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# Action of the muscarinic toxin MT7 on agonist-bound muscarinic $M_1$ receptors

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**Abstract**

The muscarinic toxin MT7 is the most selective ligand for the muscarinic $M_1$ receptors. Previous studies have shown that the toxin interacts with the antagonist–receptor complex and slows the antagonist dissociation rate, possibly by binding to an allosteric site and impeding the access to and egress from the orthosteric binding pocket. In the present study, we investigated the action of MT7 on agonist-occupied receptors in functional and radioligand binding assays of the cloned human muscarinic $M_1$ receptor expressed in Chinese hamster ovary cells. In time-course experiments, the addition of MT7 rapidly blocked the acetylcholine-stimulated guanosine-5′-*O*-(3-[$^{35}$S]thio)triphosphate binding to membrane G proteins. Similarly, in acetylcholine-treated cells MT7 completely stopped the agonist-stimulated [$^3$H]inositol phosphate accumulation. In dissociation experiments using membranes pre-equilibrated with [$^3$H]acetylcholine, the addition of MT7 increased the rate of radioligand dissociation. The data indicate that MT7, while partially stabilizing the antagonist–receptor complex, effectively destabilizes the agonist-occupied muscarinic $M_1$ receptors.


*Keywords:* *Dendroaspis angusticeps* toxins; Muscarinic $M_1$ receptor; [$^{35}$S]GTPγS binding; Phosphoinositide hydrolysis; [$^3$H]Acetylcholine binding; [$^3$H]*N*-Methyl-scopolamine binding

## 1. Introduction

The venom of the green mamba *Dendroaspis angusticeps* contains a number of 65–66 amino acid peptide toxins which possess a common three-finger fold structure and bind with high affinity and selectivity to acetylcholine muscarinic receptors (Bradley, 2000; Karlsson et al., 2000; Jerusalinsky and Harvey, 1994; Potter, 2001). One of these toxins, MT7 (also named $M_1$-toxin1) has been identified as a highly selective ligand of the muscarinic $M_1$ receptors (Adem and Karlsson, 1997; Carsi and Potter, 2000; Jolkkonen, 1996). The toxin binds to $M_1$ receptors at nanomolar concentrations, while it displays negligible affinity for the muscarinic $M_2$–$M_5$ receptors. Thus, MT7 represents the most selective ligand for the muscarinic $M_1$ receptor and has been widely employed to identify the role of this receptor subtype in different physio-pathological processes (Liang et al., 2001; Marino et al., 1998; Onali and Olianas, 2002; Rouse et al., 2000). On the other hand, the mode of action of the toxin on muscarinic $M_1$ receptor is still under investigation. A prominent feature of this toxin is that it appears to bind to the receptor in a stable manner and to block the receptor non-competitively (Max et al., 1993; Olianas et al., 2000; Bradley et al., 2003). Radioligand binding studies have shown that, when applied first to unoccupied receptors, MT7 prevents the subsequent binding of the muscarinic receptor antagonist [$^3$H]*N*-methyl-scopolamine (Max et al., 1993; Karlsson et al., 2000). However, when added after [$^3$H]*N*-methyl-scopolamine, picomolar concentrations of the toxin cause a modest reduction of radioligand binding which disappears at higher toxin concentrations (Karlsson et al., 2000). In addition, similarly to many allosteric modulators of muscarinic receptors (Tucek and Proska, 1995), MT7 markedly slows the rate of [$^3$H]*N*-methyl-scopolamine dissociation from the receptor (Max et al., 1993;

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Olianas et al., 2000). In competition experiments, the toxin fails to completely inhibit the binding of [$^3$H]*N*-methyl-scopolamine and the fraction of the radioligand not displaced by the toxin increases with increasing concentrations of [$^3$H]*N*-methyl-scopolamine (Olianas et al., 2000). This behavior suggests that MT7 may act as an allosteric antagonist by binding to a secondary allosteric site on the receptor molecule. Indeed, a number of radioligand binding studies have proposed the existence of multiple allosteric sites on muscarinic receptors (for review, see Birdsall et al., 2001; Christopoulos and Kenakin, 2002). On the other hand, some phenomena, such as the dependence on the order of addition of toxin and competitive radioligand and the slowing of radioligand dissociation, have been explained by postulating that the toxin interacts with the extracellular portion of the muscarinic $M_1$ receptor and creates a steric obstacle to the passage of ligands to as well as from the orthosteric site (Max et al., 1993; Karlsson et al., 2000). Recent studies on synthetic MT7 have shown that two amino acid residues located at the tip of the central toxin finger, Phe$^{38}$ and Arg$^{34}$, are relevant for the stability and high-affinity toxin binding to muscarinic $M_1$ receptors, whereas the C-terminal Lys$^{65}$ appears to be critical for the toxin interaction with [$^3$H]*N*-methyl-scopolamine-occupied receptors (Krajewski et al., 2001; Mourier et al., 2003).

