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MUSCARINIC RECEPTOR BINDING IN MOUSE BRAIN: REGULATION BY GUANINE NUCLEOTIDES

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The effect of guanine nucleotides on the binding properties of muscarinic receptors for cholinergic ligands was studied in several brain regions in mice. In homogenates of medulla-pons and cerebellum, GTP (50 μM) caused an 8 -fold decrease in the binding affinity of agonists but had no effect on antagonist binding. Gpp(NH)p was slightly more potent than GTP. The binding properties of both agonists and antagonists in the cortex and hippocampus were unaltered in the presence of guanine nucleotides. The nucleotide effect on agonists arises mainly from the conversion of high affinity to low affinity binding sites. The possible role of these nucleotides in the mechanism of action of muscarinic receptors is discussed.

Introduction

Previous in vitro binding experiments have favoured the assumption that the functional heterogeneity of muscarinic receptors in mouse brain results from subclasses of binding sites for ligands, which, in turn, are 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 molecule on the other hand (1-3). With regard to the topography of the muscarinic receptors, we recently observed that in several mouse brain regions and in Torpedo, the proportion of high and low affinity agonist sites could be transposed (4). Since the conformation and specificity of several neurotransmitter and hormonal receptor binding sites may be modified by guanine nucleotides (5-10), we investigated the possibility that the guanine nucleotides exert their effect through such transposition.

Materials and Methods

(^{3}H)-N-methy-4-piperidyl benzilate (4NMPB) (33Ci/mmole) and unlabeled muscarinic ligands have been described previously (1). The nucleotides were purchased from Sigma.

Male ICR mice (20-25g) were decapitated, their brains rapidly removed and the brain areas dissected and homogenised as described previously (1)(3).

For the binding assays, homogenates were incubated at 37^o in modified Krebs-Hensleit solution, pH 7.4 (1) containing the labeled ligand (2 nM), together with unlabeled competing ligands and nucleotides as indicated in Fig. 1. After various periods of incubation, the reaction was

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terminated by filtration as previously described (1). Assays were carried out in triplicate, and the specific binding defined as the total minus the non-specific binding, i.e. in the presence of 1×10^{-6}M of unlabeled 4NMPB or atropine. Protein was determined by the Lowry method using bovine serum albumin as a standard.

Results

The effect of the guanine nucleotide GTP on the binding of the antagonist (^{3}H)-4NMPB and the agonists oxotremorine and carbamylcholine was investigated in homogenates of medulla-pons, cerebellum, cortex and hippocampus. In the medulla-pons, as shown in Fig. 1A, as well as in the cerebellum (not shown), the binding of 4NMPB, an antagonist, was not altered (no change in K_d or Bmax). However, the ability of the agonists oxotremorine (Fig. 1B) and carbamylcholine (not shown) to inhibit (^{3}H)-4NMPB binding was markedly decreased, the shift to the right in the displacement curve for oxotremorine indicating an 8-fold increase in the I_{50} value. Maximal effect was observed when the reaction was allowed to proceed for 5 min. This GTP effect could be reproduced when homogenates were incubated at 30°, but longer incubation times were needed (∿12 min) to obtain the same effect as that shown in Fig. 1B. The effect of GTP on the I_{50} values was seen at a GTP concentration of 3-5 μM; half-maximal and maximal effects occurred at approximately 10 μM and 50 μM, respectively. In the homogenates of cortex, hippocampus and caudate putamen, there was little, if any, GTP effect on the binding of either antagonist (4NMPB) or agonist (oxotremorine and carbamylcholine).

The effect of GTP on the oxotremorine competition curve in the medulla-pons and cerebellum homogenates was two-fold: not only was the curve shifted to the right, but its slope became steeper. Flattened agonist/(^{3}H)-antagonist inhibition curves are well documented (1)(2)(11) and have led to the suggestion (2)(11) that agonists bind with differing affinities to two or three distinct site populations. The data obtained from inhibition of (^{3}H)-4NMPB binding by oxotremorine in the cerebellum and medulla-pons (Fig. 1B) can be fitted by the simplest model i.e. the two site model. The binding parameters for oxotremorine were evaluated from these experiments by the best fit procedure using a non-linear least squares program (4) and suggest that the increase in I_{50} values in the medulla-pons and cerebellum, after treatment with GTP, stems mainly from an increase in proportion of low affinity binding sites in these regions, from 35% (control) to about 70%. No substantial changes in dissociation constants were seen (K_L = 1.6 μM (control) and 1.1 μM (GTP treated); K_H = 0.020 μM (control) and 0.015 μM (GTP treated)).

