Thiochrome Enhances Acetylcholine Affinity at Muscarinic M₄ Receptors: Receptor Subtype Selectivity via Cooperativity Rather than Affinity

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ABSTRACT

Thiochrome (2,7-dimethyl-5H-thiacromine-8-ethanol), an oxidation product and metabolite of thiamine, has little effect on the equilibrium binding of L-[³H]N-methyl scopolamine ([³H]NMS) to the five human muscarinic receptor subtypes (M₁–M₅) at concentrations up to 0.3 mM. In contrast, it inhibits [³H]NMS dissociation from M₁ to M₄ receptors at submillimolar concentrations and from M₅ receptors at 1 mM. These results suggest that thiochrome binds allosterically to muscarinic receptors and has approximately neutral cooperativity with [³H]NMS at M₁ to M₄ and possibly M₅ receptors. Thiochrome increases the affinity of acetylcholine (ACh) 3- to 5-fold for inhibiting [³H]NMS binding to M₄ receptors but has no effect on ACh affinity at M₁ to M₃ receptors. Thiochrome (0.1 mM) also increases the direct binding of [³H]ACh to M₄ receptors but decreases it slightly at M₂ receptors. In agreement with the binding data, thiochrome does not affect the potency of ACh for stimulating the binding of guanosine 5'-(3-[³⁵S]thiotriphosphate) ([³⁵S]GTPyS) to membranes containing M₁ to M₃ receptors, but it increases ACh potency 3.5-fold at M₄ receptors. It also selectively reduces the release of [³H]ACh from potassium-stimulated slices of rat striatum, which contain autoinhibitory presynaptic M₅ receptors, but not from hippocampal slices, which contain presynaptic M₄ receptors. We conclude that thiochrome is a selective M₄ muscarinic receptor enhancer of ACh affinity and has neutral cooperativity with ACh at M₁ to M₃ receptors; it therefore demonstrates a powerful new form of selectivity, “absolute subtype selectivity”, which is derived from cooperativity rather than from affinity.

Most receptor-active ligands bind to the same site as the endogenous ligand, the so-called orthosteric site. Agonists mimic the actions of the endogenous ligand, whereas antagonists physically prevent the endogenous ligand from binding but lack its actions. The property of a ligand that determines its effect when bound to a receptor is called its efficacy. Different degrees of efficacy lead to so-called full agonists, partial agonists with a smaller maximal effect than full agonists; neutral antagonists, which occupy the active site without exerting any effect; and inverse agonists, which reduce the activity of constitutively active receptors (Kenakin, 2002). The selectivity of an orthosteric ligand for one receptor or receptor subtype is determined by its affinity for the receptor and its efficacy at that receptor. The difference in affinity between the target receptor and other receptors must be large for an orthosteric ligand to have useful selectivity. This can be difficult to achieve for receptors that show close homology at the orthosteric binding region such as the five subtypes of muscarinic receptor (M₁–M₅) (Hulme et al., 1990). Selectivity derived from efficacy can also be hard to achieve, because the effect of a partial agonist depends on properties of the tissue, such as receptor density and downstream amplification mechanisms, which vary between cells and tissues, so a ligand with no apparent functional effect on tissues in vitro may nevertheless activate tissues in vivo, leading to unacceptable side effects (Terry et al., 2002).

An alternative approach for developing selective ligands is to look for allosteric ligands that bind to a site on the receptor which is different from the site to which the endogenous ligand binds. This allows both types of ligand to bind simultaneously. If the affinity (or efficacy) of the endogenous ligand is different when it is bound to the allosteric liganded receptor compared with when it is bound to the free receptor, then the allosteric ligand exhibits positive or negative coop-
erativity with the endogenous ligand at that receptor. This is the mechanism by which benzodiazepine tranquilizers such as diazepam act: they enhance the affinity of certain GABA<sub>A</sub> receptors for GABA (Whiting, 2003). Many G-protein coupled receptors contain allosteric sites, and the best characterized are the muscarinic receptors for acetylcholine (ACh). An allosteric agent that enhances the affinity of ACh selectively at M<sub>1</sub> receptors could provide a therapy for Alzheimer’s disease (Lazareno et al., 1998; Birdsell et al., 1999).

Like orthosteric ligands, allosteric ligands can achieve selectivity through their selective affinity for the target receptor. In addition, allosteric ligands also have the potential to show a more powerful form of selectivity, derived from cooperativity rather than affinity, which we have termed “absolute subtype selectivity” (Lazareno and Birdsell, 1995). Just as orthosteric ligands can be agonists, neutral antagonists, or inverse agonists, so allosteric ligands can have positive, neutral, or negative cooperativity with an orthosteric ligand; neutrally cooperative ligands bind to the receptor simultaneously with the orthosteric ligand but do not modify its affinity (or efficacy). If an allosteric ligand shows positive or negative cooperativity with the endogenous ligand at one receptor subtype but neutral cooperativity at the other receptor subtypes, then the allosteric ligand will only have the potential to modulate the activity at that one receptor subtype, regardless of its dose or concentration or its relative affinity for the target receptor.