Most of the studies on MT7 have considered the toxin interaction with either unoccupied or antagonist-liganded muscarinic receptors, but little is known on the action of the toxin on agonist-occupied receptors. We have previously shown that pretreatment of muscarinic $M_1$ receptor-bearing cells or cell membranes with MT7 prevents subsequent receptor activation (Olianas et al., 2000), but the effects of the toxin on previously activated receptor have not been investigated. Moreover, if it is assumed that the toxin simply acts by forming a lid on the receptor surface, one may expect that it would be unable to stop receptor signalling once the receptor binding pocket is occupied by the agonist. This possibility may have relevant implication for the biological activity of the toxin, making it dependent on the receptor occupancy by the agonist.

To gain further information on the toxin–receptor interaction, we have investigated the action of MT7 on the agonist-muscarinic $M_1$ receptor complex by carrying out time-course experiments using functional and radioligand receptor assays in Chinese hamster ovary (CHO) cells stably expressing the cloned human muscarinic $M_1$ receptor.

## 2. Materials and methods

### 2.1. Materials

Guanosine-5′-*O*-(3-[$^{35}$S]thio)triphosphate ([$^{35}$S]GTPγS) (1306 Ci/mmol) was obtained from NEN-Perkin Elmer (Boston, MA, USA). Myo-[$^3$H]inositol (99 Ci/mmol) and [$^3$H]*N*-methyl-scopolamine (83 Ci/mmol) were obtained from Amersham (UK). [$^3$H]Acetylcholine ([$^3$H]ACh) (85 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO, USA). Both native MT7 purified from the venom of *D. angusticeps* (Vandermeers et al., 1995) and the synthetic peptide (Peptide International) were used with similar results. GTPγS and guanylyl-imidodiphosphate (GppNHp) were from Calbiochem (La Jolla, CA, USA) and Boehringer-Mannheim Italia, respectively. Oxotremorine M was obtained from Research Biochemical (Natick, MA, USA). Acetylcholine chloride, atropine sulfate and the other reagents were from Sigma (St. Louis, MO, USA).

### 2.2. Cell culture

CHO cells expressing either the cloned human muscarinic $M_1$ (CHO/$M_1$) (~ 10 pmol/mg protein) or $M_2$ (CHO/$M_2$) (~ 3.0 pmol/mg protein) receptor were kindly provided by Prof. A.D. Strosberg (Institut Cochin de Genetique Moleculaire, Paris, France). The cells were grown as a monolayer culture in Ham's F-12 medium (GIBCO BRL) supplemented with 10% foetal calf serum (GIBCO BRL) in a humidified atmosphere (5% $CO_2$) at 37 °C.

### 2.3. Cell membrane preparation

Cells were grown to confluency in 100 mm plastic Petri dishes, the medium was removed and the cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (PBS). The cells were then scraped into an ice-cold buffer containing 25 mM sodium phosphate buffer (pH 7.4) and 5 mM $MgCl_2$ and lysed at ice-bath temperature by the use of an Ultra-Turrax homogenizer with two 10 s bursts separated by a 1-min pause. The cell lysate was centrifuged at 32,500 × *g* for 30 min at 4 °C and the pellet was resuspended in the same buffer at a protein concentration of 2.0–2.5 mg/ml. The membrane preparations were either used immediately or stored at − 80 °C.

### 2.4. Assay of [$^{35}$S]GTPγS binding

CHO cell membranes were diluted 10-fold in an ice-cold buffer containing 10 mM HEPES/NaOH and 1 mM EDTA (pH 7.4), centrifuged at 32,500 × *g* for 30 min at 4 °C and resuspended in the same buffer containing 0.1% bovine serum albumin. The binding of [$^{35}$S]GTPγS was assayed in a reaction mixture containing 25 mM HEPES/NaOH (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 0.5 μM GDP and 100 mM NaCl. Cell membranes (final membrane protein concentration 15–20 μg/ml) were preincubated for 10 min at 30 °C in the absence (basal) and in the presence of 100 μM acetylcholine. At time 0, the reaction was started by the addition of [$^{35}$S]GTPγS (final concentration 1 nM). At the indicated time points, a 100-μl aliquot was withdrawn from each reaction tube, added to 5 ml of an

ice-cold buffer containing 10 mM HEPES/NaOH (pH 7.4) and 1 mM $MgCl_2$, and immediately filtered through a glass fibre filter (Whatman GF/C) presoaked in the same buffer. The filter was washed twice with 5 ml of buffer and the radioactivity trapped was determined by liquid scintillation spectrometry. For each time point, nonspecific binding was determined in the presence of 100 μM GTPγS.

