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HIGH AFFINITY ACYLATING ANTAGONISTS FOR MUSCARINIC RECEPTORS

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Summary

The muscarinic antagonists pirenzepine and telenzepine were derivitized as alkylamino derivatives at a site on the molecules corresponding to a region of bulk tolerance in receptor binding. The distal primary amino groups were coupled to the cross-linking reagent meta-phenylene diisothiocyanate, resulting in two isothiocyanate derivatives that were found to inhibit muscarinic receptors irreversibly and in a dose-dependent fashion. Preincubation of rat forebrain membranes with an isothiocyanate derivative followed by radioligand binding using [³H]N-methylscopolamine diminished the B_{max} value, but did not affect the K_d value. The receptor binding site was not restored upon repeated washing, indicating that irreversible inhibition had occurred. IC₅₀ values for the irreversible inhibition at rat forebrain muscarinic receptors were 0.15 nM and 0.19 nM, for derivatives of pirenzepine and telenzepine, respectively. The isothiocyanate derivative of pirenzepine was non-selective as an irreversible muscarinic inhibitor, and the corresponding derivative prepared from telenzepine was 5-fold selective for forebrain (mainly m1) vs. heart (m2) muscarinic receptors.

Muscarinic receptors mediate a wide variety of physiological effects, including neuronal transmission in the CNS and PNS, gastric acid release, parasympathetic actions on the heart, and the contraction of intestinal smooth muscles, airways, and bladder (1). At least three different pharmacologically identifiable types of muscarinic receptors, termed M₁ – M₃, have been identified (2). In addition, five distinct genes have been found to code for five molecularly distinct subtypes of muscarinic receptors, termed m1 – m5 (3–6). The forebrain contains mainly, but not exclusively, m1 receptors, and the heart contains exclusively m2 receptors (6). The density of m2 receptors has been shown to be selectively reduced in brain from patients with Alzheimer's disease (7,8), presumably due to the degeneration of cholinergic forebrain fibers originating in the nucleus basalis (9). It has been proposed that application of an m1-selective muscarinic agonist in this brain region may be useful in treating the associated cognitive deficit of Alzheimer's disease (7, 10).

An affinity label would aid both in characterizing the physiological roles and in elucidating the molecular structure of muscarinic receptors. Until recently the only available antagonist chemical affinity label for muscarinic receptors has been the non-selective propylbenzylcholine mustard, PrBCM (11), which was found to covalently crosslink to the carboxylate side chain of an aspartyl residue of transmembrane helix three of muscarinic

receptors (12). Newman et al. (13) reported an isothiocyanate derivative of the cholinolytic agent aprophen that irreversibly inhibited the acetylcholine-stimulated release of catecholamines in adrenal glands. Other affinity labels, particularly if selective, might help in mapping the binding site. In this study, we have utilized a functionalized congener approach for the synthesis of affinity labels derived from the closely related, selective muscarinic antagonists pirenzepine (1a) and telenzepine (1b). Based on previous structure activity study (14), we have coupled an isothiocyanate-bearing crosslinker (15) at the end of a chemically functionalized chain incorporated on the molecule at a region having bulk tolerance in receptor binding.

Materials and methods

Preparation and treatment of membranes

Membranes were isolated from rat brain and from rat heart as follows. Sprague-Dawley rats (male, 200 gm) were killed by decapitation following ether anesthesia and their forebrain and hearts were removed and kept on ice. After mincing in a small volume of 50 mM sodium phosphate pH 7.2 buffer, hearts and brain were homogenized in 10 volumes of buffer using a Polytron homogenized (3×20 sec, 75 % max) and centrifuged 20,000 x g for 20 min at 2°C. The resulting pellet was re-suspended and centrifuged either two more times (brain) or three times (heart), then re-suspended at 3 mg of protein per ml of buffer and stored frozen (−70°C) until use. Membranes were incubated with an affinity label (4a or 4b, dissolved as a stock solution in dimethyl sulfoxide and then diluted into buffer) in sodium phosphate buffer, then extensively rinsed by centrifugation followed by re-suspension. Non-aqueous stock solutions of the isothiocyanate derivatives were stable to storage at −20°C for several weeks.