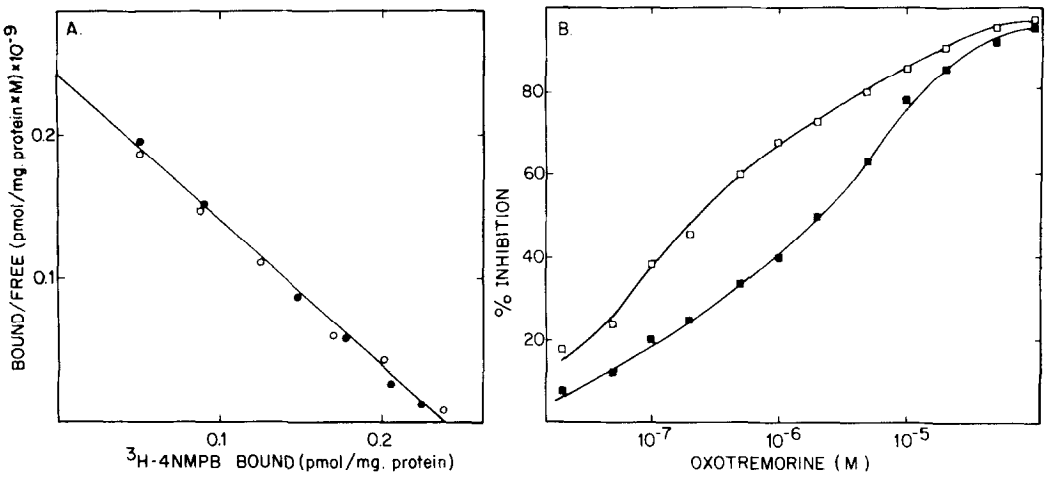

Fig. 1. Effect of GTP (50 μM) on binding of (^{3}H)-4NMPB (1A) and oxotremorine (1B) to medulla-pons homogenates, in the absence (open symbols) and presence (closed symbols) of GTP. Samples (0.05 ml) were incubated at 37° for 5 min. in 2 ml modified Krebs-Hensleit solution containing various concentrations of (^{3}H)-4NMPB (A) or 2.0 nM (^{3}H)-4NMPB and the concentration of unlabeled ligands indicated (B).

Other nucleotides were tested to see whether they produced effects similar to that of GTP (Fig. 2). It can be seen that Gpp(NH)p, a GTP analogue which is resistant to nucleotide phosphohydrolase action, was slightly more effective than GTP. The maximal effect of GTP and of Gpp(NH)p was obtained at 50 μM. GDP (50 μM) produced 50% of the effect caused by 50 μM GTP; however, the possibility that GDP was converted to GTP by the membrane preparation cannot be ruled out. Other nucleotides including ATP, ADP, cAMP and cGMP even at a concentration of 1 mM, were essentially inactive. The effects of GTP on agonist binding are relatively short-lived, being strongly dimished after 20 min., probably due to metabolisation by the membrane preparation; on the other hand, the effects of Gpp(NH)p are more persistent and last for more than 2 hours.

Preliminary experiments on the effects of Mg^{2+} suggest that the Mg^{2+} is important for the guanine nucleotide effect and cannot be replaced by Ca^{2+}. In the absence of Mg^{2+} the GTP effect is still observed but the shift in the I_{50} value is only patial i.e. 3-fold. In the presence of 0.5 mM Mg^{2+} maximal effect (10-fold shift) is observed, suggesting a sequential GTP effect which might be related to the interconversion process as discussed below.