### 2.5. Assay of [$^3$H]inositol phosphates accumulation

Cells were grown in 75 $cm^2$ flasks to ~ 80% confluency. The growing medium was removed and substituted with Ham's F-10 medium containing 1 μCi/ml of myo-[$^3$H]inositol. Cells were incubated for 20–24 h in an incubator at 37 °C in humidified atmosphere (5% $CO_2$). The medium was then removed and the cells were washed twice with PBS. The cells were detached from the tissue culture flasks by incubation in PBS containing 0.5 mM EDTA for 5 min at 37 °C followed by gentle agitation of the flasks. The cell suspension was aspirated, mixed with an equal volume of PBS containing 1 mM $MgCl_2$ and centrifuged at $300 \times g$ for 1 min. The cells were resuspended in a freshly oxygenated Krebs-HEPES buffer (pH 7.4) containing 25 mM HEPES/NaOH, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 10 mM glucose, 110 mM NaCl, 3.8 mM KCl, 1.2 mM $CaCl_2$ and 10 mM LiCl. Aliquots of the cell suspension were distributed into reaction tubes and preincubated for 20 min at 37 °C. At time 0, either vehicle or acetylcholine (final concentration 100 nM) was added and the incubation was continued at 37 °C. At the indicated time points, a 100-μl aliquot was withdrawn from each reaction tube and mixed with 940 μl of chloroform–methanol (1:2 v/v). After the samples were shaken for 10 min, 310 μl of chloroform and 510 μl of water were added. The samples were centrifuged at $1000 \times g$ for 10 min and the upper aqueous phase was applied to a column of Dowex $1 \times 8$ in the formate form. The column was washed with 20 bed volumes of $H_2O$, 20 bed volumes of 5 mM myo-inositol and 16 bed volumes of 5 mM sodium tetraborate in 50 mM sodium formate. [$^3$H]Inositol phosphates were eluted by adding 6 bed volumes of 1 M ammonium formate in 0.1 M formic acid (Berridge et al., 1983). The radioactivity present in the eluate and in the organic phase was determined by liquid scintillation counting. For each sample, the accumulation of [$^3$H]inositol phosphates was corrected for the amount of radioactivity incorporated in the organic phase. Results are expressed as percentages of the total radioactivity incorporated that was converted to [$^3$H]inositol phosphates.

### 2.6. Assay of [$^3$H]acetylcholine binding

The binding of [$^3$H]acetylcholine to muscarinic $M_1$ receptors was assayed in an incubation buffer containing 12.5 mM sodium phosphate (pH 7.4), 2.5 mM $MgCl_2$, 2.0 mM $NiCl_2$ and 50–60 μg of membrane protein. The concentration of [$^3$H]acetylcholine was 10 nM and the final assay volume was 100 μl. The incubation was carried out at 25 °C for 70–80 min in 1.5 ml Eppendorf micro tubes. Thereafter, the samples were centrifuged at $20{,}000 \times g$ for 70 s at 4 °C. The supernatants were discarded and the pellets were washed superficially with 1 ml of ice-cold 5 mM sodium phosphate buffer (pH 7.4). The total time required for the centrifugation and washing procedure was $100 \pm 5$ s. The tubes were kept inverted and the sides of the tubes carefully wiped using a cotton swab. The pellets were then solubilized and the radioactivity was determined by liquid scintillation counting. Assays were performed in duplicate. Nonspecific binding was determined in the presence of 10 μM atropine. The addition of 2 mM $NiCl_2$ was found to increase total [$^3$H]acetylcholine binding by about three-fold with little effect on nonspecific binding. In the presence of $NiCl_2$ the total and nonspecific binding corresponded to 2000–2400 (≤ 5% of the total radioactivity added) and 400–600 cpm, respectively. Preliminary time-course experiments indicated that in membranes preincubated with $NiCl_2$ for 25 min before radioligand addition the specific binding of [$^3$H]acetylcholine (10 nM) reached a steady-state after 10–15 min and remained constant for at least 3 h.

