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PHOTOAFFINITY LABELING OF SPECIFIC MUSCARINIC ANTAGONIST BINDING SITES OF BRAIN: I. PRELIMINARY STUDIES USING TWO p-AZIDOPHENYLACETATE ESTERS OF TROPINE

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SUMMARY

Possible photoaffinity probes for muscarinic acetylcholine receptors have been explored for the first time: Specific [^{3}H]-quinuclidinyl benzylate binding sites of several fractions from rat brain can be irreversibly inactivated by photoaffinity labeling with two p-azidophenylacetate esters of tropine. Inactivation of these sites depends on formation of a reversible complex with the azides prior to their photolytic conversion to the highly reactive nitrenes; it is dependent on ligand concentration and length of photolysis. Atropine and oxotremorine, but not d-tubocurarine, afford protection against photoinactivation.

These findings suggest the utility of these and related azido derivatives as potent, selective photoaffinity ligands directed against binding sites for muscarinic antagonists.

INTRODUCTION

Although some progress has been made in the isolation and characterization of the muscarinic acetyl choline receptor (mAChR)[1], our knowledge of its biochemical nature has lagged, in large part, due to the unavailability of specific, covalently linked ligands (1,2). Affinity labeling, the method of choice for this covalent attachment to the active center of receptor proteins (3) has been applied to mAChR by Fewtrell and Rang (4), Burgen *et al.* (5) and Rueß *et al.* (6) with various benzylcholine mustards.

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[1] Abbreviations used: CM, crude membranes (P_2 + P_3); mAChR, muscarinic acetylcholine receptors; ^{3}H-QNB, [^{3}H]-quinuclidinyl benzylate; SPM, synaptic plasma membranes.

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In an attempt to obtain a more facile and specific labeling of mAChR in rat brain membranes we have instituted a search for potential photoaffinity labels (7). Here we present evidence that the ^{3}H-QNB[1] binding sites of several subfractions from this source can be irreversibly inactivated by photoinsertion of p-azidophenylacetate esters of tropine. This reaction requires the prior and reversible attachment of the azido reagent to these antagonist binding sites of the mAChR and appears to be a function of both, reagent concentration and time of photolysis. The muscarinic ligands atropine (antagonist) and oxotremorine (agonist), but not d-tubocurarine, a nicotinic antagonist, afford protection against photoinsertion.

MATERIALS AND METHODS

Materials: ^{3}H-QNB (13 Ci/mmol) and ^{3}H-atropine (620 mCi/mmol) were from Amersham-Searle, Arlington Heights, Ill. Atropine sulfate was from Sigma Chemical Co., St. Louis, MO, d-tubocurarine from Cal Biochem, San Diego, CA, and oxotremorine and tropine from Aldrich Chemical Co., Milwaukee, WI.

Subcellular fractionation: Cerebral cortices were removed from decapitated, 30-35 day old, male Sprague-Dawley rats and homogenized in 0.32 M sucrose, using a glass homogenizer with motor-driven (450 rpm) pestle. The nuclear fraction was removed by centrifugation at 1000 xg for 10 min and discarded. The supernatant was centrifuged further at 80,000 xg for 90 min. The resultant pellet (crude membranes - CM[1]) was resuspended in 0.32 M sucrose and frozen (-20°C) until assayed. The synaptic plasma membrane fraction (SPM) from cortex was prepared as described previously (9).

Synthesis of p-azidophenylacetate esters of tropine: Figure 1 outlines the synthesis of Compounds I and II, two p-azidophenylacetate esters of tropine. In general one equivalent of the chloride of p-azidophenylacetic acid and one equivalent of tropine were dissolved in dioxane and the reaction mixture allowed to react at room temperature in the dark for 48 h. The solvent was evaporated and the oil obtained was purified on Alumina II, using ethyl acetate as solvent. Removal of the ethyl acetate produced an oil (Compound I). The purity of I was checked by NMR and infrared spectroscopy, which showed the characteristic azide absorption band at 2120 cm^{-1}. Using mass spectroscopy the molecular ion was found at m/e 300, calculated 300.

Compound I was dissolved in acetone and three equivalents of methyl iodide were added and the mixture allowed to react for 36 h at room temperature in the dark. The product, II, was isolated by filtration, washed several times with cold acetone and dried *in vacuo*. Both compounds were kept in the dark at 4°C until use. Under these conditions they were stable for more than four months.

