Anthroylcholine Bromide: A Fluorescent Ligand for the Muscarinic Receptor

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Abstract. The action of anthroylcholine bromide, a new fluorescent probe, has been studied at the cellular (contraction of intestinal muscle) and subcellular levels (binding of ^{3}H-quinuclidinyl benzilate to brain cortex membranes, fluorescence and enzyme activity) with the following results: 1. Anthroylcholine bromide competitively antagonized the contractile effect of acetylcholine in isolated rat duodenum (pA2 = 6.12), but had no effect either on the concentration response curves to histamine or to noradrenaline in isolated guinea pig ileum and rat vas deferens. 2 Anthroylcholine bromide displaced competitively ^{3}H-quinuclidinyl benzilate from brain cortex membranes (K_i = 0.77 μmol/l). 3. Direct binding to the muscarinic site could be measured by exploiting the fluorescence properties of the probe. Binding displaceable by atropine (approximately 20 % change in fluorescence) had an apparent affinity constant similar to that found with indirect methods. In contrast, d-tubocurarine did not displace the probe from its site, and atropine- or d-tubocurarine-sensitive binding of anthroylcholine bromide to *Torpedo marmorata* electric organ membranes, rich in nicotinic receptors, was not observed. These properties suggest the applicability of the probe to study the distribution, structure and/or kinetic properties of the muscarinic receptor.

Key words: Anthroylcholine bromide—Muscarinic ligands—mAChR markers—Muscarinic antagonists—Muscarinic receptor—Fluorescent ligands

Introduction

Since the introduction of 1-dimethylaminonaphtalene-5-sulfoamidoethyltrimethylammonium perchlorate (Weber et al. 1971), fluorescent ligands have been widely used to explore the structure and function of nicotinic cholinergic receptors (Heidman and Changeux 1978, Barrantes 1979). Regardless of the physiological impor-

tance of muscarinic synapses (Krnjevic 1974), much attention has been focused on molecular biology of nicotinic receptors. However, in the last decade, there has been a renewal of interest in central muscarinic receptors. Most of the studies have addressed the muscarinic receptor subtypes and their molecular biology. In contrast, little attention has been paid to searching for ligands with physical properties which would allow to study either the distribution of the muscarinic receptor or its structure-to-function relationships.

The use of extrinsic probes has advanced our understanding of the structure, distribution and function of the nicotinic receptor. The aim of this paper is to introduce a new fluorescent ligand with high specificity for the muscarinic receptor which might be useful in the study of this receptor.

Abbreviation: QNB - quinuclidinylbenzilate

Materials and Methods

Isolated organs: Wistar rats (200–250 g) and guinea pigs (approx. 500 g) were used. The animals were killed by head blow and bled. Longitudinal segments, approx. 3 cm long, of rat duodenum or guinea pig ileum or rat vas deferens were suspended in 20 ml organ bath. The contraction of all preparations other than the vas deferens were measured isotonically by means of an Ugo Basile 7006 force-displacement transducer and a Geminis model Ugo Basile recorder. The initial tension was 1 g and each preparation was allowed to rest in the bath for at least 30 min. The bathing solution for all tissues was Tyrode of the following composition (in mmol/l): NaCl 136.7, KCl 2.7, $CaCl_2$ 1.4, $MgSO_4$ 0.04, KH_2PO_4 0.04, $NaHCO_3$ 11.9, glucose 5.5. The bath temperature was maintained at 31 °C for rat vas deferens. The organ bath was bubbled with 95% O_2 plus 5% CO_2. After the rest period, cummulative concentration response curves to acetylcholine (rat duodenum), histamine (guinea pig ileum) and noradrenaline (rat vas deferens) in the absence and presence of anthroylcholine bromide were obtained. Anthroylcholine was applied into the bath 15 min before the responses to acetylcholine, histamine or noradrenaline were tested.

