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Inhibition of acetylcholine muscarinic M_1 receptor function by the M_1-selective ligand muscarinic toxin 7 (MT-7)

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1 MT-7 (1–30 nM), a peptide toxin isolated from the venom of the green mamba *Dendroaspis angusticeps* and previously found to bind selectively to the muscarinic M_1 receptor, inhibited the acetylcholine (ACh)-stimulated [^{35}S]-guanosine-5′-O-(3-thio)triphosphate ([^{35}S]-GTPγS) binding to membranes of Chinese hamster ovary (CHO) cells stably expressing the cloned human muscarinic M_1 receptor subtype.

2 MT-7 failed to affect the ACh-stimulated [^{35}S]-GTPγS binding in membranes of CHO cells expressing either the M_2, M_3 or M_4 receptor subtype.

3 In N1E-115 neuroblastoma cells endogenously expressing the M_1 and M_4 receptor subtypes, MT-7 (0.3–3.0 nM) inhibited the carbachol (CCh)-stimulated inositol phosphates accumulation, but failed to affect the CCh-induced inhibition of pituitary adenylate cyclase activating polypeptide (PACAP) 38-stimulated cyclic AMP accumulation.

4 In both CHO/M_1 and N1E-115 cells the MT-7 inhibition consisted in a decrease of the maximal agonist effect with minimal changes in the agonist EC_{50} value.

5 In CHO/M_1 cell membranes, MT-7 (0.05–25 nM) reduced the specific binding of 0.05, 1.0 and 15 nM [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) in a concentration-dependent manner, but failed to cause a complete displacement of the radioligand. Moreover, MT-7 (3 nM) decreased the dissociation rate of [^{3}H]-NMS by about 5 fold.

6 CHO/M_1 cell membranes preincubated with MT-7 (10 nM) and washed by centrifugation and resuspension did not recover control [^{3}H]-NMS binding for at least 8 h at 30°C.

7 It is concluded that MT-7 acts as a selective noncompetitive antagonist of the muscarinic M_1 receptors by binding stably to an allosteric site.

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Keywords: *Dendroaspis angusticeps* toxin; muscarinic receptor subtypes; [^{35}S]-GTPγS binding; phosphoinositide hydrolysis; cyclic AMP accumulation; [^{3}H]-NMS binding; Chinese hamster ovary cells; N1E-115 cells; noncompetitive antagonism

Abbreviations: ACh, acetylcholine; BSA, bovine serum albumin; CCh, carbachol; CHO, Chinese hamster ovary; CHO/M_1-M_4 cells, CHO cells stably expressing the cloned human M_1-M_4 receptors; GTPγS, guanosine-5′-O-(3-thio)triphosphate; IPs, inositol phosphates; KIU, kallikrein inhibitor unit; MT-7, muscarinic toxin 7; NMS, N-methylscopolamine; PACAP, pituitary adenylate cyclase activating polypeptide; PBS, phosphate buffered saline