For the experiments measuring the dissociation of [$^3$H]acetylcholine, the binding was first allowed to proceed for 70 min at 25 °C under the above conditions. The amount of [$^3$H]acetylcholine specifically bound at this time was taken as $t = 0$. Atropine without and with the test compounds was then added and the samples were centrifuged at specific times after the addition of the dissociating agent. The non-specific binding, which was subtracted from each experimental point, was determined throughout in a parallel experiment where 10 μM atropine was added at the beginning of the first incubation period. In Figs. 4–6, the time scale at time points $t > 0$ were frame shifted by the amount of time required for the centrifugation and washing of the pellet.

The binding of [$^3$H]acetylcholine to muscarinic $M_2$ receptors was performed using the same experimental procedure employed for the muscarinic $M_1$ receptors, with the exception that the concentration of [$^3$H]acetylcholine was 5 nM. Total and nonspecific binding (determined in the presence of 10 μM atropine) corresponded to 3000–3500 and 250–350 cpm, respectively.

### 2.7. Assay of [$^3$H]N-methyl-scopolamine binding

For equilibrium binding assays, CHO/$M_1$ cell membranes (50–60 μg of membrane protein) were incubated in buffer containing 12.5 mM sodium phosphate (pH 7.4), 2.5 mM $MgCl_2$, 5.0 nM [$^3$H]*N*-methyl-scopolamine and 2.0 mM $NiCl_2$ to maintain the assay conditions similar to those of [$^3$H]acetylcholine binding. At this concentration, the metal failed to affect either total or nonspecific [$^3$H]*N*-

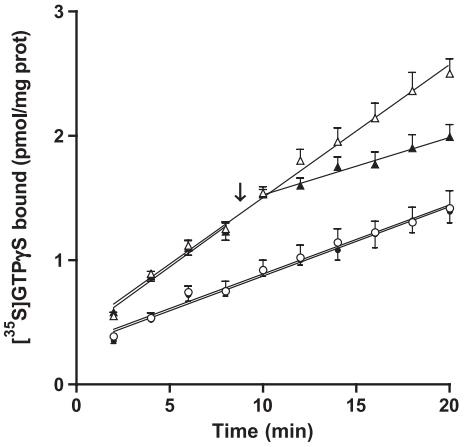


Fig. 1. Blockade of acetylcholine stimulation of [$^{35}$S]GTPγS binding by MT7 in membranes of CHO/$M_1$ cells. Cell membranes were preincubated for 10 min at 30 °C in the absence (circles) and in the presence of 100 μM acetylcholine (triangles). At 0 time, the reaction was started by the addition of [$^{35}$S]GTPγS (1 nM) and aliquots were withdrawn from each reaction tube at the indicated times. MT7 (1 μM) (filled symbols) or vehicle (open symbols) was added at the time point indicated by the arrow. Values are the mean ± S.E.M. of four experiments.

methyl-scopolamine binding. The incubation was carried out at 25 °C for 70 min and was stopped by adding 4 ml of ice-cold buffer followed by immediate filtration through glass fibre filters (Whatman GF/C) presoaked in 0.1% polyethylenimine. The filters were washed twice with the same buffer, dried and the bound radioactivity was counted by liquid scintillation spectrometry. Nonspecific binding was determined in the presence of 10 μM atropine and corresponded to 4–5% of total binding. Assays were performed in duplicate.

For dissociation experiments, 10–12 μg of membrane protein were incubated with 1.0 nM [$^3$H]*N*-methyl-scopolamine in a buffer containing 15 mM sodium phosphate (pH 7.4), 3 mM $MgCl_2$ and 2.0 mM $NiCl_2$ in a final assay volume of 500 μl. The incubation was carried out at 30 °C for 90–100 min and was stopped as previously described at different time points following the addition of the dissociating agent. Nonspecific binding was determined in the presence of 1 μM atropine and corresponded to 3–4% of total binding. Assays were performed in duplicate.

Protein content was determined by the method of Bradford (1976), using bovine serum albumin as a standard.

### 2.8. Statistical analysis

Results are reported as means ± S.E.M. The data were analysed by using the computer program PRISM (GraphPad, USA). Radioligand binding data were analysed by using the computer programs LIGAND (Munson and Rodbard, 1980) and KINETIC (McPherson, 1985) (both obtained from Biosoft, UK). Statistical significance of the difference between means was determined by Student's *t*-test.

