An $[3^H]$Oxotremorine Binding Method Reveals Regulatory Changes by Guanine Nucleotides in Cholinergic Muscarinic Receptors of Cerebral Cortex

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A rapid, reliable filtration method for $[3^H]$oxotremorine binding to membranes of the cerebral cortex that allows the direct study of regulation by guanine nucleotides of muscarinic receptors was developed. $[3^H]$Oxotremorine binds to cerebral cortex membranes with high affinity ($K_D$, 1.9 nM) and low capacity ($B_{max}$, 187 pmol/g protein). These sites, which represent only about 18% of those labeled with $[3^H]$quinuclidinyl benzilate, constitute a population of GTP-sensitive binding sites. Association and dissociation binding experiments revealed a similar value of $K_D$ (2.3 nM). Displacement studies with 1–4000 nM oxotremorine showed the existence of a second binding site of low affinity ($K_D$, 1.2 µM) and large capacity ($B_{max}$, 1904 pmol/g protein). Gpp(NH)p, added in vitro, produced a striking inhibition of $[3^H]$oxotremorine binding with an IC 50 of 0.3 µM. Saturation assays, in the presence of 0.5 µM Gpp(NH)p, revealed a non-competitive inhibition of the binding with little change in affinity. These results are discussed from the viewpoint of conflicting reports in the literature about guanine nucleotide regulation of muscarinic receptors in reconstituted systems and membranes from different tissues.

KEY WORDS: $[3^H]$Oxotremorine; muscarinic receptors; guanine nucleotides; receptor regulation; high- and low-affinity sites.

INTRODUCTION

Cholinergic muscarinic receptors are widely distributed in the central and peripheral nervous systems, and also as in those tissues innervated by parasympathetic neurons. Radioligand binding studies have revealed that, while muscarinic antagonists bind to a single class of sites, agonists interact with heterogeneous population of sites having different affinities (1, 3). This different behavior of agonists and antagonists for the receptor is also reflected in the regulatory action of mono- and divalent cations (4, 16, 22), sulphhydril reagents such as N-ethylmaleimide or p-chloromercuribenzoate (8, 18) and guanine nucleotides (2, 8, 15). These regulatory effects occur essentially on the binding of agonists; however, most investigations have been carried out by using $^3H$-antagonists in displacement experiments with agonists. More recently, some studies have also been done using $^3H$-agonists, such as oxotremorine (17), cis-methylidioxolane (7), and acetylcholine (11).

Guanine nucleotides tend to inhibit agonist binding, an effect that is more pronounced in heart, ileum, and cerebellum, than in the cerebral cortex, corpus striatum, and hippocampus. These brain regions show little or no change in agonist binding in the presence of the nucleotide (23). This differential

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regulatory effect of guanine nucleotides in the various tissues has been interpreted by some as favoring the existence of muscarinic receptor subtypes M₁ and M₂, as can be detected by pirenzepine binding (12, 25).

The differential regulation by guanine nucleotides could also depend on whether or not the receptor can be coupled to a regulatory protein (23). Furthermore, the modulation by GTP could be related to the inhibition of adenylate cyclase by way of the muscarinic receptor agonists (21). The fact that in the intact membranes of cerebral cortex the interaction between muscarinic receptors and GTP regulatory proteins is not readily observed (10) might be due to the existence of only a small population of GTP-sensitive receptors. This hypothesis led us to study the regulatory effect mediated by GTP, using a specific muscarinic agonist such as [³H]oxotremorine.

In the present work we have characterized the binding properties of the agonist [³H]oxotremorine to crude synaptosomal membranes of the cerebral cortex of bovine or rat brain. A rapid filtration technique was developed, which provides a selective method for studying the high-affinity agonist state of the muscarinic receptor in this area of the brain. With this technique, at variance with the data from the literature, marked modulatory effects of guanine nucleotides on the binding of [³H]oxotremorine could be demonstrated and the properties analyzed.