Photolabeling: Protein solutions (1 ml; 1 mg/ml) in 50 mM Na^+-K^+ phosphate, pH 7.4, were incubated with varying concentrations of reagents I and II in

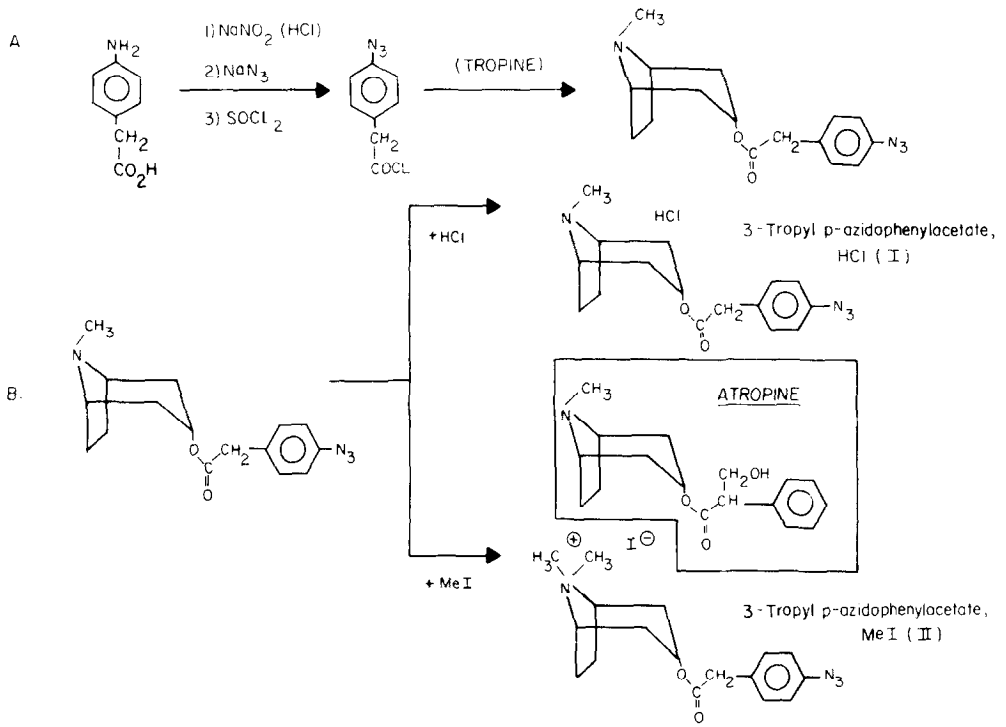

Fig. 1. Outline of Synthesis of Compounds I and II

the presence or absence of competitors (atropine, oxotremorine) at room temperature for 60 min in the dark. The reaction mixture was cooled to 4°C and photolyzed with a short-wavelength (254 nm) UVS-11 Mineralight (Ultraviolet Products, San Gabriel, CA) at a distance of 4 cm for different times. In some cases (Fig. 3A and B) a Pen-Ray Lamp (Ultraviolet Products, San Gabriel, CA) was used. In this case the photolabeling procedure was as described, except for a distance of 12 cm, and stirring of the reaction mixture.

Other methods: ^{3}H-QNB and ^{3}H-atropine binding assays were performed as described earlier (8,9).

RESULTS

a) Compounds I and II inhibit binding of [^{3}H]-QNB and [^{3}H]-atropine: Both Compound I and II were able to inhibit the binding of ^{3}H-QNB and ^{3}H-atropine to CM and SPM fractions from rat brain. In Fig. 2 we show such data for CM as a function of ligand concentration. The ID_{50}, the concentrations required to reduce ^{3}H-QNB binding to 50% of the control values, were 8.5×10^{-6} M (I) and 1.0×10^{-6} M (II), corresponding to apparent dissociation constants (10) of 3.5 and 0.42×10^{-6} M respectively. The use of [^{3}H]atropine as the primary