Here we report that thiochrome (Fig. 1), an oxidation product and metabolite of thiamine (Petrov, 1992), shows absolute subtype selectivity with regard to M<sub>1</sub> to M<sub>4</sub> receptors in allosterically enhancing the binding and actions of ACh at human M<sub>4</sub> receptors.

**Materials and Methods**

1-[N-methyl-<sup>3</sup>H]Scopolamine methyl chloride ([<sup>3</sup>H[NMS], 81–86 Ci/mmol) and [<sup>3</sup>H]choline chloride (82 Ci/mmol) were from Amersham (England). [3H]acetylcholine iodide (60 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO), [35S]GTP<sub>S</sub> (0.1–100 Ci/mmol) was from Perkin Elmer Life and Analytical Sciences (Boston, MA), and thiochrome (Fig. 1) and other compounds were from Sigma Chemical (Poole, Dorset, UK).

**Cell Culture and Membrane Preparation.** CHO cells stably expressing cDNA encoding human muscarinic M<sub>1</sub> to M<sub>5</sub> receptors (Buckley et al., 1989) were generously provided by Prof. N. J. Buckley (University of Leeds, Leeds, UK). These were grown in α-minimal Eagle’s medium (Invitrogen, Paisley, UK) containing 10% (v/v) newborn calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine, at 37°C under 5% CO<sub>2</sub>. Cells were grown to confluence and harvested by scraping in a hypotonic medium (20 mM HEPES + 10 mM EDTA, pH 7.4). In some cases, 5 mM sodium butyrate was added to the medium 24 h before harvesting to increase receptor expression. Membranes were prepared at 0°C by homogenization with a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland) followed by centrifugation (40,000g for 15 min), washed once in 20 mM HEPES plus 0.1 mM EDTA, pH 7.4, and stored at −70°C in the same buffer at protein concentrations of 2 to 5 mg/ml. Protein concentrations were measured with the Bio-Rad reagent (Bio-Rad, Hemel Hempstead, UK) using bovine serum albumin as the standard.

[<sup>3</sup>H]NMS Binding Assays. Unless otherwise stated, frozen membranes were thawed, resuspended in incubation buffer containing 20 mM HEPES, 100 mM NaCl, and 10 mM MgCl<sub>2</sub>, pH 7.4, and incubated with radioligand and unlabeled drugs for 2 h at 30°C in a volume of 1 ml. Membranes were collected by filtration over glass fiber filters (Whatman GF/B) presoaked in 0.1% polyethylenimine, using a Brandel cell harvester (Semat, Herts, UK), extracted overnight in scintillation fluid (ReadySafe; Beckman Coulter, High Wycombe, UK), and counted for radioactivity in LS6000 scintillation counters (Beckman Coulter). Membrane protein concentrations (5–50 μg/ml) were adjusted so that not more than approximately 15% of added radioligand was bound. Nonspecific [<sup>3</sup>H]NMS binding was measured in the presence of 10<sup>−6</sup> M QNB (an antagonist with picomolar potency) and accounted for 1 to 5% of total binding. GTP was present at a concentration of 2 × 10<sup>−5</sup> M in assays containing unlabeled ACh or as stated. Data points were usually measured in duplicate. CHO cell membranes do not possess cholinesterase activity (Lazareno and Birdsell, 1993; Gnagrey and Ellis, 1996), so ACh could be used in the absence of a cholinesterase inhibitor. In some [<sup>3</sup>H]NMS binding assays, thiochrome was dissolved in dimethyl sulfoxide which, at a final concentration of 1%, seemed to have no effect on binding, but thiochrome was usually dissolved in water or buffer.

[<sup>3</sup>H]ACh Binding Assays. Membranes containing M<sub>2</sub> or M<sub>4</sub> receptors (8–20 μg protein) were incubated with [<sup>3</sup>H]ACh in the absence and presence of thiochrome in incubation buffer in a final volume of 0.25 ml at 30°C for 30 min and were collected by filtration over glass fiber filters (Whatman GF/B) presoaked in 0.1% polyethylenimine. Total binding was measured in duplicate, and nonspecific binding in the presence of 10<sup>−6</sup> M QNB was measured in singleton. To measure the reversibility of thiochrome binding, two sets of tubes, A and B, received 10 μl of M<sub>2</sub> membranes and 10 μl of various concentrations of thiochrome at twice its final concentration (set A) or 10 μl buffer (set B). After 15-min incubation, 80 μl of [35S]GTP<sub>S</sub> and 100 μl of buffer (set A) or thiochrome (set B) were added, and the incubation proceeded for a further 30 min. The final concentration of thiochrome in the set A tubes was therefore 10-fold less than the concentration to which the membranes were initially exposed.