**EXPERIMENTAL PROCEDURE**

**Membrane Preparation.** Bovine brains provided by a local slaughter house were put on ice and brought to the laboratory. The cerebral cortex was dissected in the cold and homogenized in 10 vol of ice-cold 0.32 M sucrose using a glass teflon homogenizer (15 strokes). The homogenate was centrifuged at 1000 g for 10 min, the supernatant was separated and the pellet was again rehomogenized in sucrose and centrifuged as above. Pellet (P₁) was discarded and the two supernatants were pooled and centrifuged at 11,000 g for 30 min. The pellet (P₂) obtained was submitted to an osmotic shock in 13 vol of distilled water for 20 min and centrifuged at 20,000 g for 30 min. The pellet obtained (P₃) was separated and resuspended in a buffer of 10 mM Tris-CI, pH 7.4, and kept frozen at -60°C until used for binding assay. Such a P₃ pellet can be considered a crude synaptosomal membrane fraction.

**[³H]Oxotremorine Binding Assay.** Treatment of the Glass Filters. For the binding of [³H]oxotremorine (84.9 Ci/mm, NEN) the membranes were resuspended in the above buffer plus 5 mM MgCl₂ to reach a concentration of 0.75 nmol/mL, as determined by the Folin reagent (19). For each assay, aliquots of 0.25 or 0.5 ml of the membrane suspension were incubated using various concentrations of the [³H]-ligand. The incubations were carried out at 4°C for 3 hr and the assays were done in triplicate. The assay was terminated by the addition of 5 ml of ice-cold buffer and the separation of the bound ligand was done by rapid filtration on GF/B glass fiber filters (Whatman) under vacuum, followed by washing twice with 5 ml of the same buffer. The specific binding was determined by subtracting from the total binding the part that was not displaced by 10 μM atropine. The filters were dried and, after the addition of 3 ml of PPO-toluene as scintillation fluid, were counted in a Tracor spectrometer with a 30% efficiency.

To measure the binding parameters of oxotremorine at higher concentrations (1-4000 nM), drug displacement experiments were done using unlabeled oxotremorine. These assays were carried out as described above, but samples were incubated with 1 nM [³H]oxotremorine and varying concentrations of the cold ligand.

In normal assay conditions, [³H]oxotremorine tends to bind pseudospecifically to the glass fiber filters, showing a considerable atropine displaceable binding (see Table I). To avoid this artifact we tried several variations of the assay, such as changing types and concentrations of buffer, and using different glass fiber filters. Finally, the problem was solved by presoaking the GF/B filters, for 60 min, with a solution of 0.01% polyethyleneimine, a treatment that has been successfully used for other [³H]-ligands (24) (see Results).

**Kinetic Experiments.** For the kinetic experiments the procedure was essentially the same. Association assays were carried out under pseudo first-order conditions. Samples were incubated with 0.5, 0.8, or 1.2 nM [³H]oxotremorine and filtered at different time intervals.

For dissociation experiments, either unlabeled oxotremorine (10 μM) or 25 μl of ice-cold buffer (10 mM Tris-Cl, 5 mM MgCl₂, pH 7.4) were added to samples that had reached equilibrium after incubation in the presence of 1 nM [³H]oxotremorine; then samples were filtered at different time intervals.

**[³H]QNB Binding Assay.** Muscarinic cholinergic receptors were also analyzed using [³H]-quinuclidinyl benzylate (QNB, 33.1 Ci/mm, NEN) and the assay conditions routinely used in our laboratory (9).

**Data Analysis.** Data obtained from the binding experiments were analyzed with the aid of an IBM PC computer, using a nonlinear least-squares curve fitting program called LIGAND, whose theory was described in detail by Munson and Rodbard (20). The kinetic experiments were analyzed with the aid of another computer program called ESTRIP, a polyexponential curve fitting program suitable for complex dissociation curves in radioligand binding experiments (5). The significance of the data was determined using the Student’s “t” test.

**RESULTS**

In Table I, it is shown that [³H]oxotremorine binds pseudospecifically (i.e., displaceable by atropine) to the GF/B filters. This artifact can be successfully eliminated by the use of a previous immersion of the filters in polyethyleneimine. With this treatment the binding of [³H]oxotremorine to the filters is almost nil and the non-specific binding to