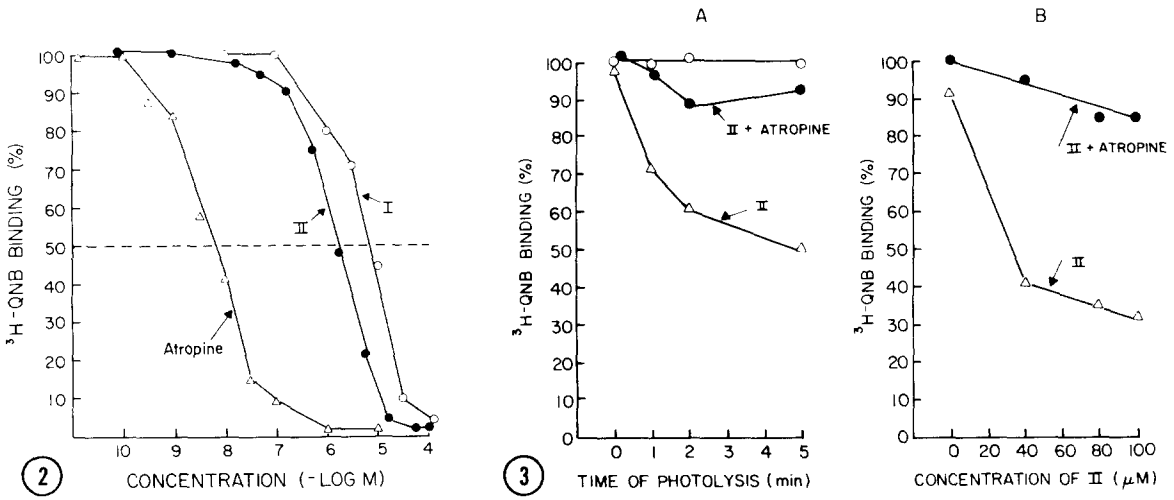

Fig. 2. Inhibition of ^{3}H-QNB binding to CM by atropine, I and II. Experiments performed as described under Methods, in the dark for 60 min at room temperature. K_D, the apparent dissociation constants were calculated from $K_D = ID_{50}/L \times K_L$ (13), where ID_{50} is the concentration of the ligand needed to inhibit 50% of the ^{3}H-QNB binding, L is the concentration of ^{3}H-QNB used in the assay (1.8 nM), and K_L equals K_D for ^{3}H-QNB (0.76 nM) (6,7). The results reported are the means of three experiments, each in triplicate, and agreeing within 5-15%.

Fig. 3. A) Irreversible Inactivation of ^{3}H-QNB Binding Sites as a Function of Time of Photolysis. Membranes alone (O—O), + Compound II (6×10^{-5} M) (Δ—Δ), + atropine (2×10^{-6} M) and Compound II (6×10^{-5} M) (●—●), were incubated for 60 min at room temperature in the dark and then irradiated, as described in Methods. The washing procedure was as described in the legend to Table 1, with the final pellet resuspended in 1 ml of Na^+/K^+ phosphate buffer pH 7.4, and assayed for ^{3}H-QNB (1.8 μM) binding. Results are expressed as % of the values obtained with membranes alone with no irradiation, and are means of four determinations agreeing within 5-10%.

B) Irreversible Inactivation of ^{3}H-QNB Binding Sites as a Function of Concentration of Photoaffinity Reagent II. See legend to Fig. 3A for details.

ligand generated similar results (data not shown), with an apparent dissociation constant of 3.4×10^{-6} M for I, in total agreement with that found by competition with ^{3}H-QNB.

Similar results (not shown) were obtained with SPM fractions. Compound I was able to inhibit the binding of ^{3}H-QNB with an apparent dissociation constant of 2.7×10^{-6} M ($ID_{50} = 5 \times 10^{-5}$ M). Irradiation of the mixture of SPM and I, just prior to the [^{3}H]-QNB binding assay, resulted in displacing the titration

curve to the left (not shown), lowering the apparent dissociation constant to 4.2×10^{-7} M, suggesting that a fraction of Compound I had become inserted into its binding sites, the presumed receptor.

b) Photolysis leads to irreversible inactivation of QNB-binding sites: To try to determine whether the two ligands were indeed capable of irreversible inactivation of such QNB binding sites, CM fractions were incubated with several concentrations of the reagents for 60 min at room temperature in the dark, followed by irradiation with short wave UV light. The samples were then diluted and washed by centrifugation (see legend to Table I and Fig. 3 for details), and again assayed for ^{3}H-QNB binding. Two controls were performed in parallel: a) membranes without added ligand but subjected to irradiation, and b) membranes plus I or II added at a higher concentration, but without irradiation. Both controls gave values for ^{3}H-QNB binding agreeing within 5% of those found in the standard binding assay for this ligand. The results are presented in Table Ia and permit the inference that both compounds (at 5×10^{-5} M) are potential photoaffinity probes, at least for the antagonist-binding subsites of the mAChR. It is also evident that for the irreversible inactivation of these sites, the ligands require photochemical conversion to the highly reactive nitrene, a dose-dependent reaction, proportional to length of exposure to (Fig. 3A) and concentration of photoaffinity reagent (Fig. 3B).

c) Muscarinic ligands protect the receptor against photoinsertion: The muscarinic antagonist atropine was found to protect receptor sites against this irreversible loss of ability to bind ^{3}H-QNB, as shown in Table Ib and Ic and Figs. 3A and B. Similar results were obtained when oxotremorine, a muscarinic agonist, was used as the protective agent; in contrast, d-tubocurarine, a nicotinic antagonist, was completely incapable of preventing the reaction.

