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INDUCED INTERCONVERSION OF AGONIST AFFINITY STATES IN MUSCARINIC RECEPTOR FROM MICE BRAIN: EFFECTS OF TEMPERATURE AND SUGARS

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In homogenates of mouse medulla-pons and cerebellum, thermal exposure (50°, 5 min.) caused a 10-12 fold decrease in binding affinity of muscarinic agonists with no effect on antagonist binding, but in cortical preparations both agonist and antagonist binding were unaltered. The thermal effect on agonist reflects a conversion of high to low affinity binding sites and could be prevented using sugars in the medium. Incubation of mouse cortex homogenates at 0° for 3 hours decreased the I_{50} values for agonist binding with no effect on antagonist binding. The relationships between factors affecting agonist affinity states and the mode of action of muscarinic receptors are discussed.

The complex nature of agonist binding to muscarinic receptors has been described by Birdsall et al. (1 and refs. therein), who suggested the presence of a heterogeneous population of agonist binding sites, including high and low affinity binding sites. Interconversion between the high and the low affinity muscarinic receptor binding sites has recently been inferred from agonist binding studies in mammalian brain and in Torpedo (2). It seemed reasonable to assume that conformational changes leading to such interconversion may be temperature-dependent. In order to investigate this possibility we examined the binding of antagonists and agonists to membrane bound receptors obtained from mouse brain. The two regions selected for these studies were the medulla-pons and the cortex, the former having a large and the latter a small population of high affinity binding sites (2).

MATERIALS AND METHODS

Full details of the preparation of (^{3}H) N-methyl-4-piperidyl benzilate (4NMPB) (33 Ci/mmole), the homogenates used and the binding assay using the filtration method have been given elsewhere (3). Binding of (^{3}H)-4NMPB that was inhibited by 10 μM unlabeled 4NMPB or atropine was considered to be specific. Binding of agonists was inferred from their ability to inhibit specific binding of (^{3}H)-4NMPB (3). Membranes (~5 mg protein/ml) were incubated at different temperatures, and aliquots were withdrawn for binding assays which were carried out at 25° in the case of samples incubated at 50° and at 0° in the case of samples incubated at 0°. Protein was determined by the Lowry method using bovine serum albumin as a standard.

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RESULTS AND DISCUSSION

Raising the temperature of the medulla-pons and cerebellum (prepared in 0.32 M sucrose) homogenates to 55° indicated that while the K_d of (^{3}H)-4NMPB binding was not affected the I_{50} values for oxotremorine and carbamylcholine were markedly increased. The observed effect was a function of both temperature and incubation time. As mentioned above, we were interested in comparing binding site properties in the cortex and the medulla-pons. In preliminary experiments the former showed greater sensitivity to heat (i.e. a diminution above 50° in both antagonist and agonist binding sites). We therefore selected for further studies the optimal incubation conditions of 5 min at 50°.

As shown in Fig. 1, the competition curve for oxotremorine binding to medulla-pons preparation incubated at 50° for 5 min and assayed at 25° was shifted 10-12 fold to the right, while the K_d of (^{3}H-)-4NMPB was unaltered with only very slight increase in Bmax. Similar results were obtained using other ligands, e.g., using QNB or atropine as antagonists and carbamylcholine and acetylcholine as agonists. A similar observation was recently reported for the dopamine receptor, where thermal exposure (53°, 2 min) indicated that agonist high affinity sites are heat labile while antagonist sites are heat stable (4). Binding of either agonists or antagonists to cortex, striatum, hippocampus and thalamus preparations incubated at 50° for 5 min showed no significant change, e.g., in the cortex a 1.5 fold shift to the right was seen in agonist binding (data not shown). We believe this is due to the fact that these regions have a small population of high affinity sites under our standard assay conditions.

The effect of thermal exposure on the binding site properties of the medulla-pons and cerebellum homogenates was independent of pH in the range of 5.5-9.3. Thoroughly washing the preparation after thermal exposure did not reverse the observed effect. Furthermore, similar results were obtained when the preparation used was crude synaptosomal fraction (P_2) or purified synaptosomal fraction (sucrose gradient), thus eliminating the possibility that the effect was due to factors not directly related to the receptor membrane. For the sake of convenience, we therefore used whole homogenates in further studies.