^{3}H-QNB binding to rat brain membranes: Membranes from the rat brain cortex were prepared according to the method described by Aronstam et al. (1977). Protein content of the membranes was determined using a modification of the method of Lowry et al. (1951), using bovine serum albumin as standard. Binding to rat brain cortex membranes was carried out following a filtration technique modified from Yamura and Snyder (1974): 0.4 ml of membranes (0.1 mg of protein per ml) were incubated with 0.6 ml of 50 mmol/l phosphate buffer, pH 7.4, containing ^{3}H-QNB. After incubation at 37 °C for 1 h, the samples were filtered in a Millipore sintered glass filtration apparatus through Whatman GF/B filters. The filters were washed twice with 8 ml ice cold buffer and placed in scintillation vials; 10 ml of picofluor 15 (Packard) were added to each vial and left overnight at room temperature before counting in a LKB Wallac Liquid Scintillation counter. Every determination was performed in triplicate. Scintillation counting efficiency was 43.5%. Specific binding is defined as the difference between binding in the presence and in the absence of 1 μmol/l atropine.

Fluorescence measurements: Fluorescence measurements were performed in a Perkin-

Elmer MPF-44 fluorimeter, using 1.5–2 ml samples in 5 ml glass cuvettes modified with a stirring device in order to prevent the settling of membranes in the cuvette. Unless otherwise specified, fluorescence measurements were performed in a medium containing 0.1 mg of protein/ml membranes, in 50 mmol/l phosphate buffer. Slit widths were set at 2 nm for the excitation monochromator and at 15 nm for the emission monochromator.

Acetylcholinesterase: Acetylcholinesterase activity was measured in the presence and absence of 5 μmol/l anthroylcholine following the method described by Ellman et al. (1961).

Data analysis: An iterative computerized procedure (Basulto et al. 1978) was used to fit the contraction-response curve and calculate the maximum effect (E_m) and the concentration producing 50% of the maximum effect (EC_{50}). Evaluation of drug antagonism was performed following the pA2 method as reported by Schild (1947). A non-linear least square regression analysis of ^{3}H-QNB binding data was performed using a program modified from Duggleby (1981). All the values are given as mean ± S.E. Student's t-test was used to compare differences in the values.

Materials: Anthroylcholine bromide was obtained from Molecular Probes (Eugene, Oregon, USA). ^{3}H-QNB (42 Ci/mmol) was from Amersham. Acetylcholine bromide, atropine, histamine dihydrochloride and noradrenaline bitartarate were from Sigma. All drug concentrations were calculated as free base. All chemicals used were of reagent grade.

Results

Structure and spectroscopic properties

Anthroylcholine bromide showed four peaks in the excitation spectrum (λ_1 = 256 nm, λ_2 = 350 nm, λ_3 = 368 nm, λ_4 = 385 nm) and one large band with a

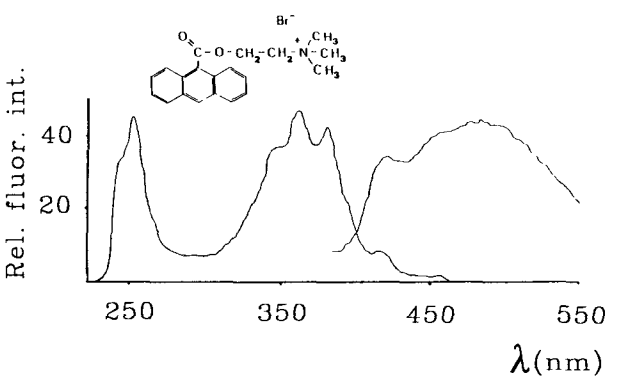

Figure 1. Structure of anthroylcholine bromide (M.w. = 388), together with the uncorrected excitation (λ_{em} = 485 nm) and emission (λ_{ex} = 365 nm) of a 10 μmol/l solution of the probe in 50 mmol/l phosphate buffer, pH 7.4, temperature 21 ± 1 °C. The dotted line (left) corresponds to the excitation spectrum.

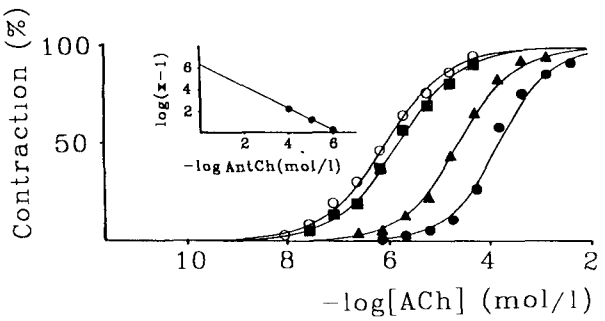

Figure 2. Log concentration-response curve to acetylcholine on isolated rat duodenum in the absence (o) and presence of 1 μmol/l (■), 10 μmol/l (▲) and 100 μmol/l (●) anthroylcholine. Each point represents mean of at least 6 values. Inset: representative Schild plot showing the antagonism of acetylcholine for anthroylcholine on the contractile response of rat duodenum.

maximum at 485 nm in the emission spectrum (Fig. 1). Fluorescence emission vs. concentration is linear (correlation coefficient = 0.99, 18 points) below 35 mmol/l.