[<sup>35</sup>S]GTP<sub>S</sub> Binding Assays. Membranes from CHO cells expressing muscarinic receptors M<sub>1</sub> to M<sub>4</sub> (5–20 μg/ml) were incubated with [35S]GTP<sub>S</sub> (0.1 nM), GDP (10<sup>−7</sup> M for M<sub>1</sub> and M<sub>3</sub> receptors, 10<sup>−8</sup> M for M<sub>2</sub> and M<sub>4</sub>) and ligands in incubation buffer in a volume of 1 ml for 30 to 60 min at 30°C. Bound label was collected over glass fiber filters presoaked with water. Thiochrome was dissolved in water or buffer, because when it was made up in dimethyl sulfoxide, it reduced stimulated [35S]GTP<sub>S</sub> binding.

Data Analysis. General data preprocessing, as well as the “affinity ratio” calculations and routine plots of the semiquantitative equilibrium assay, were performed using Minitab (Minitab Ltd., Coventry, UK). The other assays were analyzed with nonlinear regression analysis using the fitting procedure in SigmaPlot (SPSS Inc., Erkrath, Germany) or Prism 4 (GraphPad Software Inc., San Diego, CA).

**Equilibrium Binding Assays for Estimation of the Affinity of an Allosteric Agent for the Receptor and the Magnitude of Its Cooperativity with [3H]NMS and ACh.** The simple ternary complex allosteric model has been used to understand allosteric effects at muscarinic receptors (Ehler, 1988; Lazareno and Birdsell, 1995; Christopoulos, 2002). It consists of a receptor with two binding sites: one for the orthosteric ligand and the other for the allosteric ligand, and the only effect of the binding of one type of ligand is to alter the affinity of the other type of ligand for its site on the receptor. The experimental design and analyses have been described in detail.
previously (Lazareno and Birdsall, 1995; Lazareno et al., 1998). Briefly, specific binding of a low concentration of [3H]NMS (1–2 times the $K_d$) was measured in the presence of a number of concentrations of test agent, all in the absence and presence of one or more concentrations of ACh. Specific binding of a high concentration of [3H]NMS (5–10 times $K_d$) was also measured. Nonlinear regression analysis was used to fit the data to the equation

$$B_{LAX} = \frac{B_{max} \times L \times K_L \times (1 + \alpha \times X \times K_X)}{[1 + X \times K_X + (A \times K_A)^n \times (1 + \beta \times X \times K_X) + L \times K_L \times (1 + \alpha \times X \times K_X)]}$$  (1)

where $B_{LAX}$ is observed specific bound radioligand, $L, A$, and $X$ are concentrations of [3H]NMS, ACh, and allosteric agent, respectively, $K_L, K_A$, and $K_X$ are affinity constants for the corresponding ligands and the receptor; $\alpha$ and $\beta$ are allosteric constants of $X$ with [3H]NMS and ACh, respectively; and $n$ is a logistic slope factor to describe the binding of ACh.

If only a single concentration of ACh was used, the data were visualized with "affinity ratio" plots, in which the affinity ratio is the apparent affinity of the "primary" ligand ([3H]NMS or ACh) in the presence of a particular concentration of test agent divided by the apparent affinity of the primary ligand in the absence of test agent. Theoretically, the $EC_{50}$ or $IC_{50}$ of the affinity ratio plot corresponds to the $K_a$ of the test agent at the free receptor, and the asymptotic level corresponds to the cooperativity constant for the test agent and primary ligand (Lazareno and Birdsall, 1995). Affinity ratios were calculated from the specific binding data as follows (Lazareno and Birdsall, 2000; Lazareno et al., 2002):

The affinity ratio of [3H]NMS in the presence of a single concentration of test agent is

$$r_L = \frac{B_{LX} \times (B_{L1} - B_L)}{B_{L1} \times (B_L - q \times B_{L1})}$$  (2)

The affinity ratio of ACh the presence of a single concentration of test agent is

$$r_A = \frac{B_L \times B_{LA} \times (B_{L1} - B_L) \times (B_{LX} - B_{LAX})}{B_{LAX} \times (B_L - B_{LAX}) \times [B_{L1} \times B_L \times (1 - q) - B_{LX} \times (B_L - q \times B_{L1})]}$$  (3)

where $B_L$ is binding in the presence of the low [3H]NMS alone; $B_{L1}$ is binding in the presence of the high [3H]NMS; $B_{LAX}$ is binding in the presence of the low [3H]NMS and ACh; $B_{L1X}$ is binding in the presence of the high [3H]NMS and ACh.