The data obtained for the oxotremorine inhibition curves were analysed according to the two-site model (1) by the best fit procedure using a non-linear least squares program (2). The data obtained suggest that the increase in I_{50} values stems from the increase in proportion

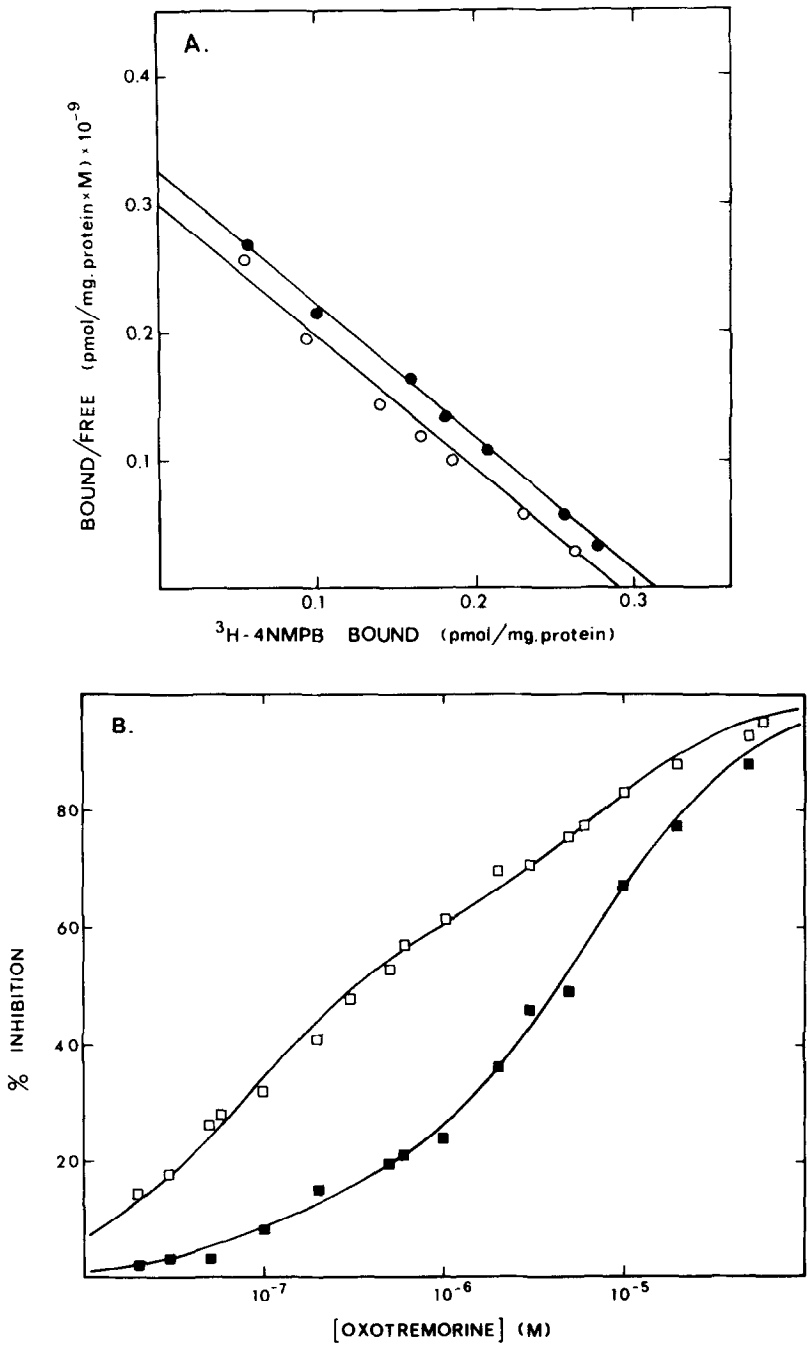


Fig. 1. The binding of (^{3}H)-4NMPB (A) and oxotremorine (B) to control (open symbols) and thermal exposed (5 min 50°; closed symbols) medulla-pons homogenates. Samples (0.05 ml) were assayed at 25° for 30 min in 2 ml modified Krebs-Hensleit solution containing various concentrations of (^{3}H)-4NMPB (A) or 4.0 nM (^{3}H)-4NMPB and the concentration of oxotremorine indicated (B). Concentrations of (^{3}H)-4NMPB binding sites were in all cases < 0.1 nM. The solid lines in (B) are the best fit lines obtained by a nonlinear least-squares program (2).

of low affinity binding sites in the medulla-pons from 35% (control) to about 80%. No substantial changes in dissociation constant were seen, (e.g. K_L = 1.6 μM (control) and 1.3 μM (thermal exposure); K_H = 0.02 μM (control) and 0.03 μM (thermal exposure).

The change(s) at 50° seems to be irreversible or, if reversible, the reverse reaction is extremely slow, since subsequently incubating the preparation at 25° for 3-4 h did not change the proportion of high and low affinity sites. On the other hand, it should be pointed out that where thermal exposure resulted in almost no alteration in the proportion of high and low affinity sites (e.g. in the cortex), the possibility of a fast reversible interconversion could not be dismissed altogether.