Introduction

Muscarinic toxins are small peptides of 64–66 amino acids isolated from mamba snake venoms on the basis of their ability to bind to acetylcholine (ACh) muscarinic receptors (Adem *et al*., 1988; Karlsson *et al*., 1994; Jerusalinsky & Harvey, 1994; Adem & Karlsson, 1997). Nine muscarinic toxins (termed MT-1 to MT-7, ml-toxin and m2-toxin) have been isolated from the venom of the Eastern green mamba *Dendroaspis angusticeps* and three (termed MT-α, β and γ) from the venom of the black mamba *Dendroaspis polylepsis* (Adem & Karlsson, 1997; Carsi *et al*., 1999). Although the toxins generally display a high sequence homology and likely possess a similar 'three-finger' structure (Segalas *et al*., 1995), they show notable differences in their selectivity for the distinct muscarinic receptor subtypes. For instance, muscarinic toxin 1 (MT-1), 4 (MT-4) and 5 (MT-5) bind with high affinity to both muscarinic M_1 and M_4 receptor subtypes but display low affinity for the M_2, M_3 and M_5 subtypes (Adem & Karlsson, 1997). Muscarinic toxin 3 (MT-3) (Karlsson *et al*., 1994) shows high affinity for the M_4, a low affinity for the M_1 and a very low affinity for the M_2, M_3 and M_5 receptor subtypes (Jolkkonen *et al*., 1994; Olianas *et al*., 1999), whereas m1-toxin binds with high affinity to the M_1 and with a lower affinity to the M_4 subtype (Max *et al*., 1993a; Potter *et al*., 1993). The recently isolated m2-toxin fully blocks radioligand binding to M_2 receptors, has no effect on M_4 receptors, and slightly increases radioligand binding to M_1 receptors (Carsi *et al*., 1999). Important differences are also found in the functional activities of the muscarinic toxins. Thus, in an inhibitory learning task in rats MT-2 showed muscarinic agonist-like action (Jerusalinsky *et al*., 1993), and in different peripheral tissues MT-1 and MT-2 acted as selective agonists at M_1 receptors (Jerusalinsky & Harvey, 1994). Conversely, MT-3 was found to behave as a competitive and reversible antagonist at the cloned and native M_4 receptors (Olianas *et al*., 1996; 1999) whereas m1-toxin was reported to act as a pseudo-irreversible allosteric antagonist of the M_1 receptor (Max *et al*., 1993b).

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MT-7 is a 65 amino acid peptide toxin which has been identified as the first high-affinity and selective ligand for M_1 receptors (Jolkkonen, 1996; Adem & Karlsson, 1997). In radioligand binding studies employing the human cloned muscarinic receptor subtypes expressed in Chinese hamster ovary (CHO) cells, MT-7 bound to the M_1 subtype with a potency in the low nanomolar range and did not bind to the other receptor subtypes at micromolar concentrations (Adem & Karlsson, 1997).

To further characterize the pharmacological profile of MT-7, in the present study we have investigated the activity of the toxin in functional assays of the cloned human M_1-M_4 receptors expressed in CHO cells and of the native M_1 and M_4 receptors present in murine N1E-115 neuroblastoma cells. In addition, we have examined the nature of the toxin interaction with the M_1 receptor by examining the toxin effects on [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) binding. Part of this work has previously been presented in an abstract form (Onali *et al*., 1999).

Methods

CHO cell culture and membrane preparation

CHO cells stably expressing the cloned human M_1-M_4 receptors (CHO/M_1-M_4 cells) were kindly provided by Professor 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. Cells were grown to ~80 % confluency in plastic Petri dishes (Falcon), the medium was removed, and the cells were washed with 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 by the use of an Ultra-Turrax homogenizer. The cell lysate was centrifuged at $32{,}500 \times g$ for 30 min at 4°C and the pellet was resuspended in the same buffer at a protein concentration of ~3 mg ml^{-1}. The membrane preparations were either used immediately or stored at −75°C.

Assay of guanosine -5′-O-(3-[^{35}S]-thio)triphosphate ([^{35}S]-GTPγS) binding

CHO cell membranes were diluted 10 fold in an ice-cold buffer containing HEPES/NaOH (10 mM), EDTA (1 mM) (pH 7.4), centrifuged and resuspended in the same buffer supplemented with 0.1% bovine serum albumin (BSA). The binding of [^{35}S]-GTPγS was assayed in a reaction mixture (final volume, 100 μl) containing (mM) HEPES/NaOH 25 (pH 7.4), $MgCl_2$ 10, EDTA 1, GDP (0.1 μM) for M_1 and M_3 and GDP (1 μM) for M_2 and M_4 receptor activities (Lazareno *et al*., 1993), NaCl 100, 10 kallikrein inhibitor units (KIU) of aprotinin. The membranes (1.5–2.0 μg of protein) were preincubated in the presence of the indicated concentrations of ACh and MT-7 at 30°C for 30 min. The samples were then placed on ice and the reaction was started by the addition of 10 μl of [^{35}S]-GTPγS (final concentration, 1.0–1.5 nM). The samples were incubated for 45 min at 30°C. The reaction was stopped by the addition of 5 ml of ice-cold buffer containing HEPES/NaOH (10 mM) (pH 7.4) and $MgCl_2$ (1 mM), immediately followed by rapid filtration through glass fibre filters (Whatman GF/C) pre-soaked in the same buffer. The filters were washed twice with 5 ml of buffer and the radioactivity trapped was determined by liquid scintillation spectrometry. Nonspecific binding was determined in the presence of 100 μM GTPγS. Assays were performed in duplicate.