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**Fig. 2.** A, effect of thiochrome on [3H]NMS binding to M₁ to M₅ receptors alone and in the presence of a fixed concentration of ACh. Individual data points are shown. The lines in the plots for M₁ to M₅ receptors were derived from nonlinear regression to the allosteric model. B, affinity ratios with ACh. Affinity ratios were calculated as described under Materials and Methods. At M₁ to M₅ receptors, the concentrations of [3H]NMS were 0.20, 0.42, 0.21, 0.16, and 0.84 nM, respectively; the estimated $K_a$ values were 0.12, 0.46, 0.19, 0.08, and 0.72 nM, respectively; the fixed concentrations of ACh were 22, 1, 10, 3, and 13 μM, respectively.
Fig. 3. A, effect of increasing concentrations of thiochrome on the inhibition by Ach of 0.32 nM [3H]NMS binding to M₄ receptors in the presence of 2.10⁻¹⁰ M GTP. Individual data points are shown. Parameters were estimated by nonlinear regression to the allosteric model, and the lines were derived from the fit. B, effect of 3.10⁻¹⁰ M thiochrome on the inhibition by Ach of 0.24 nM [3H]NMS binding to M₄ receptors in the absence and presence of 2.10⁻¹⁰ M GTP. The points are mean ± range/2 of duplicate observations. Parameters were estimated by nonlinear regression to a logistic function, and the lines were derived from the fit.

presence of the low [³H]NMS and a particular concentration of test agent; B_LAX is binding in the presence of the low [³H]NMS, Ach, and the same concentration of test agent; L is the low [³H]NMS concentration; L₃ is the high [³H]NMS concentration; and q is the ratio of low and high [³H]NMS concentrations, L₃/L₁.

Off-Rate Assay to Estimate the Affinity of Thiochrome for the [³H]NMS-Occupied Receptor. A high concentration of membranes (2–4 mg protein/ml) was incubated with a high concentration of [³H]NMS (5 nM) for approximately 15 min. Then 10-μl aliquots were distributed to tubes that were empty or contained 1 ml of 10⁻⁶M QNB alone and in the presence of a number of concentrations of thiochrome (typically n = 4). Nonspecific binding was measured in separately prepared tubes containing 10 μl of membrane and 2 μl of [³H]NMS plus QNB. Some time later, approximately 2.5 dissociation half-lives, the samples were filtered. The data were transformed to observed rate constants, k_obs, using the formula k_obs = ln(B₀/Bₜ)/t, where B₀ is initially bound radioligand and Bₜ is bound radioligand remaining after t min dissociation. These values were expressed as a percentage of the true [³H]NMS dissociation rate constant k₀ = kₐ½/kₐ½ (in the absence of allosteric agent) and fitted to a logistic function using nonlinear regression analysis. Theoretically the curves should have slopes of 1 and mirror the occupancy curves of the allosteric agents at the [³H]NMS-occupied receptors, regardless of whether the change of [³H]NMS dissociation is caused by an allosteric change in the shape of the receptor or the trapping of the [³H]NMS in its binding pocket by the bound allosteric agent (Lazareno and Birdsall, 1995). Initially the curve was fitted without constraints. If the slope factor was not different from 1, and the maximal effect (E_max) was not less than 0, then the curve was constrained to 1 and the E_max was fitted. If the fitted E_max was less than 0 dissociation (a physical impossibility, apart from experimental variation or error) then the E_max was constrained to 0 and the slope fitted.

Brain Slice Superfusion. Wistar male rats, 2 to 3 months old, were killed by cervical dislocation and decapitation. Striata and hippocampi were dissected, and slices were prepared using a McIlwain-type tissue chopper set at the slice width 0.35 mm. Next, 10 to 12 striatal slices or 20 to 24 hippocampal slices were prelabeled with 10 μCi of [³H]choline in 2 ml of Krebs' buffer (final concentrations, 123 mM NaCl, 3 mM KCl, 1 mM MgSO₄, 1.3 mM CaCl₂, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, and 10 mM glucose, saturated with 95% oxygen/5% CO₂, pH 7.4) for 30 min at 37°C. The slices were then washed in fresh buffer and loaded into a six-chamber superfusion apparatus (one slice per chamber for striatal slices and two slices per chamber for hippocampal slices) and superfused using Krebs’ buffer containing 10 μM hemicholinium-3 (to prevent reuptake of choline) at a rate of 0.5 ml/min and at 37°C. After a 1-h washout period, 4-min

<table>
<thead>
<tr>
<th>Subtype</th>
<th>pK</th>
<th>Coop NMS</th>
<th>Coop Ach</th>
<th>n</th>
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<tbody>
<tr>
<td>M₁</td>
<td>4.07 ± 0.23</td>
<td>0.74 ± 0.16</td>
<td>1.07 ± 0.17</td>
<td>4</td>
</tr>
<tr>
<td>M₂</td>
<td>3.92 ± 0.23</td>
<td>0.94 ± 0.25</td>
<td>0.90 ± 0.28</td>
<td>5</td>
</tr>
<tr>
<td>M₃</td>
<td>4.44 ± 0.21</td>
<td>0.62 ± 0.13</td>
<td>0.82 ± 0.24</td>
<td>6</td>
</tr>
<tr>
<td>M₄</td>
<td>4.01 ± 0.12</td>
<td>0.72 ± 0.16</td>
<td>3.90 ± 0.59</td>
<td>7</td>
</tr>
<tr>
<td>M₅</td>
<td>N.E.</td>
<td></td>
<td></td>
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</table>