[³H]N-Methylscopolamine (NMS) binding

For Scatchard analysis, membranes (approximately 150 μ g protein) were incubated with 6 concentrations of [³H]NMS between 0.02 pM and 1.2 nM in 50 mM sodium phosphate buffer at pH 7.4 for 120 min at 37°C. The reaction was terminated by rapid filtration over GF/C filters, which were then washed three times with 25 ml of ice-cold 0.9% NaCl. The filters were equilibrated in Econofluor scintillation counting fluid, then counted at 47% efficiency in a scintillation counter. For single point determinations, membranes were incubated with 0.5 nM [³H]NMS in sodium phosphate buffer for 60 min at 37°C then processed for scintillation counting as above. Non-specific binding was determined with 1 μ M atropine.

Data analysis

All binding data are means of triplicate determinations from experiments that were repeated at least three times. Means $\pm$ standard deviation from each of these determinations is generally given in the Results. Scatchard and binding data was analyzed using the Graphpad InPlot Version 3.0 (GraphPAD Software, San Diego, CA) computer program.

Results

The synthesis of isothiocyanate derivatives of potent tricyclic muscarinic antagonists is outlined in Figure 1. The details of these syntheses will be presented elsewhere (16). Both pirenzepine, 1a (17), and telenzepine, 1b (18), contain side chains having the N-methylpiperidine structure. The N-methyl group was removed using α -chloroethyl chloroformate by a procedure applied previously to pirenzepine (14). The resulting secondary amines, 2a and 2b, were alkylated with 1, 10-dibromodecane, to give bromodecane derivatives, that were not isolated but were treated with ammonia to give

primary amine congeners, 3a and 3b. The n-decyl amine congener of pirenzepine (PAC), 3a, was previously reported (19) as a high affinity, muscarinic antagonist. Now we have extended this series to include the analogous amine congener of more potent selective muscarinic antagonist telenzepine (TAC), 3b.

The affinity of TAC, 3b, for muscarinic receptors (K_i) was determined in radioligand binding assays using membranes from cells expressing pure subtypes to be 2.4 nM at m1 receptors, 3.7 nM at m2 receptors, 7.5 at m3 receptors, and 1.3 nM at m4 receptors (16). In comparison, the K_i values for PAC, 3a, were 16 nM at m1 receptors and 12 nM at m2 receptors (14).

Both amine congeners reacted readily with the crosslinker, meta-phenylene diisothiocyanate (m-DITC), which was used previously to prepare high affinity, irreversible inhibitors of A_1 (18) and A_2 adenosine receptors (20). The isothiocyanate products of the final step were shown to have the structures shown in Fig. 1 (4a and 4b, for m-DITC-PAC and m-DITC-TAC, respectively).

The initial experiment to determine whether m-DITC-PAC and m-DITC-TAC were irreversible inhibitors of muscarinic receptors, was to incubate membranes containing receptors with these derivatives, then to wash the membranes repeatedly prior to radioligand binding. Thus, aliquots of membrane (1.5 mg) were incubated with 10 μ M of m-DITC-PAC for 30 min at room temperature in 50 mM sodium phosphate buffer, pH 7.4. Excess buffer (20 ml) was then added to the tubes, and the tubes were centrifuged at 18,000 $\times$ g for 20 min at 2°C. The resulting pellet was re-suspended in 25 ml of fresh buffer and incubated for 5 min at room temperature to allow dissociation of any reversibly bound compound. The tubes were re-centrifuged and this entire process was repeated up to 5 times. [3 H]NMS binding was then determined as described above by adding [3 H]NMS (0.5 nM, final conc.) and incubating another 60 min at 37°C. As shown in Figure 2, incubation of membranes with 10 μ M of m-DITC-PAC reduced [3 H]NMS binding to less than 5% of that seen with untreated membranes. Furthermore, repeated washing and incubation of these membranes in fresh buffer failed to restore any [3 H]NMS binding, indicating that m-DITC-PAC bound irreversibly to muscarinic receptors. Similar results were obtained with m-DITC-TAC (data not shown). In order to estimate the kinetics of association of m-DITC-PAC, we incubated membranes with 0.25 μ M m-DITC-PAC for various periods of time, then assayed the specific [3 H]NMS binding. There was a rapid loss in receptor binding within the first minute of incubation, followed by a slower decrease in [3 H]NMS bound, which continued throughout an incubation of one hour (data not shown).

