° for 5 min (in 0.32 M sucrose) did indeed prevent the manifestation of the manganese effect in the subsequent assay, as shown in Fig. 3. Thermal exposure results in a 3.5 fold shift in I_{50} value for carbamylcholine binding assayed in the presence of Mn^{2+}, in contrast to a 1.3-fold shift in the presence of Mg^{2+}. These shifts stem from a decrease from 39% to 22% in the proportion of high affinity sites assayed with Mn^{2+}, while there are no substantial changes in either the high or the low affinity constants.

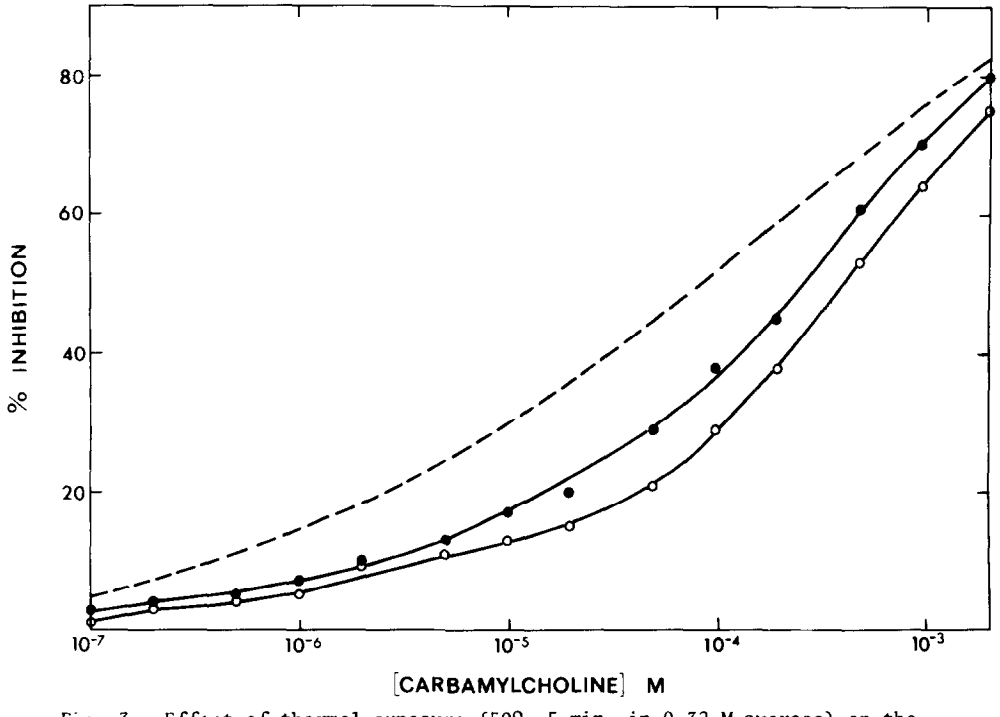

Fig. 3. Effect of thermal exposure (50°, 5 min, in 0.32 M sucrose) on the binding of carbamylcholine to cortex homogenate in the presence of 1 mM Mg^{2+} (open symbols) or 1 mM Mn^{2+} (closed symbols). Assays were carried out as described in legend to Fig. 1B. Dashed line is the same as in Fig. 2.

DISCUSSION

The results of this investigation lend further support to the concept that the binding of muscarinic agonists to their specific receptors is a function of the structure of the ligand on the one hand and the topography and environmental restraints of the binding sites in the receptor complex on the other hand (1,4,8,10). Thus, for example, Mn^{2+}, Ni^{2+} and Co^{2+} converted low affinity agonist binding sites to a high affinity state showing sensitivity to guanine nucleotides, a property not shared by antagonists. The ready reversibility of the Mn^{2+} induced interconversion indicated that the effect of these divalent ions is likely to occur directly at the more hydrophilic site of the membranal receptor complex rather than indirectly as a result of, for example, the oxidation of membrane lipids or of protein sulfhydryl moieties. In fact, oxidation of sulfhydryl groups on the membrane containing the neural muscarinic receptors (11) or treatment with Cd^{2+} (12) or PCMB (13) produce an opposite effect, i.e. a reduction in agonist affinity. It should be pointed out that since the Mn^{2+} - induced conversion to high affinity state is extremely sensitive to submicromolar concentrations of guanine nucleotides, which induce an opposite effect and are probably present here as endogenous components of the membrane preparation (14), it is most likely that only a minimal effect was observed in these experiments. Since the concentrations of the various divalent ions are much higher than the GTP concentration it seems unlikely that the GTP effect on cortical preparation is a nonspecific chelation of manganese from the receptor preparation. This is also evident from the ineffectiveness of ATP.

The effects of Mn^{2+} as well as the previously and presently reported guanine nucleotide effect on muscarinic receptors seem to be analogous to the α- and some of the β-noradrenergic receptor systems (5,15), but they differ somewhat from those reported for the opiate receptor interactions (6,7). It was recently suggested that manganese augments opiate agonist binding through the activation of membrane-bound phosphatase thus leading indirectly to hydrolysis of GTP bound to regulatory subunits (7). This is unlikely to explain our results, as manganese exerted its effect on muscarinic receptors only when present during receptor assay, and the effect was not time-dependent, and was unaltered by co-incubation with ATP. Following the pioneering studies of Rodbell and others (reviewed (16) and references therein), it is now recognized that guanine nucleotides have an important role in the interactions between hormones and neurotransmitters with their respective receptors. By analogy, we have recently suggested (1,3) that the muscarinic complex also contains a separate membrane unit which is guanine nucleotide sensitive and which initiates a response via some form of complex("low affinity") between the receptor, the agonist and some other component(s), e.g. a stimulatory or inhibitory cyclase or Ca^{2+} channel. This

unit should be denoted as N_x according to Rodbell's recent notation (16). The process of receptor regulation appears to result in a selective interconversion of high to low affinity agonist sites, and in vitro treatments (e.g. using transition metal ions) which enhance the proportion of high affinity sites will now allow observation of the GTP regulation effect which will thus reverse the reaction.

Due to the close resemblance in the effects induced by guanine nucleotides and by the thermal exposure on agonist binding to medulla-pons homogenates, and to the fact that the effect of the two treatments is almost non-additive, we suggested (1) that there was some overlap in target areas, and that the heat-labile component was part of the muscarinic complex. This conclusion is further supported by recent experiments in which 100 μM oxotremorine was found to afford partial protection from the thermally induced interconversion when present during the heat exposure, an effect which could be blocked by antagonists (i.e. 1 μM atropine) (D. Gurwitz and M. Sokolovsky, unpublished observations). There was however essentially no effect of thermal exposure on agonist binding in brain regions enriched with low affinity sites such as the mouse cortex (1). As reported here, the artificial increase in high affinity receptor population in the cortical homogenate renders this preparation sensitive to thermal exposure and to guanine nucleotides. Further elucidation of the "new" high affinity binding sites induced by transition metal ions and which we regard in this report as the "normal" high affinity state is clearly needed. Since the concentrations of the transition metal ions inducing the above effects are non-physiological, it would appear either that they mimic the action of a possible endogenous modulator or that they alter the hydrophobic interactions at the receptor complex as suggested previously (1) or from both.

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