## 3. Results

### 3.1. Blockade of acetylcholine-stimulated [$^{35}$S]GTPγS binding

As shown in Fig. 1, time-course experiments indicated that in CHO/$M_1$ cell membranes preincubated for 10 min with 100 μM acetylcholine there was a rapid stimulation of [$^{35}$S] GTPγS binding, which was evident 2 min after the radioligand addition (the earliest time point investigated) and linear with time for at least 18 min. Under these conditions, acetylcholine increased the rate of [$^{35}$S]GTPγS binding by about two-fold when compared to the basal rate (from $0.055 \pm 0.003$ to $0.108 \pm 0.010$ pmol/min/mg protein, mean ± S.E.M., $P < 0.01$). The addition of MT7 (final concentration 1 μM) 8 min after the beginning of the reaction caused a rapid blockade of the acetylcholine stimulatory effect, reducing the binding rate to basal value. When added to membranes preincubated in the absence of acetylcholine, MT7 failed to change the [$^{35}$S]GTPγS binding rate.

### 3.2. Blockade of acetylcholine-stimulated [$^3$H]inositol phosphate accumulation

In CHO/$M_1$ cells prelabeled with myo-[$^3$H]inositol and incubated in the presence of 10 mM LiCl, the addition of acetylcholine (100 nM) caused a marked stimulation of [$^3$H]inositol phosphate accumulation. Time-course experi-

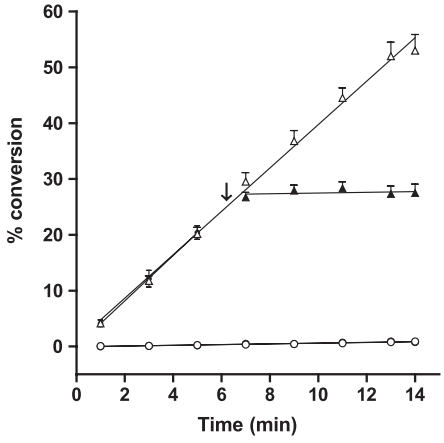


Fig. 2. Inhibition of acetylcholine stimulation of [$^3$H]inositol phosphate accumulation by MT7 in CHO/$M_1$ cells. Cells prelabelled with myo-[$^3$H]inositol were incubated with either vehicle (circles) or 100 nM acetylcholine (triangles) added at 0 time. At the indicated times aliquots were withdrawn from each experimental tube and mixed with chloroform–methanol. At the time point indicated by the arrow, MT7 (1 μM) (closed symbols) or vehicle (open symbols) was added and the incubation continued for the indicated time. Values are the mean ± S.E.M. of three experiments.

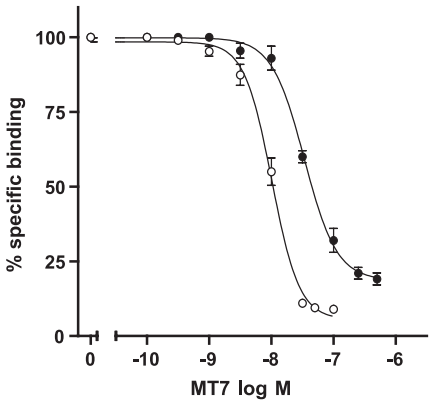


Fig. 3. Effect of MT7 on the specific binding of [$^3$H]acetylcholine (○) and [$^3$H]*N*-methyl-scopolamine (●) to muscarinic $M_1$ receptors at equilibrium. CHO/$M_1$ cell membranes (50–60 μg of protein) were incubated in the presence of either [$^3$H]acetylcholine (10 nM) or [$^3$H]*N*-methyl-scopolamine (5 nM) and the indicated concentrations of MT7 and the reaction was allowed to continue for 70 min at 25 °C. Thereafter, the incubation was stopped by either centrifugation or filtration as described under Section 2. Values are the mean ± S.E.M. of three experiments.

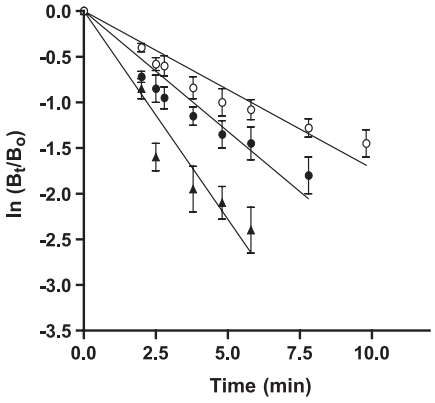


Fig. 5. Effects of MT7 on [$^3$H]acetylcholine dissociation from muscarinic $M_1$ receptors. CHO/$M_1$ cell membranes were incubated for 70 min at 25 °C in the presence of 10 nM [$^3$H]acetylcholine. Radioligand dissociation was initiated by the addition of atropine (10 μM) without (○) and with 50 (●) and 200 (▲) nM MT7 and the binding of [$^3$H]acetylcholine was followed with time as described in Section 2. [$^3$H]Acetylcholine dissociation is plotted on a semilogarithmic scale, where $B_t$ and $B_o$ represent the amount of radioligand specifically bound at $t$ and $t=0$, respectively. Values are the means ± S.E.M. of three to four experiments.

