Binding of \(^3\text{H}\)-Pilocarpine to Membranes from Rat Cerebral Cortex

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Summary. Binding of \(^3\text{H}\)-pilocarpine to synaptic membranes in rat cerebral cortex was investigated, pilocarpine binding was also studied by competition of unlabelled pilocarpine with the \(^3\text{H}\)-labelled muscarinic antagonist, \(^3\text{H}\)-N-methyl-4-piperidinyl benzilate.

1. \(^3\text{H}\)-pilocarpine binding sites are of protein nature, and the highest specific activity of binding is found in the synaptosomal fraction of all subcellular fractions.

2. Competition studies show that only muscarinic drugs inhibit \(^3\text{H}\)-pilocarpine binding in their pharmacologically active concentration range.

3. Binding of \(^3\text{H}\)-pilocarpine is influenced by GMPP-(NH)P (0.1 mM) similarly to the binding of other muscarinic agonists.

4. Examination of pilocarpine binding with \(^3\text{H}\)-pilocarpine and the competition experiments with unlabelled pilocarpine indicate the presence of three sites with different affinities: 5 nM, 0.2 μM and 30 μM respectively.

5. Experiments with \(^3\text{H}\)-4-NMPB and \(^3\text{H}\)-pilocarpine indicate that there are more \(^3\text{H}\)-pilocarpine binding sites than \(^3\text{H}\)-4-NMPB binding sites in rat cerebral cortex.

Key words: Pilocarpine — Agonist binding — Muscarinic receptor — Cerebral cortex

Introduction

\(^3\text{H}\)-labelled antagonists: \(^3\text{H}\)-3-quinuclidinyl benzilate (Yamamura and Snyder 1974), \(^3\text{H}\)-propyl benzilyl choline mustard (Burgen et al. 1974), \(^3\text{H}\)-atropine (Alberts and Bartfai 1976) and \(^3\text{H}\)-N-methyl-4-piperidinyl benzilate (Kloog and Sokolovsky 1978) have been extensively used to characterize muscarinic receptors (cf. for review Birsdall and Hulme 1979). Binding of antagonists and agonists appeared to obey different molecular mechanisms. Whereas antagonist binding curves could be described by a single rectangular hyperbola, agonist binding curves had to be fitted with models describing at least two receptor populations with different binding capacities and affinities (Kloog and Sokolovsky 1977; Hedlund and Bartfai 1979). Recently, however, heterogeneity among antagonist binding sites has also been reported (Hammer et al. 1980; Hedlund 1981; Mukherjee et al. 1980).

Lack of high specific activity, high-affinity muscarinic agonists forced most investigators to study receptor-agonist interactions indirectly, by measuring inhibition of the binding of \(^3\text{H}\)-antagonists by the agonist. The study of direct binding of \(^3\text{H}\)-N-methyloxotremorine by Birdsall and his colleagues (Birdsall et al. 1978) and of \(^3\text{H}\)-cis-methyl dioxolane (Ehler et al. 1980) constitute exceptions to this approach.

Recently, \(^3\text{H}\)-pilocarpine became commercially available to provide a high specific activity (10 Ci/m mole) agonist for the study of muscarinic receptors. The stimulatory effects and agonist-like properties of pilocarpine are well documented (Christie and Overstreet 1979; Lyson 1979). We have decided to investigate the binding of this \(^3\text{H}\)-agonist (Furchgott and Bursztyn 1967) to muscarinic receptors from rat cerebral cortex.

Materials and Methods

\(^3\text{H}\)-pilocarpine (10 Ci/m mole) was purchased from New England Nuclear Co., Boston, MA, USA.

\(^3\text{H}\)-N-methyl-4-piperidinyl benzilate (\(^3\text{H}\)-4-NMPB) (33 Ci/m mole or 70 Ci/m mole) and N-methyl-4-piperidinyl benzilate (4-NMPB) were generously donated by Professor Mordechai Sokolovsky, Tel Aviv University, Israel. All other chemicals were of reagent grade and from Sigma except oxotremorine and Lumagel which were from Aldrich, Milwaukee, WI, USA, and Lumac, Basel, Switzerland, respectively.

Male Sprague Dawley rats, from Anticimex, Stockholm, Sweden, weighing 200–250 g, were decapitated and the brains rapidly removed. Membranes were prepared as earlier described (Hedlund and Bartfai 1979). The crude mitochondrial pellet (P2) was resuspended in a modified Krebs Ringer’s buffer (137 mM NaCl, 2.68 mM KC1, 1.80 mM CaCl2, 1.05 mM MgCl2, 5 mM Hepes, 1 g/l glucose and 10 μM phenyl methyl sulfonl fluoride, pH = 7.4) and used for binding studies (in the kinetic studies and denaturation experiments) without further treatment.