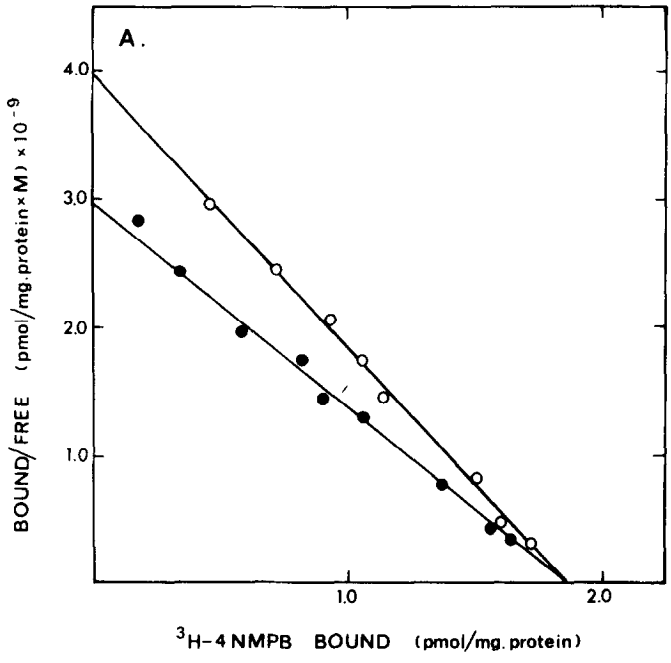
Increasing the temperature is usually thought to strengthen hydrophobic interactions. It has been known for many years that sugars stabilize proteins against heat. We therefore investigated the effect of temperature when 2-3 M glucose was present in the incubation medium during the thermal exposure. The presence of glucose did, in fact, prevent interconversion, and the binding properties of antagonists and agonists were similar for thermal and control preparations. Sucrose, sorbitol and glycerol (50% w/v) were about equally effective in inhibiting interconversion. It should be noted that 3 M glucose, when added to pretreated membrane preparation (after incubation for 5 min at 50°) did not affect the "new" binding properties. Thus, glucose exerts its effect only during the heat induced interconversion process and does not alter equilibrium between the two affinity sites. Hence, as suggested recently (5), the primary effect of sugars and polyols is likely to be through their effect on the structure of water, which in turn determines the strength of hydrophobic interaction between pairs of hydrophobic groups. This phenomenon indicates that the reactivity of muscarinic and probably of other receptors as well may be mediated by the presence of local high concentrations of polyhydroxy compounds within the microenvironment of the membranes containing the receptor complex. It follows that caution should be exercised in ligand-receptor interaction studies carried out in the presence of high concentrations of sugars and other polyols.

If we assume that the induced interconversion results from hydrophobic influences in the membrane preparation and is characteristic of regions which are rich in high affinity agonist sites, it might be expected that in regions where low-affinity sites predominate under standard assay condition, lowering the temperature might reveal the opposite effect, i.e. interconversion from low to high affinity state. We there-

fore examined this possibility using a cortical preparation, for which agonist/(^{3}H)-antagonist inhibition studies had indicated that about 80% of the oxotremorine binding sites were in the low affinity state (2). Homogenates of mouse cortex were incubated at 0°. Due to the slow rate of association of ligands at 0° (6), and in order to follow the reaction at this temperature, (^{3}H)-4NMPB 4 nM) in the presence and absence of various concentrations of oxotremorine was included in the reaction mixture, which was incubated at 0° for 3 h. Under these conditions antagonist binding showed some minor alteration (Fig. 2A) in contrast to the considerable alteration seen in agonist binding. As shown in Fig. 2B, the I_{50} values decreased. Analysis of the curves revealed that there was no change in the population of high and low affinity sites. However, while only small changes were seen in the low affinity state: K_L = 2.5 μM (25°) as compared to 0.8 μM (at 0°) in the K_H values we observed much greater changes of about two orders of magnitue which were indicative of the presence of super high affinity state (1). In order to obtain a more accurate measure of these parameters a different methodology is required (1) and this is currently being worked out in our laboratory. Subsequent incubation of the above cortical preparation at 25° indicated that the reaction was reversible, i.e., the binding properties were identical to the control (at 25°). In the medulla-pons homogenates incubated for 3 h. at 0°, there was little if any effect on the binding properties of either antagonist (4NMPB) or agonist (oxotremorine).

These experiments demonstrate that investigation of the reaction to thermal exposure in the presence and absence of polyhydroxy compounds in muscarinic receptors (and probably in other receptor systems) *in vitro* might be a useful technique for investigating the differences in structure and function, if any, between pre- and post-synaptic receptors as well as between extra- and sub-synaptic receptors, and for studying their evolution during synapse formation.