N.E., no effect.
samples of superfusate were collected, and the outflow of radioactivity was determined using liquid scintillation counting and expressed as a percentage of radioactivity present in the slice at the beginning of the respective collection period. The evoked release of radioactivity represents the release of labeled ACh (Richardson and Szerb, 1974; Dolezal et al., 1992; Dolezal and Tucek, 1998, 1999), whereas the resting liberation of radioactivity represents a mixture of labeled choline and its metabolites. The release of ACh was stimulated by potassium depolarization (1-min superfusion with medium containing 50 mM potassium chloride, iso-osmotically replaced at the expense of sodium chloride, at the beginning of the third and the ninth fraction) or by electrical stimulation (2-ms rectangular monopolar pulses with a current strength of 35 mA at the beginning of the 3rd, 8th, and 13th fractions; for details of frequency and number of pulses, see figure legends). Tested drugs were added to the superfusion medium 8 min before the second or third stimulation as indicated and remained present until the end of superfusion. The evoked release of ACh was calculated as the release of radioactivity over background by subtracting values immediately preceding (B1) and after the evoked liberation. The influence of drugs on the evoked release of radioactivity was estimated from the changes of the ratio second or third stimulation (S2 or S3; in the presence of absence of tested drugs)/first stimulation (S1; always the control stimulation).

Results

Thiochrome (Fig. 1) had only small effects on the equilibrium binding of [3H]NMS to M1 to M5 receptors (Fig. 2). It also had little effect on [3H]NMS binding in the presence of ACh at M1, M2, M3, or M5 receptors. At M4 receptors, however, in the presence of ACh, thiochrome inhibited [3H]NMS binding (Fig. 2), suggesting that thiochrome allosterically enhanced the affinity of ACh at M4 receptors. This effect is seen more clearly when the data are transformed into affinity ratios, i.e., the apparent affinity of ACh in the presence of thiochrome divided by its apparent affinity alone (Fig. 2B); according to the allosteric model, the EC50 of the affinity ratio plot corresponds to the Kd of thiochrome at the free receptor, and the asymptotic value corresponds to the cooperativity of the system. It can be seen that thiochrome had little effect on ACh affinity at M1, M2, M3, or M5 receptors but increased it up to 4-fold at M4 receptors. The effect of thiochrome on the potency of ACh at M4 receptors was studied in more detail (Fig. 3A), and it was found that thiochrome increased the potency of ACh without affecting the slope of the inhibition curves. The data from Figs. 2 and 3A and similar assays were fitted to the allosteric model, and the parameter estimates are shown in Table 1. Thiochrome had no effect at M5 receptors at concentrations up to 3 × 10-4 M and had a log affinity of approximately 4 at M1 to M4 receptors, although the errors in the parameter estimates were relatively large at M4 to M3 receptors because of the small effects of thiochrome on [3H]NMS binding both in the absence and presence of ACh. Thiochrome had neutral or low negative cooperativity with [3H]NMS at M1 to M4 receptors and neutral cooperativity with ACh at M3-M5 receptors; it had 4-fold positive cooperativity with ACh at M4 receptors. These assays were conducted in the presence of 2 × 10-4 M GTP, but thiochrome also increased ACh potency at M4 receptors in the absence of GTP (Fig. 3B). GTP reduced the potency of ACh and increased the slope to a similar extent in the absence and presence of thiochrome, whereas thiochrome increased the potency of ACh to a similar degree in the absence and presence of GTP.

Like most other allosteric agents at muscarinic receptors, thiochrome inhibited the dissociation of [3H]NMS essentially completely (Fig. 4, Table 2). It showed similar potencies at M1, M2, and M3 receptors, a slightly lower potency at M4 receptors, and much lower potency at M5 receptors. According to the allosteric model, the IC50 of thiochrome for this effect corresponds to the Kd of thiochrome for the [3H]NMS-occupied receptor. In theory, the same parameter can be estimated from the equilibrium assays summarized in Table 1; the comparison between the two estimates is shown in Table 2. Where there is a clear signal, at M4 receptors in the presence of ACh, the predicted affinity at the [3H]NMS-occupied receptor differs less than 2-fold from the value measured directly in the off-rate assay, supporting the allosteric model as the mechanism underlying both sets of observations. It should be noted, however, that the slopes of the

![Figure 4](https://example.com/image.png)

**Figure 4.** Effect of thiochrome on the dissociation of [3H]NMS from M1 to M5 receptors measured at a single time. For M1 to M4 receptors in this assay, the times were 20, 4, 25, and 45 min, respectively, and the dissociation rate constants (koff) were 0.061, 0.35, 0.059, 0.055, and 0.023 min⁻¹, respectively, corresponding to dissociation half times of 11, 2, 12, 13, and 30 min, respectively. The points are mean ± range/2 of duplicate observations, and the lines were derived from nonlinear regression to a logistic function. Parameter estimates for these and similar experimental data are summarized in Table 2.