Discussion

The data presented here clearly show: (i) a selective effect of guanine nucleotides upon agonist interactions with muscarinic receptors by means of

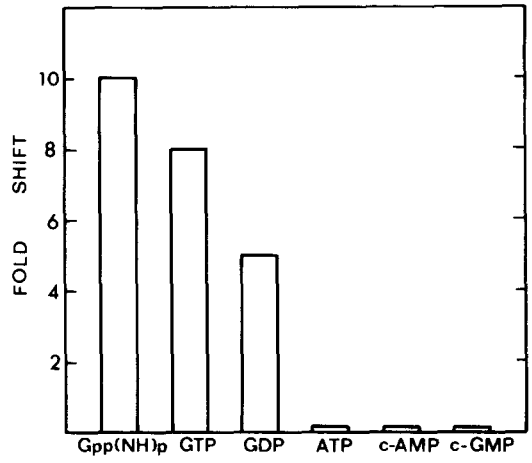

Fig. 2. Effects of various nucleotides on oxotremorine inhibition of specific (^{3}H)-4NMPB binding. The shift in the oxotremorine inhibition of specific (^{3}H)-4NMPB binding was determined as described in Fig. 1B. GTP, GDP and Gpp(NH)p concentration was 50 µM while other nucleotides were at 1 mM. Values shown are means of 3 experiments.

lowering the receptor-ligand affinity. These effects are quite similar to those described for muscarinic receptors in rat heart (12)(13) and for several other neurotransmitter and hormonal receptors (5-10). As in those systems, we also observed that GTP had no effect on the binding of muscarinic antagonists; hence the effect of guanine nucleotides can be used to discriminate between agonist and antagonist interactions with muscarinic receptors. (ii) GTP induces interconversion of high to low affinity agonist binding sites. The effect is observed in those brain regions which,under our standard assay conditions,have a relatively large proportion of high affinity binding sites (medulla-pons and cerebellum),while in regions enriched in low affinity sites (cortex, hippocampus) no such effect is seen (4). It should be noted that the effect of guanine nucleotides on the rat heart muscarinic receptors are larger (12) than those reported here for mouse brain. This difference might result from the presence of large population of high affinity sites in the rat heart as compared to mouse medulla-pons.

The question arises whether the GTP effect is a general phenomenon for all muscarinic receptors in the CNS and the fact that we have obtained positive results only in the medulla-pons and cerebellum is due to limitations of the methodology employed. Alternatively, the GTP effect might be confined to regions whose binding characteristics resemble those of the medulla-pons and cerebellum, thus affording further insight into muscarinic receptor heterogeneity. In this context it should be noted that we recently suggested that these two brain regions are enriched with presynaptic muscarinic recep-

tors (4)(14), and it is therefore tempting to suggest that the GTP effect is augmented by presynaptic receptors. However, recent experiments in our laboratory have indicated that interconversion between high and low affinity sites can be influenced by a variety of factors such as temperature, sugars, pH etc. Hence, conclusions about the nature of these receptors and their "region-specificity" should await further and more detailed experimentation, i.e. using labeled agonists.

It would appear that a modulating system located on the muscarinic receptor complex is distal to the antagonist binding site, since the latter is unaltered by guanine nucleotides. By analogy to other neurotransmitter systems, it is also tempting to suggest that the muscarinic complex 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 system, or Ca^{2+} channel. The process of receptor regulation appears to result in a selective interconversion of high to low affinity binding sites, and treatments blocking the formation of low affinity agonist sites (experiments to be published) will therefore prevent regulation of muscainic receptors. These suggestions, which are of course still speculative, are in accordance with the following observations: (i) Regulation of adenylate cyclase activity is mediated by muscarinic receptors in, e.g. NG108-15 hybrid cells (15) and rabbit myocardium (16). (ii) Muscarinic agonists increased cGMP levels in mammalian superior cervical ganglion (17), secretory systems (18), brain tissue slices (11)(19) and cultured neurons (20)(21). It is noteworthy that Strnage et al. (21) suggested that muscarinic activation of cGMP formation corresponds only to the occupancy of the low-affinity population of muscarinic receptors in neuroblastoma cells. (iii) Desensitisation of muscarinic receptor-mediated cGMP formation was observed in cultured nerve cells (22). Experiments to verify this hypothesis are currently under investigation in our laboratory.

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