ments showed that the acetylcholine stimulation maintained a constant rate for at least 14 min (Fig. 2). The addition of MT7 (final concentration 1 μM) to CHO/$M_1$ cells 6 min after the exposure to acetylcholine caused a rapid and complete reversal of the agonist stimulatory effect. The basal [$^3$H]inositol phosphate accumulation was not affected by the addition of MT7. Like acetylcholine, the muscarinic receptor agonist oxotremorine M (100 nM) elicited a marked stimulation of [$^3$H]inositol phosphate accumulation. The addition of MT7 (1 μM) 6 min after caused a complete and rapid blockade of the oxotremorine M effect (results not shown).

### 3.3. Effects on [$^3$H]acetylcholine binding

Equilibrium binding experiments with [$^3$H]acetylcholine (10 nM) performed in the presence of increasing concentrations of unlabelled ligand (from 5 nM to 3 μM) yielded a monophasic competition curve, indicating the presence of a single class of saturable binding sites. The estimated equilibrium dissociation constant ($K_D$) and maximal binding capacity ($B_{max}$) values were 70 ± 12 nM and 2.62 ± 0.43 pmol/mg protein, respectively ($n=3$; results not shown). In these membranes, the $B_{max}$ value of [$^3$H]*N*-methyl-scopolamine was about 10 pmol/mg protein. When 1 mM GTP was present during the incubation, the specific binding of

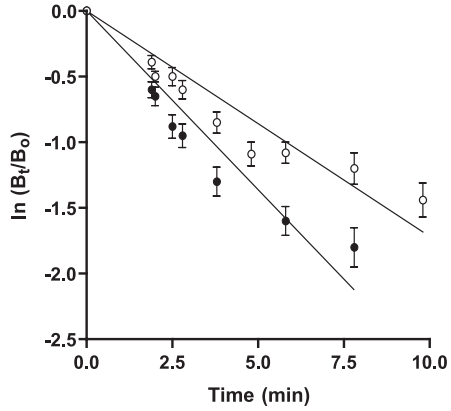


Fig. 4. Dissociation of [$^3$H]acetylcholine from muscarinic $M_1$ receptors. CHO/$M_1$ cell membranes were incubated for 70 min at 25 °C in the presence of 10 nM [$^3$H]acetylcholine. At this point ($t=0$) atropine (10 μM) without (○) and with (●) 100 μM Gpp(NH)p was added and the [$^3$H]acetylcholine binding was followed with time as described in Section 2. [$^3$H]Acetylcholine dissociation is plotted on a semilogarithmic scale, where $B_t$ and $B_o$ represent the amount of radioligand specifically bound at $t$ and $t=0$, respectively. Values are the means ± S.E.M. of five experiments.

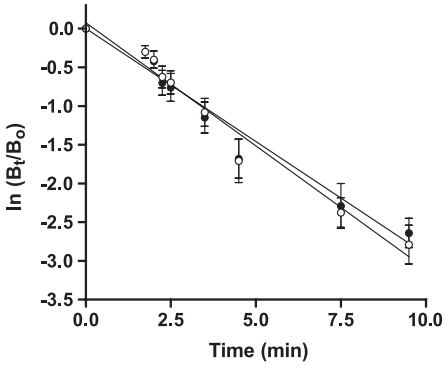


Fig. 6. Lack of effect of MT7 on [$^3$H]acetylcholine dissociation from muscarinic $M_2$ receptors. CHO/$M_2$ cell membranes were incubated for 60 min at 25 °C in the presence of 5 nM [$^3$H]acetylcholine. At this point ($t=0$), atropine (10 μM) without (○) and with (●) 100 nM MT7 was added and the binding of [$^3$H]acetylcholine was followed with time as described in Section 2. [$^3$H]Acetylcholine dissociation is plotted on a semilogarithmic scale, where $B_t$ and $B_o$ represent the amount of radioligand specifically bound at $t$ and $t=0$, respectively. Values are the means ± S.E.M. of three experiments.

[$^3$H]acetylcholine was reduced by 75 ± 8% ($n$ = 3; results not shown).