In order to validate that the loss of binding due to pre-incubation of membranes with these compounds was due to covalent labeling of the receptor, and not due to very slow dissociation rates, we performed Scatchard analysis of membranes pre-treated with varying concentrations of m-DITC-TAC and m-DITC-PAC. As shown in Fig 3, incubation of membranes with either 50 or 500 nM of m-DITC-PAC reduced the B_{max} without significantly affecting the K_d , further indicating that m-DITC-PAC had covalently labeled the receptor. On the other hand, if the label were binding reversibly in a competitive manner, then the K_d and not the B_{max} should be affected. As shown in Fig. 3, incubation of the brain membranes with 50 nM of m-DITC-PAC resulted in a significant decrease in the density of [3 H]NMS binding sites (541 vs. 951 fmol/mg protein) without significantly affecting the K_d value (K_d = 0.69 nM for control and 0.57 nM for treated membranes). Incubation of membranes with 500 nM m-DITC-PAC caused a nearly total loss of [3 H]NMS binding sites, while the K_d value for radioligand binding to the residual sites differed from the original value by only a factor of 1.6 (K_d = 1.13 nM).

The concentration dependence of irreversible inhibition of rat brain muscarinic receptors by m-DITC-PAC is shown in Figure 4. In this figure, the percent reduction in $B_{\max}$ was plotted against the concentration of m-DITC-PAC, giving an IC_{50} for this inhibition of 15.3 ± 7 nM. Since m-DITC-PAC is a derivative of the M_1 -selective pirenzepine, we next determined whether the subtype selectivity of pirenzepine was retained following derivatization. We compared the ability of both compounds to inhibit [3 H]NMS binding in forebrain (largely m1) vs. cardiac (m2) muscarinic receptors. In these experiments, an aliquot of membrane was pre-incubated with the indicated concentration of either m-DITC-PAC (Fig. 5a) or m-DITC-TAC (Fig. 5b), then washed extensively before being assayed for [3 H]NMS binding using a single concentration of [3 H]NMS. As shown in Fig 5a, m-DITC-PAC inhibited [3 H]NMS binding to forebrain membranes (largely m1) with a similar dose-dependency as to cardiac membranes (m2), indicating that m-DITC-PAC had lost the m1-selectivity observed with underivatized pirenzepine. m-DITC-TAC, on the other hand, exhibited considerable m1-selectivity: it was selective for brain receptors over heart receptors by a factor of 7 (Fig. 5b). The IC_{50} values for m-DITC-TAC-mediated inhibition of binding was 0.29 ± 0.08 μ M in heart membranes, and 0.06 ± 0.02 μ M for brain membranes, indicating that in contrast to m-DITC-PAC, m-DITC-TAC had not lost all of the m1-selectivity of the parent compound telenzepine.

Discussion

We present evidence that two novel isothiocyanate derivatives (m-DITC-PAC and m-DITC-TAC) are irreversible inhibitors of rat muscarinic receptors. The isothiocyanate group was included as the chemically reactive group designed to acylate a nucleophilic residue of the receptor protein based on use of this group in covalent inhibitors of other receptors (13,15,20). The isothiocyanate group is relatively stable in aqueous medium (during the experimental time required) and does not require a chemical activation step as do the mustard type affinity labels (14).