Assay of [^{3}H]-NMS binding

The binding of [^{3}H]-NMS to CHO/M_1 cell membranes was assayed in a buffer containing sodium phosphate (25 mM) (pH 7.4), $MgCl_2$ (5 mM), 0.1% BSA, and 10–12 μg of membrane protein. The final assay volume was 1.0 ml. In competition experiments, the MT-7 concentration ranged from 50 pM to 30 nM and the [^{3}H]-NMS concentrations were 0.05, 3.0 and 15 nM. The incubation was carried out at 30°C for 90 min. When the rate of dissociation of [^{3}H]-NMS was studied, the membranes were incubated with 1.0 nM [^{3}H]-NMS for 60 min before the addition of either vehicle or MT-7 (3 nM). After 20 min, atropine (10 μM) was added to each sample and the incubation was stopped at different time intervals after the atropine addition over a total period of 60 min. To investigate the stability of MT-7 binding to M_1 receptors, CHO/M_1 cell membranes were preincubated with either vehicle or MT-7 (100 nM) for 45 min at 30°C. Thereafter, the samples were centrifuged at $32{,}500 \times g$ for 20 min at 4°C and resuspended in fresh buffer. Aliquots of the membrane suspension were incubated in the presence of 1.5 nM [^{3}H]-NMS for the indicated times over a total period of 8 h. The incubation was stopped by adding 4 ml of ice-cold buffer without BSA to each sample followed by immediate filtration through glass fibre filters presoaked in 0.1% polyethylenimine for at least 18 h. 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. Assays were performed in triplicate.

N1E-115 neuroblastoma cell culture

Cells were obtained from European Collection of Cell Cultures (U.K.). The cells were grown in Dulbecco's modified Eagle's medium containing 2 mM glutamine and 10% foetal calf serum in 75-cm^2 flasks (Falcon). The medium (20–30 ml) was changed on day 2 of subculture and every subsequent day. Confluent cell cultures (6–8 days postpassage) were used for the experiments.

Assay of [^{3}H]-inositol phosphates ([^{3}H]-IPs) accumulation

N1E-115 cells were prelabelled with myo-[^{3}H]-inositol (1 μCi ml^{-1}) in Dulbecco's modified Eagle medium for 24 h at 37°C in an incubator. The medium was then removed and the cells were washed twice with PBS. The cells were detached from the tissue culture flask by incubation in PBS containing EDTA (0.5 mM) for 5 min at 37°C followed by gentle agitation of the flask. The cell suspension was aspirated, mixed with an equal volume of PBS containing $MgCl_2$ (1 mM) and centrifuged at $300 \times g$ for 1 min. The cells were resuspended in a freshly oxygenated Krebs-HEPES buffer containing (mM) HEPES/NaOH 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 10, NaCl 110, KCl 3.8, $CaCl_2$ 1.2 and LiCl 10. Aliquots of the cell suspension were distributed into Bio-vials (Beckman, Ireland) and incubated for 30 min at 37°C in the presence and in the absence of MT-7. Thereafter, carbachol (CCh) was added as indicated and the incubation was continued for 45 min. The final incubation volume was 300 μl. The incubation was terminated by adding 940 μl of chloroform-methanol