Biological activity on isolated organs

As shown in Fig. 2, 1, 10 and 100 mmol/l anthroylcholine shifted the concentration-response curves of acetylcholine on isolated rat duodenum to the right in a parallel and concentration dependent manner without depressing the maximum. The plot of log (dose-ratio − 1) versus log (anthroylcholine concentration) yielded a straight line with a slope (1.08) not significantly different from unity. Histamine (dihydrochloride) produced a concentration dependent contraction of the isolated guinea pig ileum, the EC_{50} value being 1.3 ± 0.16 μmol/l. Antroylcholine at a concentration of 10 μmol/l failed to affect the contractile response to histamine (EC_{50} after incubation with anthroylcholine was 1.46 ± 0.22 μmol/l). Noradrenaline produced concentration-dependent contraction of the isolated vas deferens, EC_{50} was 3.0 ± 0.7 μmol/l. Anthroylcholine (10 μmol/l) failed to affect the contractile response to noradrenaline (EC_{50} after incubation with anthroylcholine was 2.9 ± 0.9 μmol/l).

Competitive inhibition of ^{3}H-QNB binding

Binding of ^{3}H-QNB to rat brain cortex membranes closely agreed with those previously reported by Yamamura and Snyder (1974). Apparent saturation of specific binding sites occurred at approximately 3 nmol/l ^{3}H-QNB. Non linear squares regression gave an estimate of the apparent dissociation constant (K_d) of 0.21 ± 0.01 nmol/l (mean ± S.D.) and a maximum receptor density of 1061 ± 100 fmol/mg membrane protein. Incubation of membranes (0.05 mg protein per ml) with ^{3}H-QNB (1 nmol/l) in the presence of anthroylcholine produced a displacement of

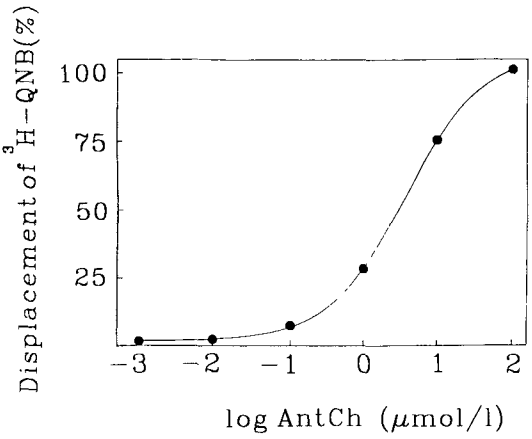

Figure 3. Displacement of ^{3}H-QNB binding to rat brain cortex membranes by anthroylcholine bromide. ^{3}H-QNB concentration = 1 nmol/l, 0% displacement corresponds to B_{max} (3 nmol of ^{3}H-QNB/g of membrane protein), Hill coefficient estimated from the fitted curve = 0.82.

^{3}H-QNB bound to the receptor (Fig. 3). Anthroylcholine at concentrations of 100 μmol/l was able to completely prevent the binding of 1 nmol ^{3}H-QNB. The IC_{50} value (the concentration of drug which inhibits binding by 50 %) determined from a log-probit plot using six concentrations of the inhibitor, was 1.54±0.26 μmol/l. The K_i value obtained using the Cheng and Prusoff (1973) equation was 0.77 μmol/l. In an independent series of experiments, it could be shown that the inhibitory effect of the probe on ^{3}H-QNB binding was reversible. At equilibrium, binding of increasing concentrations of ^{3}H-QNB to rat brain membranes was inhibited by anthroylcholine bromide in a competitive way (Fig. 4), the values of K_i were similar to those reported above.