TABLE I

EFFECTS OF PHOTOAFFINITY PROBES I AND II ON [^{3}H]-QNB BINDING TO MEMBRANE FRACTIONS

Experiment	*Ligand*	*[^{3}H]-QNB Binding (% of control)*	
		unirradiated	*irradiated*
A	I	95	49
(n=4)	II	96	43
		- atropine	*+ atropine*
B	I	42	74
(n=4)	II	50	81
C (n=4)	I (50 μM)	48	74
	I (25 μM)	64	100
	I (10 μM)	59	100

A) Irreversible Inactivation of ^{3}H-QNB Binding by Photoaffinity Reagents I and II

Experiment A): CM were incubated (see Methods) with I and II (5×10^{-5} M). Experimental samples were irradiated for 30 min at 4°C while controls were kept in the dark at 4°C during this time. Samples were then diluted with 5 ml of buffer, centrifuged at 30,000 x g x 30 min, pellets resuspended in 2 ml of buffer and diluted to 6 ml final volume and centrifuged again. This procedure was repeated three more times. Final pellets were resuspended in 1 ml of buffer and assayed for QNB binding using 1.8 nM [^{3}H]-QNB. The results are expressed as % of a control in which no photoaffinity reagents were added, and are means of four determinations agreeing within 10-15%.

B) Membrane samples (see A) were incubated in the presence or absence of 50 μM atropine. After 30 min of incubation at room temperature, I (or II), final conc = 50 μM was added and the incubation continued for 60 min in the dark. All samples were then irradiated for 30 min at 4°C. The washing procedure and [^{3}H]-QNB binding were performed as described in A.

Controls: 1) Membranes incubated with atropine (50 μM) and then washed as described above. 2) As in 1) plus irradiation, and 3) not incubated with atropine, unirradiated. The results of these three different controls agreed within 5%.

C) See A and B. Three different concentrations of photoaffinity reagent I were used.

DISCUSSION

A specific photoaffinity probe for the ligand-binding site of a biologically active protein, such as an enzyme or receptor, must imitate its natural

counterpart in both structure and function (7,11). In the case of the mAChR, atropine is known to be highly specific in its binding to these sites (12). We have therefore used this particular molecule as a model in our attempts to synthesize photoaffinity probes for its receptors. We have prepared Compounds I and II, two p-azidophenylacetate esters of tropine, and found them to be useful photoaffinity probes for mAChR sites in rat brain. In the dark, both reagents are bound reversibly to the ^{3}H-QNB binding sites, which can then be inactivated irreversibly upon photolysis. Exposure of membrane preparations to photolysis conditions in the absence of ligands does not result in inactivation. Specific occupancy of the receptors by muscarinic ligands such as atropine (antagonist) and oxotremorine (agonist) eliminates or reduces the photoinactivation by the probes, while d-tubocurarine is without effect.

The structure of atropine and its derivatives I and II are compared in Fig. 1. It is apparent that the photoaffinity probes lack the $-CH_2-OH$ group of atropine which is required for its binding to antagonist sites with high affinity (11,13). The differences in apparent dissociation constants for I and II (3.5 μM and 0.43 μM; Fig. 1) - virtually identical to those of the unsubstituted parent compounds (11,12) - compared to atropine (0.3 nM), are probably a reflection of this structural feature. The azido function therefore does not influence binding, but the introduction of a second methyl group on the nitrogen atom of tropine in Compound II increases its affinity for the receptors by a factor of two, again in agreement with values reported for atropine and its derivatives (12,13).

The two principal features of the acetylcholine molecule believed to be responsible for its interaction with the muscarinic receptor are: 1) $-\overset{+}{N}(CH_3)_3$, its cationic head, and 2) its methyl group (14). The introduction of azido functions into these two groups of a cholinergic ligand should permit the specific photoaffinity labeling of these two complementary sites. In this paper, we have presented evidence that suggests the possibility of

photoaffinity labeling the mAChR in a region responsible for the second of these interactions, i.e., van der Waals contacts (14) with the methyl group of acetylcholine or the benzene ring of atropine and its relatives. In a subsequent paper (15) we will provide evidence for photoaffinity labeling of the second region in the receptor, responsible for its electrostatic interactions with the cationic head of acetylcholine, atropine, and similar ligands. We are also exploring the effect of incorporating a $-CH_2OH$ group in the methylene carbon of Compound II.

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