**Table 2.** Parameter estimates from [3H]NMS off-rate assays

<table>
<thead>
<tr>
<th>Subtype</th>
<th>pEC50</th>
<th>Slope</th>
<th>Emax</th>
<th>Predicted %</th>
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<tr>
<td>M1</td>
<td>3.40 ± 0.04</td>
<td>1.7 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>3.94</td>
</tr>
<tr>
<td>M2</td>
<td>3.52 ± 0.06</td>
<td>1.6 ± 0.1</td>
<td>1.7 ± 1.7</td>
<td>3.87</td>
</tr>
<tr>
<td>M3</td>
<td>3.17 ± 0.06</td>
<td>1.7 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>4.23</td>
</tr>
<tr>
<td>M4</td>
<td>3.59 ± 0.03</td>
<td>1.5 ± 0.1</td>
<td>0.6 ± 0.6</td>
<td>3.77</td>
</tr>
<tr>
<td>M5</td>
<td>2.81 ± 0.17</td>
<td>1.3 ± 0.3</td>
<td>15 ± 15</td>
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</table>
curves in off-rate assays were greater than 1, whereas the allosteric model predicts slopes of 1.

The binding of [3H]ACh can be measured only at M2 and M4 receptors in the high ionic strength buffer used here. In the absence of GTP, [3H]ACh binds to the high-affinity, G-protein bound form of the receptor. Thiochrome increased the binding of low concentrations of [3H]ACh approximately 2-fold to the M4 receptor but decreased [3H]ACh binding to the M2 receptor by approximately 20% (Fig. 5A). In saturation assays with [3H]ACh (Fig. 6), 10^-4 M thiochrome had little effect at M2 receptors and decreased the Kd at M4 receptors by approximately 50% (see legend to Fig. 6). Binding of thiochrome to M4 receptors was fully reversible (see legend to Fig. 5B).

The effect of 10^-4 M thiochrome on the stimulation by ACh of [35S]GTPgammaS binding to membranes containing muscarinic receptors is shown in Fig. 7. Thiochrome (made up in water or buffer, see Materials and Methods) did not affect basal activity, the slopes of the curves, or the maximal effect of ACh. Thiochrome increased ACh potency by 2- to 5-fold at M4 receptors and did not affect ACh potency at the other receptor subtypes.

To study the effect of thiochrome in a more physiological preparation, we measured its effects on ACh release from superfused brain slices. In the first set of experiments, we investigated the effect of thiochrome on 50 mM potassium stimulation-evoked liberation of ACh from rat striatal cholinergic interneurones, which have been shown to possess inhibitory presynaptic M4 muscarinic receptors (Dolezal and Tucek, 1998; Zhang et al., 2002). Depolarization with 50 mM potassium directly depolarizes nerve terminals and precludes the propagation of action potentials. As shown in Fig. 8A, thiochrome inhibited the evoked release of ACh in a concentration-dependent manner by 15 and 25% at concentrations of 0.3 and 1 mM, respectively. The inhibitory effect on release was mediated by muscarinic receptors because it was abolished by the muscarinic receptor antagonist N-methylscopolamine (NMS). Thiochrome at a concentration of 1 mM slightly but significantly attenuated basal outflow of radioactivity by 15% (Table 3). For this reason, it was used in the subsequent experiments at a concentration of 0.3 mM, which significantly inhibited the evoked release of ACh but did not influence the basal outflow of radioactivity.

The release of ACh in hippocampus, which is innervated by cholinergic neurons from basal forebrain, is regulated by presynaptic muscarinic receptors of the M2 subtype (Allen and Brown, 1993, 1996; Kitaichi et al., 1999a,b; Zhang et al., 2002). Thiochrome at a concentration of 0.3 mM did not influence either resting outflow of radioactivity (Table 3) or potassium stimulation-evoked release of ACh (Fig. 8B). The functionality of inhibitory muscarinic receptors was shown by the 40% inhibition of evoked release in the presence of 0.1 mM carbachol. The concentration of ACh at the receptors was sufficient to exert an inhibitory effect, as shown by the increase in evoked release in the presence of NMS.

To discriminate between a direct agonist action and an indirect allosteric effect (increasing the affinity of the muscarinic receptor for endogenous ACh), we investigated the influence of thiochrome on the release of ACh from striatal slices evoked by a fixed number of electrical pulses delivered at increasing frequencies (Fig. 9A). At a concentration of 0.3 mM, thiochrome had no influence on the release of ACh evoked by 40 pulses delivered at 1 Hz, whereas it inhibited the release when stimulated at a frequency of 2 Hz. The release of ACh was strongly autoinhibited when evoked by stimulation at a frequency of 5 Hz, and in this case, thiochrome had no further significant effect on the release (Fig. 9B). Apparently, shorter spacing of action potentials results in an increased residual concentration of ACh in the synaptic cleft at the time when the next action potential reaches the terminal, and at a certain level, it enables the allosteric potentiation of ACh action at M4 muscarinic receptor by thiochrome to be manifest.