MT7, added at the beginning of the reaction, caused a concentration-dependent inhibition of specific [$^3$H]acetylcholine binding with an $IC_{50}$ value of 12.2 ± 1.5 nM and a Hill coefficient of 1.9 ± 0.2 (Fig. 3). The maximal inhibitory effect was less than 100% and corresponded to 90 ± 2% ($n$ = 3) reduction of specific binding. Under similar assay conditions, MT7 inhibited [$^3$H]*N*-methyl-scopolamine binding with an $IC_{50}$ value of 31.5 ± 3.0 nM and a Hill coefficient of 1.6 ± 0.3. The maximal inhibitory effect corresponded to 81 ± 2% ($n$ = 3).

The addition of atropine (10 μM) to CHO/$M_1$ cell membranes preincubated for 70 min with 10 nM [$^3$H]acetylcholine, induced a monophasic dissociation of the radioligand with a $t_{1/2}$ = 4.2 ± 0.2 min (Fig. 4). The nonhydrolyzable GTP analogue GppNHp (100 μM) increased the dissociation rate to 2.5 ± 0.1 min ($P$ < 0.05). When the effects of MT7 were investigated, it was found that the addition of the toxin at 50 and 200 nM increased the [$^3$H]acetylcholine dissociation rate from 4.1 ± 0.2 min of control to 2.6 ± 0.2 and 1.5 ± 0.2 min, respectively ($P$ < 0.05) (Fig. 5). To check the specificity of the toxin effect, we investigated the action of MT7 on the dissociation of [$^3$H]acetylcholine from muscarinic $M_2$ receptors. [$^3$H]Acetylcholine dissociation yielded a monoexponential kinetic with $t_{1/2}$ of 2.2 ± 0.3 min. The addition of the toxin (100 nM) failed to affect the dissociation of the radioligand (Fig. 6).

### 3.4. Effects on [$^3$H]*N*-methyl-scopolamine dissociation

The effect of MT7 on the dissociation of [$^3$H]*N*-methyl-scopolamine from the muscarinic $M_1$ receptor was examined in the presence of 2 mM $NiCl_2$. The antagonist dissociated with a rate constant of 0.045/min, which was similar to that previously obtained in the same cells in the absence of $NiCl_2$ (Olianas et al., 2000). The addition of MT7 (20 nM) markedly decelerated the dissociation of [$^3$H]*N*-methyl-scopolamine from muscarinic $M_1$ receptors (rate constant = 0.0058/min) (Fig. 7).

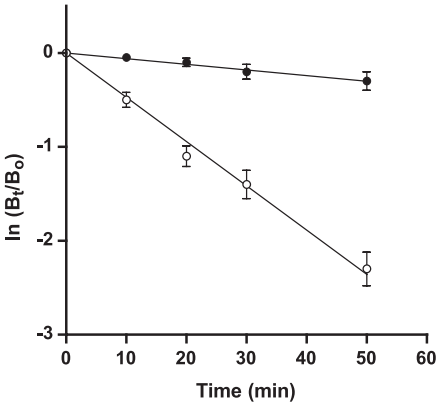


Fig. 7. Deceleration of [$^3$H]*N*-methyl-scopolamine by MT7 from muscarinic $M_1$ receptors. CHO/$M_1$ cell membranes were incubated for 90 min at 30 °C in the presence of 1 nM [$^3$H]*N*-methyl-scopolamine in a sodium phosphate buffer containing 3 mM $MgCl_2$ and 2.0 mM $NiCl_2$. At this point ($t$ = 0), 10 μM atropine without (○) and with (●) 20 nM MT7 was added and the binding of [$^3$H]*N*-methyl-scopolamine was followed with time as described in Section 2. [$^3$H]*N*-methyl-scopolamine dissociation is plotted on a semilogarithmic scale, where $B_t$ and $B_o$ represent the amount of radioligand specifically bound at $t$ and $t$ = 0, respectively Values are the mean ± S.E.M. of three experiments.

## 4. Discussion

We have investigated the action of MT7 on agonist-occupied muscarinic $M_1$ receptors by both functional and radioligand binding assays. In functional studies, time-course experiments were performed to examine whether the toxin could change ongoing agonist-induced receptor signalling. The receptors were pre-exposed to maximally effective concentrations of acetylcholine and the reaction was monitored within the linear portion of the time-course. The addition of MT7 induced a rapid reversal of acetylcholine-induced stimulation of either [$^{35}$S]GTPγS binding or [$^3$H]inositol phosphate accumulation, indicating that the toxin disrupted the agonist-activated state of the receptor. The toxin behaved similarly in intact and broken cell assays, which indicates that MT7 has no unspecific effects on intracellular molecules of the signalling pathway, such as G proteins or phospholipase C. Moreover, the toxin reversed the receptor activation elicited by oxotremorine M, a compound structurally different from acetylcholine, indicating that its action was independent of the agonist used.