Fluorescence measurement of anthroylcholine binding to rat brain membranes

Addition of rat brain membranes (0.1 mg protein per ml) to a medium containing 10 μmol/l anthroylcholine bromide resulted in an enhancement (approx. 300 %) of fluorescence emission at 485 nm when excited at 260 and 370 nm. A 4 nm red shift (from 256 to 260 nm) in the first peak of excitation spectrum was also observed (Fig. 5). The red shift and the enhancement of fluorescence were taken as indicative of bound dye to membranes. Thus, binding was measured by a direct method. Specific binding to muscarinic receptor is defined as the decrease in fluorescence produced by previous incubation with 5 μmol/l atropine for 1 h. Steady state (Langmuir) saturation isotherms were furnished at 260 and 370 nm. Atropine produced a 20 % change in fluorescence emission at 485 nm (λ_{ex} = 260 nm). As seen from Fig. 6 direct binding of anthroylcholine bromide to rat brain cortex membranes had a K_d (0.84 μmol/l) similar to that found by displacement of ^{3}H-QNB binding to rat brain membranes (K_i = 0.77 μmol/l) and by inhibition of the contractile effect of acetylcholine in isolated rat duodenum ($pA2$ = 6.12). D-tubocurarine at a concentration of 1 μmol/l produced no decrease in fluorescence.

Furthermore, no binding (displaceable by atropine or d-tubocurarine) to nicotinic receptor rich membranes (obtained from *Torpedo marmorata* electric organ) was appreciated.

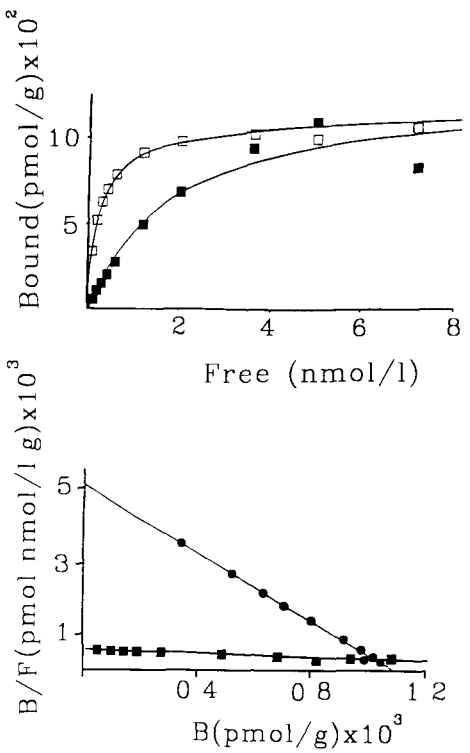

Figure 4. (a) Equilibrium binding of ^{3}H-QNB to rat brain membranes in the presence (▪) and absence (□) of anthroylcholine bromide (1.5 μmol/l); (b) Scatchard plot: control (•) and in the presence of 11.5 μmol/l anthroylcholine bromide (▪)

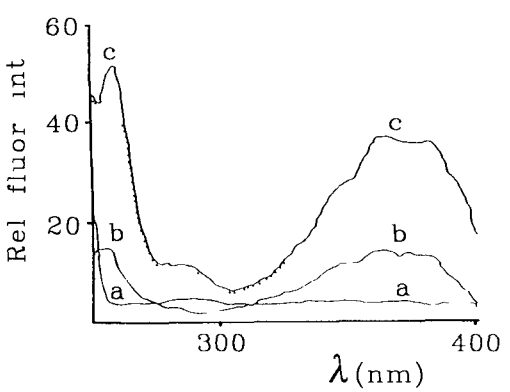

Figure 5. Uncorrected excitation spectra of membranes (0.1 mg/ml) (a), 10 μmol/l free anthroylcholine (b), membranes plus 10 μmol/l anthroylcholine (c) and membranes plus 10 μmol/l anthroylcholine plus 5 μmol/l atropine (dotted line), solved in 50 mmol/l phosphate buffer. Emission measured at 485 nm. Temperature: 20 ± 1 °C.