The inhibitory effect of thiochrome on the electrically
evoked release of ACh depended not only on the frequency of stimulation but also on the duration of stimulation (Fig. 9). The inhibitory effect of thiochrome on ACh release was also found when the stimulation at 1 Hz was prolonged to 120 pulses, perhaps because the longer stimulation resulted in a small increase of residual ACh in the synaptic cleft.

**Discussion**

This article characterizes the subtle effects of thiochrome on muscarinic receptors. By a number of criteria, thiochrome seems inactive at muscarinic receptors. It has little or no effect on equilibrium [3H]NMS binding at any receptor subtype, nor on the potency or efficacy of ACh at M1, M2, M3, or M5 receptors (Figs. 2 and 7). However its inhibition of [3H]NMS dissociation from M1 to M4 receptors, presumably by binding to the [3H]NMS-occupied receptors, provides evidence that thiochrome probably does bind to these subtypes under equilibrium conditions. The major effect of thiochrome on equilibrium binding is the 3- to 5-fold increase in the apparent affinity of ACh to compete with [3H]NMS binding at M4 receptors, both in the presence and absence of GTP (Fig. 3B). This effect is not an artifact because it is subtype-specific and it can be seen in other types of assay: thiochrome increases the direct binding of [3H]ACh to M4 receptors (Figs. 5 and 6) and increases the functional potency of ACh to stimulate [35S]GTPγS binding (Fig. 7) and inhibit ACh release from striatal nerve endings via M4 receptors (Figs. 8 and 9).

The behavior of other allosteric agents at muscarinic receptors is generally consistent with the simple ternary complex model, in which the endogenous ligand (or a different orthosteric ligand) can bind simultaneously with an allosteric ligand at the receptor, and the difference in affinity of the orthosteric ligand for the free and allosteric liganded receptors constitutes the cooperative effect. One test of consistency is that the affinity of the allosteric agent for [3H]NMS-ligated receptors should be the same whether it is measured directly in an off-rate assay or indirectly as the product of affinity and cooperativity with [3H]NMS from equilibrium assays. Thiochrome partly survives this test. The comparison between the two estimates is shown in Table 2, and it can be seen that only at M4 receptors is there good consistency between the two parameter estimates. This is probably caused by the very small effects of thiochrome on [3H]NMS.
binding + ACh at M₁ to M₃ receptors (Fig. 2), leading to inaccurate parameter estimates (thiochrome was inactive in equilibrium studies at M₂ receptors). Where there is a clear signal, at M₄ receptors in the presence of ACh, the log affinity of thiochrome at free M₄ receptors was estimated with more precision, and the predicted affinity at the [³H]NMS-occupied receptor differs less than 2-fold from the value measured directly in the off-rate assay, supporting the allosteric model as the mechanism underlying both sets of observations.

One result that is not compatible with the simple allosteric model is the relatively steep slope of the curves describing the inhibition of [³H]NMS dissociation from M₁ to M₄ receptors. Other allosteric ligands have been found to bind with steep slopes, including tacrine, Duo3, and a series of pentacyclic compounds (Potter et al., 1989; Gharagozloo et al., 2002; Tränkle et al., 2003), and explanations such as binding to both the orthosteric and allosteric sites, binding to two or more allosteric sites, and the involvement of receptor dimerization have been suggested to account for the phenomenon. In one respect, however, the behavior of these compounds is different from that of thiochrome, because they have steep slopes in equilibrium assays as well as in off-rate assays, whereas the curves of thiochrome increasing both the affinity of ACh in competition with [³H]NMS at M₄ receptors and the binding of [³H]ACh to M₄ receptors are consistent with a slope of 1 (Figs. 2, 3A, and 5). It is possible that the highest concentration of thiochrome used in the off-rate assays, 1 mM, has nonspecific effects on [³H]NMS dissociation in addition to its allosteric effect.

The effects of thiochrome on the saturation binding of [³H]ACh to M₂ receptors were small and nonsignificant. At
M₄ receptors, where the effects were larger, the $B_{\text{max}}$ was never significantly altered by thiochrome, although there was a trend for a small increase, and the $K_I$ was reduced by 40 to 50%. An effect of thiochrome on the $K_I$ of $[^3\text{H}]\text{ACh}$ is consistent with the simple allosteric model, whereas a lack of effect on $B_{\text{max}}$ may indicate that thiochrome does not alter the efficacy of ACh; if the $B_{\text{max}}$ were increased by thiochrome, then this might imply that thiochrome had increased the affinity of the ACh-occupied receptor for the G protein, which could in turn lead to an increase in the efficacy of ACh (Tota and Schimerlik, 1990).