To investigate whether MT7 blocked agonist-induced receptor signalling by inducing the dissociation of the agonist from the receptor, we performed radioligand binding experiments using [$^3$H]acetylcholine as a radioligand. The study of [$^3$H]acetylcholine binding to muscarinic $M_1$ receptors was greatly facilitated by incubating the cell membranes in the presence of $NiCl_2$, which markedly increased total binding without significantly affecting nonspecific binding. The stimulatory effect of $Ni^{2+}$ on agonist binding to muscarinic receptors of brain membranes has been previously documented by several investigators and explained as the result of a possible ion-induced increase in receptor–G protein coupling (Gurwitz et al., 1984, 1985; Hulme et al., 1983; Nukada et al., 1983). Under these assay conditions, [$^3$H]acetylcholine labelled a single class of binding sites with an estimated $B_{max}$ value corresponding to approximately 25% of that displayed by [$^3$H]*N*-methyl-scopolamine. The equilibrium binding of [$^3$H]acetylcholine was markedly reduced by GTP, indicating that [$^3$H]acetylcholine mostly labelled a G protein-coupled state of the receptor. MT7 inhibited equilibrium binding of [$^3$H]acetylcholine and [$^3$H]*N*-methyl-scopolamine with steep curves, as expected from the non-competitive and stable binding of the toxin to muscarinic $M_1$ receptors. Moreover, the toxin failed to completely block the specific binding of either [$^3$H]acetylcholine or [$^3$H]*N*-methyl-scopolamine, supporting the con-

cept that the toxin modulates the orthosteric site allosterically. The absence of 100% inhibition of specific [$^3$H]*N*-methyl-scopolamine binding was also observed in previous studies (Olianas et al., 2000; Krajewski et al., 2001).

In membranes pre-equilibrated with [$^3$H]acetylcholine in the presence of $Ni^{2+}$, the addition of atropine induced a monophasic dissociation of the radioligand which was accelerated by GppNHp, a stable G protein activator and receptor–G protein uncoupler (Gilman, 1987). The accelerating effect of guanine nucleotides on agonist dissociation has previously been documented in other muscarinic receptor systems (Harden et al., 1983; Vickroy et al., 1983; Waelbroeck et al., 1982). When the effect of MT7 was investigated, it was found that the toxin significantly increased the rate of [$^3$H]acetylcholine dissociation. On the other hand, the toxin, at a similar concentration, had no effect on the dissociation kinetics of [$^3$H]acetylcholine from muscarinic $M_2$ receptors, indicating a receptor subtype-dependent specificity of the toxin action. Moreover, as previously reported (Olianas et al., 2000), MT7 caused a deceleration of the dissociation of [$^3$H]*N*-methyl-scopolamine from muscarinic $M_1$ receptors, indicating that addition of $Ni^{2+}$ did not alter this toxin property.

The present finding that MT7 differentially affects agonist and antagonist dissociation kinetics suggests a different mode of interaction with agonist- and antagonist-occupied muscarinic $M_1$ receptors. The observation that the toxin accelerates agonist dissociation strongly supports an allosteric action of the toxin. It has been postulated that MT7 binds to extracellular portions of the receptor creating a cap that blocks the access to and the egress from the orthosteric binding site. According to this model the toxin should slow down the dissociation of all ligands, antagonists as well as agonists, from the orthosteric site. While this model can explain the slowing effect on antagonist dissociation, it is clearly not consistent with the ability of the toxin to enhance agonist dissociation. Previous studies have shown that allosteric modulators can affect agonist and antagonist dissociation either similarly or differently. For instance, Gnagey and Ellis (1996) reported that gallamine slowed the dissociation of both [$^3$H]acetylcholine and [$^3$H]*N*-methyl-scopolamine from muscarinic $M_2$ receptors. On the other hand, Leppik et al. (1998) and Leppik and Birdsall (2000) found that amiloride analogs differentially affects agonists and antagonists dissociation from $\alpha_{2A}$-adrenergic receptors. While the latter studies indicate that the action of MT7 is not unique, the reason of the opposite effects of the toxin on agonist and antagonist dissociation kinetics remains to be elucidated. Further studies aimed to the identification of the toxin binding site(s) on the muscarinic $M_1$ receptor molecule may help to clarify this issue.

In conclusion, the present study demonstrates that the muscarinic toxin MT7 can rapidly interrupt agonist-induced muscarinic $M_1$ receptor signalling and accelerate agonist dissociation. Further, we confirm earlier results that the toxin decelerates antagonist dissociation. Thus, the toxin has a different mode of action on muscarinic $M_1$ receptors occupied by agonists or antagonists.

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