Synaptosomes were prepared using a Ficoll/sucrose gradient. In this case the crude mitochondrial pellet was resuspended in 17% (w/v) Ficoll in 10% (w/v) sucrose and the membrane suspension transferred to Beckman SW-27 centrifuge tubes. An equal volume of 6% (w/v) Ficoll in 10% (w/v) sucrose was overlaid. After centrifugation at 100,000 X g for 2 h three fractions were collected: the myelin fraction on top of the tube, the brown mitochondrial fraction in the bottom and the synaptosomal fraction at the interface of the gradient. The three fractions were spun down, after dilution, in a cooled centrifuge to remove Ficoll and sucrose and the pellets obtained this way were resuspended in Krebs Ringer’s buffer and used for binding studies.

Equilibrium binding studies with the \(^3\text{H}\)-antagonist were carried out by the filtration method described by Yamamura and Snyder (1974). The incubation was carried out in glass tubes at room temperature for 45 min for \(^3\text{H}\)-4-NMPB. Filtration and washing of the filters with at least 10 ml of ice cold buffer was completed within 20 s. Specific binding of the antagonist was defined as the difference in binding of the labelled ligand in the absence and presence of excess (10 μM) unlabelled atropine.

Under the same assay conditions \(^3\text{H}\)-pilocarpine binds to glass fiber filters. Binding to Whatman GF/B filters is characterized by a Kd value of 1.5 μM (cf. Fig. 1) and could be reduced by presoaking the filters with carbacol or oxotremorine.

We refrained from filtration assay and used a centrifugation assay to study the binding of \(^3\text{H}\)-pilocarpine: In brief: membranes and \(^3\text{H}\)-
pilocarpine were incubated in 1.5 ml plastic centrifuge tubes (LKB-Beckman) together with appropriate ligands in a total volume of 200–500 μl at room temperature for 30–40 min.

Bound ligand was separated from free ligand by centrifugation for 30 s in a Beckman Microfuge B. The supernatant was discarded and the bottom of the tube containing membranes and bound [3H]-pilocarpine, punched out into a scintillation vial.

5 ml Lumagel scintillation cocktail was added and the vials were shaken before they were counted for radioactivity on the next day in a liquid scintillation spectrometer at an efficiency of 45–50%.

Specific binding of [3H]-pilocarpine was defined as the difference in binding in the absence and presence of 1 mM unlabelled oxotremorine, a concentration which did completely block [3H]-4-NMPB-binding.

All K₁-values were calculated graphically.

The protein concentration was determined according to Lowry et al. (1951) using bovine serum albumin as standard.

Results

Characterization of the Kinetics of [3H]-Pilocarpine Binding

As membranes (P2) and synaptosomes showed similar association and dissociation kinetics the association rate of [3H]-pilocarpine to muscarinic receptors was studied under pseudo-first order conditions using membranes (P2) of rat cerebral cortex containing approximately 1 picomole of receptor (determined by 3H-antagonist binding) and 25 picomoles or 100 picomoles [3H]-pilocarpine, respectively, in 1 ml volume. Saturation of the specific binding was reached within 30–40 min at 25°C at both pilocarpine concentrations. The association rate constant was 2.66 × 10⁸ min⁻¹ M⁻¹. Dissociation of the receptor-[3H]-pilocarpine complex was studied following addition of a large excess of oxotremorine (10⁻³ M) to the incubation mixture. The dissociation rate constant was 0.08 ± 0.02 min⁻¹. Within 25–30 min 100% of the specific [3H]-pilocarpine binding was displaced, indicating that the binding was fully reversible. The calculated K₂ is 3.0 ± 0.5 × 10⁻⁴ M in good agreement with one of the dissociation constants found in equilibrium binding studies.

Protein Nature of [3H]-Pilocarpine Binding Sites: Experiments with Trypsin and Heating

Heat denaturation experiments (using a membrane preparation (P2) carried out at 60°C showed that the decay of the specific [3H]-pilocarpine binding sites and of [3H]-4-NMPB binding sites (determined for both ligands in the absence and presence of oxotremorine (10⁻² M) were identical (Fig. 2). A total heat denaturation of the specific binding capacity was reached after 10 min incubation at 60°C. After incubation of the membranes with trypsin (10 μg/ml for 30 min at 37°C) specific binding of both [3H]-4-NMPB and [3H]-pilocarpine were totally abolished (data not shown).

Specificity, Density and Affinity of [3H]-Pilocarpine Binding Sites

Inhibition of the binding of [3H]-pilocarpine (0.5 μM) to synaptosomes by cholinergic drugs was studied with the centrifugation assay. Figure 3A shows the results of com-