Thiochrome also acts as a selective allosteric enhancer of ACh at rat, as well as human, M₄ receptors. Rat striatum is known to contain presynaptic M₄ receptors, which inhibit ACh release, whereas the autoinhibitory presynaptic muscarinic receptors in rat hippocampus are of the M₂ subtype. Thiochrome inhibited ACh release from striatum stimulated by 50 mM KCl in a concentration-dependent manner, without affecting basal release (at concentrations lower than 1 mM), and the effect was blocked by NMS, indicating the involvement of muscarinic receptors. This effect had to occur at the level of presynaptic terminals because high potassium stimulation does not allow the spreading of action potentials. In contrast, thiochrome had no effect in hippocampus, although the directly acting agonist carbachol was effective in reducing release, and endogenous ACh was active at the inhibitory receptors because the antagonist NMS increased release. When ACh release from striatum was elicited electrically, thiochrome was ineffective at inhibiting release caused by 40 pulses at a frequency of 1 Hz, whereas release elicited by 2 Hz stimulation was strongly inhibited, as was the release caused by 120 pulses at 1 Hz. With 5-Hz stimulation, ACh release was strongly autoinhibited, and thiochrome did not cause further significant inhibition. These results demonstrate that thiochrome was not acting as a direct agonist but required the continuing presence of non-saturating concentrations of ACh at the receptor to be effective.

In conclusion, thiochrome may not bind to M₅ receptors but does seem to bind weakly to muscarinic M₁ to M₄ receptors.

**TABLE 3**
The influence of thiochrome on the resting outflow of radioactivity from striatum and hippocampus

An effect of drugs is given as a ratio of resting efflux expressed as fractional basal release (B2 and B1) immediately before S2 and S1, respectively. B1 (percentage of tissue content) was $1.30 \pm 0.04$ (41) for striatum and $1.00 \pm 0.04$ (47) for hippocampus. Data represent mean ± S.E.M. of the number of observations given in parentheses.

<table>
<thead>
<tr>
<th>Potassium Stimulation: B2/B1</th>
<th>Striatum</th>
<th>Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.12 ± 0.03 (18)</td>
<td>0.92 ± 0.03 (18)</td>
</tr>
<tr>
<td>Thio 0.1 mM</td>
<td>1.24 ± 0.06 (5)</td>
<td>0.88 ± 0.02 (15)</td>
</tr>
<tr>
<td>Thio 0.3 mM</td>
<td>1.10 ± 0.01 (6)</td>
<td>1.05 ± 0.07 (3)</td>
</tr>
<tr>
<td>Thio 1 mM</td>
<td>0.95 ± 0.03 (6)*</td>
<td>0.88 ± 0.02 (15)</td>
</tr>
<tr>
<td>NMS 1 µM</td>
<td>1.12 ± 0.05 (3)</td>
<td>1.05 ± 0.07 (3)</td>
</tr>
<tr>
<td>Thio 0.3 + NMS</td>
<td>0.98 ± 0.05 (3)</td>
<td>0.98 ± 0.05 (3)</td>
</tr>
</tbody>
</table>

* $p < 0.05$ from control by analysis of variance followed by Dunnett’s multiple comparison test.

Thio, thiochrome.

It enhances the affinity of ACh 3- to 5-fold at M₄ receptors only, while having negligible effects on ACh at the other four muscarinic receptor subtypes. Physiological consequences of this property were observed in rat striatum, where thio-
chrome enhanced the effect of neuronally released ACh. Despite its low potency and lack of selective affinity for M₁ to M₄ receptors, thiochrome shows absolute receptor subtype selectivity as an allosteric enhancer of ACh affinity at M₂ receptors and may prove to be a useful tool in the characterization of functional muscarinic responses.

References


Buckley NJ, Bonner TI, Buckley CM, and Brann MR (1989) Antagonist binding receptors, thiochrome shows absolute receptor subtype selectivity with respect to its low potency and lack of selective affinity for M₁ to M₄.-

References


Kitaichi K, Day JC, and Quirion R (1999a) A novel muscarinic M₄ receptor antago-
nist provides further evidence of an autoreceptor role for the muscarinic M₄ subtype. Eur J Pharmacol 368:53–56.

Kitaichi K, Hori T, Srivastava LK, and Quirion R (1999b) Antisense oligodeoxynucle-

otides against the muscarinic M₂, but not M₄, receptor supports its role as an autoreceptor in the rat hippocampus. Brain Res Mol Brain Res 67:98–106.


Lazareno S, Gharaguzloo P, Kuonen D, Popham A, and Birdsell NJM (1998) Sub-


W84 at the common allosteric site of muscarinic M₂ receptors. Mol Pharmacol 64:180–189.


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tonhole Lane, Mill Hill, London NW7 1AD, United Kingdom. E-mail: sebastian.lazareno@tech.mrc.ac.uk