Agonist binding sites are more sensitive to environmental restraints than antagonist sites, e.g., they are hydrophobic for low affinity sites and hydrophilic for high affinity sites, and interconversion between high and low affinity states can be induced. It should be noted that in *Torpedo* muscarinic receptors we have been able to observe interconversions within agonist states by manipulating both the ionic strength and the buffer used (2). Moreover, oxidation and reduction of sulfhydryl and disulfide groups on the membrane containing neural muscarinic receptor respectively decreases and increases agonist affinity without affecting antagonist binding (7-9).



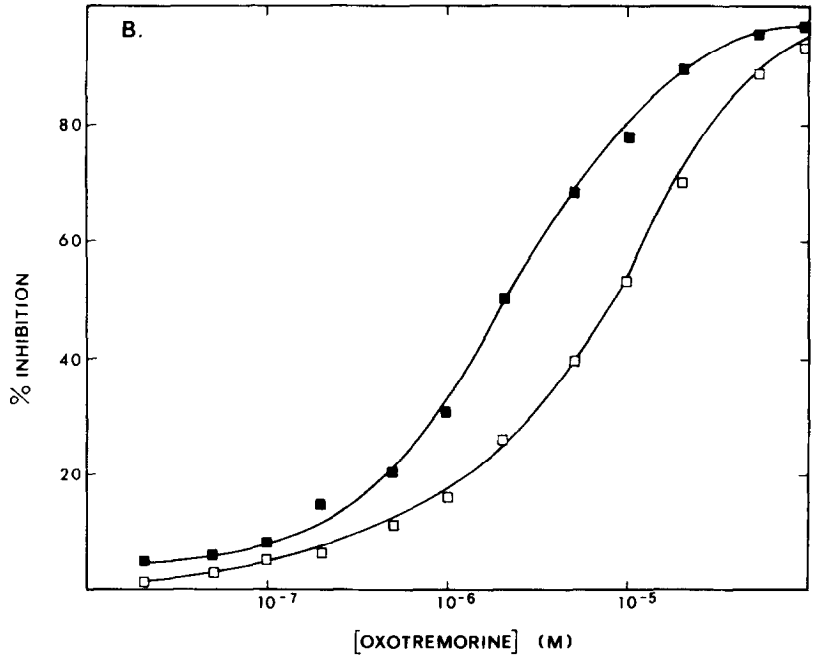


Fig. 2. Effect of temperature (0°) on binding of (^{3}H)-4NMPB (A) and oxotremorine (B) to cortex homogenates. Samples (0.05 ml) were assayed at 25° for 30 min (open symbols) or at 0° for 3 h (closed symbols) in 2 ml modified Krebs-Hensleit solution containing the muscarinic ligands as indicated in legend to Fig. 1.

These results raise several intriguing possibilities: Does the interconversion described here result from an indirect effect, i.e., a change in the membrane fluidity, or is it the result of a direct effect either on the muscarinic receptor itself or on another component that is part of the muscarinic complex? We favour the assumption that the muscarinic complex is the target, for the following reasons: (i) The binding of agonists and antagonists is competitive and mutually exclusive; since the effects reported here are virtually confined to agonist sites, it is easier to envisage that a component that modulates or is modulated by agonist sites is involved. (ii) Recent studies with guanine nucleotides and muscarinic receptors in the mouse medulla-pons showed a similar shifting of high affinity to low affinity sites (10). These results indicated the involvement of a separate membrane unit which is sensitive to guanine nuecleotides and which plays a part in receptor regulation. Treating the medulla-pons preparation at 50^{o} for 5 min resulted in a 10-fold shift to the right in the agonist binding curve; subsequent treatment with Gpp(NH)p (50 μM) resulted only in a further 2-fold shift, whereas treatment of unheated control receptors with Gpp(NH)p alone produced a 10-12 fold shift (10). This seems to indicate that the target molecule was the same or at least that there was some overlap in target areas for both the heat and the guanine nucleotide treatments. (iii) Desensitisation has long been thought to involve the agonist receptor complex. Richelson (11) has described the specific desensitisation of muscarinic receptors in cultured nerve cells, a process which is temperature dependent. Thus, desensitisation at 37^{o} was markedly reduced at 22^{o} and did not occur at 0^{o}, and the observation is reminiscent of results reported here. (iv) Affinity labeling measurements of rat jejunum (12) suggest that modification occurred at two sites, one being the muscarinic receptor proper and the other a regulatory site. Further experimentation is clearly needed to verify the hypotheses discussed here.

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