We prepared amine congeners PAC, 3a, and TAC, 3b, as synthetic intermediates for reaction with chemical crosslinking agents. The telenzepine derivative TAC was more potent than PAC in inhibiting [3 H]NMS binding, by factors of approximately 7 and 3 at m1 and m2 receptors, respectively. The crosslinking agent chosen for this study was meta-phenylene diisothiocyanate, by analogy to a set of purines that contain this group and were shown to bind covalently to adenosine receptors (15, 18). The xanthine derivatives in the previous studies were also prepared in radioactive form in order to visualize the A_1 -adenosine receptor protein on an SDS electrophoretic gel. In order to further demonstrate that derivatives 4a and 4b indeed couple to the m1 and m2 receptor proteins covalently, as suggested here in binding and washing experiments, it will be useful to carry out similar radioactive labeling studies.

The isothiocyanate group reacts preferentially with primary amino and thiol groups. Another affinity label for muscarinic receptors, PrBCM, which has aided in mapping of the receptor binding site, was found to react with a carboxylate group of the receptor protein (12). Thus, one may expect that there is a high probability that the sites of covalent modification by the current pair of isothiocyanate derivatives and by PrBCM are different. By probing the site of attachment of m-DITC-PAC and m-DITC-TAC to muscarinic receptors, through enzymatic degradation, chemical modification of the inhibited receptor or through site-directed mutagenesis, one may gather further structural or conformational knowledge of muscarinic receptors.

m-DITC-TAC was modestly selective for brain receptors vs. heart (m2) receptors. Thus, a one hour incubation with m-DITC-TAC at a concentration of 0.1 μ M inhibited

approximately half of the brain muscarinic receptors, while leaving the cardiac receptors essentially unaffected. Whether this is sufficient selectivity to delineate m1 and m2 receptors physiologically remains to be determined. Certainly m-DITC-TAC constitutes a valuable lead in the design of even more selective irreversible inhibitors of m1 muscarinic receptors.

It is noteworthy that the selectivity of telenzepine (28-fold for m1 vs. m2 receptors, 14) was abolished upon conversion to the amine congener 3b, but partially restored in the phenylisothiocyanate 4b. The loss of m1 selectivity was previously noted for the amine congener 3a in comparison to its parent drug, pirenzepine, which is 21-fold selective for m1-receptors (14). Perhaps further structural elaboration of the amino site of 3b will lead to enhanced selectivity.

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Fig. 1. Synthesis of irreversible inhibitors of muscarinic receptors derived from pirenzepine, 1a, or telenzepine, 1b. The structures of m-DITC-PAC and m-DITC-PAC are 4a and 4b, respectively.

Fig. 2.

Effect of washing on radioligand binding to m-DITC-PAC-treated membranes. Rat brain membranes were either left untreated (○) or were treated (●) with 10 μ M m-DITC-PAC affinity label for 30 min at room temperature, then washed for the indicated number of times with 25 ml of sodium phosphate buffer, and incubated in fresh buffer, before being assayed for [3 H]NMS binding. Data are means from triplicate determinations whose S.D. was less than 4% of the mean.

Fig. 3.

Scatchard analysis of untreated rat brain membranes (●) and membranes treated with 0.05 μM (▲) and 0.5 μM (■) m-DITC-PAC. Membranes were incubated with affinity label for 60 min at room temperature, then were washed twice with large volumes of buffer before being assayed for [^3H]NMS binding.

Fig. 4. Effect of varying concentrations of m-DITC-PAC on the density of muscarinic antagonist binding sites, indicated by $B_{\max}$ values. Membranes were incubated with the indicated concentration of the affinity label, m-DITC-PAC and washed extensively. The treated membranes were then assayed for [3 H]NMS binding and subjected to Scatchard analysis. The resulting $B_{\max}$ is plotted as a function of the concentration of the affinity label.

Figure 5a

Figure 5b

Figure 5.

Effect of varying concentrations of m-DITC-PAC (a) and m-DITC-TAC (b) on $[^3\text{H}]\text{NMS}$ binding in membranes from rat brain (■) and from rat heart (●). Membranes were treated with the indicated concentration of compound for 60 min at room temperature, washed extensively, and assayed for $[^3\text{H}]\text{NMS}$ binding using a single point determination. Each data point is the mean of three separate determinations whose standard deviation did not exceed 7% of the mean.