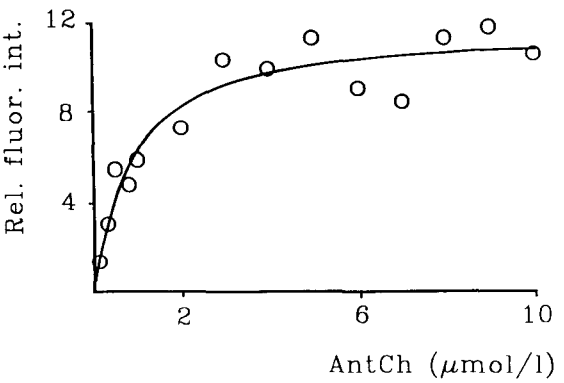

Figure 6. Specific binding (measured as atropine-sensitive changes in the emission spectra) of anthroylcholine to rat brain cortex membranes measured at 485 nm when excited at 260 nm. Temperature: 20 ± 1 °C.

Absence of effects on acetylcholinesterase activity

The well-known inhibitor of acetylcholinesterase paraoxon, at a concentration of 200 nmol/l did not change the effective concentration of anthroylcholine needed to inhibit ^{3}H-QNB binding to rat brain membranes. Furthermore, incubation during 10 min with anthroylcholine (5 μmol/l) produced an approx. 16 % decrease in acetylcholinesterase activity in rat brain cortex, suggesting that 1. the ligand was not processed by acetylcholinesterase and 2. there was a weak interaction between anthroylcholine bromide and acetylcholinesterase. Therefore, the concentration of anthroylcholine needed to inhibit acetylcholinesterase is several times higher than that needed to inhibit ^{3}H-QNB binding or the contractile response induced by acetylcholine.

Discussion

The results presented in the previous section indicate a specific interaction of the probe with muscarinic receptors. Displacement caused by anthroylcholine bromide on acetylcholine-induced contraction of rat duodenum suggest that the fluorescent ligand is a competitive antagonist of the muscarinic receptor (Fig. 2). This hypothesis was further demonstrated by radioligand binding studies. Anthroylcholine bromide displaces ^{3}H-QNB from its binding site (Figs. 3 and 4). Furthermore, the K_i reported is in the same range as that observed in assays with isolated organs. The fact that there was no displacement of anthroylcholine bromide by 1 μmol/l d-tubocurarine, together with the absence of specific binding to Torpedo electric organ membranes rich in nicotinic receptor, reinforces the selective interaction with muscarinic type of cholinergic receptor. The anthroylcholine bromide binding site has not been characterized pharmacologically (i.e. exploring the spectrum of affinities for a number or antagonists). However, since concentration-response curves to

histamine and noradrenaline were not modified in a significant manner by anthroylcholine bromide we can assume that the drug is devoid of spasmolytic effects. Then, we can conclude that anthroylcholine bromide is a specific ligand of the muscarinic receptor and can be classified as a weak antagonist. The low affinity, in comparison with other muscarinic antagonists which bind to the receptor in the nanomolar range, is probably due to the presence of a large hydrophobic moiety which, in contrast, confers the drug its interesting fluorescent properties and allows to measure specific binding of the drug to the muscarinic sites (in spite of the low density of binding sites) using atropine-sensitive changes in fluorescence. This method does not allow to obtain the value of B_{max}; it only yields ligand affinity which is in the submicromolar range and very close to that determined from radioligand binding studies (0.84 μmol/l and 0.77 μmol/l, respectively). The fluorescence properties may also supply some information on the chemico-physical properties of the binding site: e.g., the bathochromic shift in passing from the free form in water (λ_{ex} = 256 nm) to the bound state (λ_{ex} = 260 nm) suggests that the probe in lits bound form is in a highly polarizable environment. The binding site of anthroylcholine and, by inference, the antagonist recognition site, might be highly polarizable due to the presence of an electron rich moiety. A similar observation has also been reported for fluorescent antagonists of the nicotinic receptors (Tan et al. 1980).

Anthroylcholine bromide possesses several properties which make it particularly attractive for studies of the cholinergic system: 1. It is the first fluorescent ligand reported to interact specifically with the muscarinic acetylcholine receptor; 2. The effect on muscarinic induced contraction of rat duodenum and binding of ^{3}H-QNB indicates that the probe has a reversible antagonist effect; and 3. The spectroscopic properties of the anthroyl group may be used to explore the characteristics of the binding site environment. In summary, the properties of the ligand introduced in the present work suggest its applicability to structural or kinetic studies exploiting the sensitivity and versatility of fluorescent techniques.

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