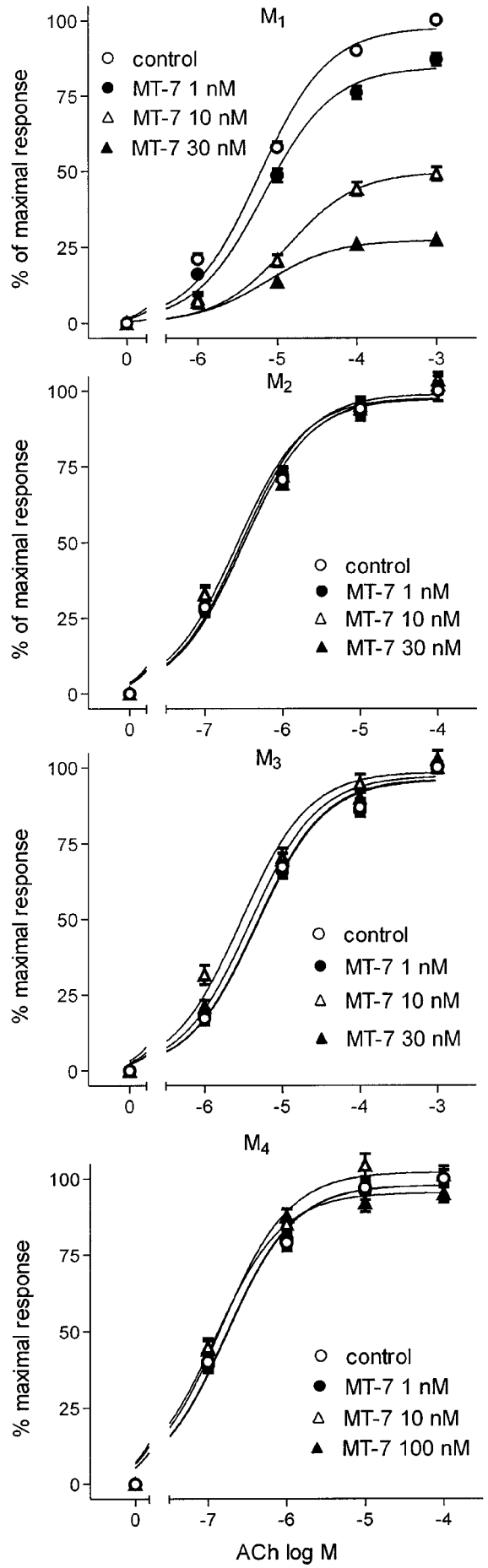

Figure 1 Effects of MT-7 on ACh stimulation of [^{35}S]-GTPγS binding to membranes of CHO cells expressing the cloned human M_1 to M_4 receptors. The [^{35}S]-GTPγS binding stimulated by ACh was measured in the absence (control) and in the presence of the indicated concentrations of MT-7. Data are the mean$\pm$s.e.mean of three experiments.

(1 : 2 v v^{-1}). After the samples were shaken for 10 min, 310 μl aliquots of chloroform and 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 60 mM sodium formate. [^{3}H]-IPs 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]-IPs was corrected for the amount of myo-[^{3}H]-inositol incorporated in the organic phase. Assays were performed in triplicate.

Assay of [^{3}H]-cyclic AMP accumulation

N1E-115 cells grown in 36-mm plastic dishes were incubated in Dulbecco's modified Eagle medium containing 2 μCi ml^{-1} of [^{3}H]-adenine for 1 h at 37°C in an incubator. Thereafter, the medium was removed and the cells were incubated in an oxygenated Krebs-HEPES buffer containing 3-isobutyl-1-methylxanthine (1 mM) in the absence and in the presence of MT-7 for 30 min at 37°C. Pituitary adenylate cyclase activating polypeptide (PACAP) 38 and CCh were then added as indicated and the incubation was continued for 10 min. The incubation was stopped by the aspiration of the medium and the addition of an ice-cold solution containing 6% (w v^{-1}) perchloric acid and 0.1 mM [^{14}C]-cyclic AMP ($\sim$3000 c.p.m.). After 30 min at ice-bath temperature, the solution was neutralized by the addition of ice-cold 0.6 M KOH and left on ice for additional 30 min. Following centrifugation at 15,000 $\times$ *g* for 5 min, the supernatant was collected, and [^{3}H]-cyclic AMP was isolated according to Salomon (1979). The recovery of [^{3}H]-cyclic AMP from each sample was corrected on the basis of the recovery of [^{14}C]-cyclic AMP. Assays were performed in triplicate.

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

Statistical analysis

Results are given as mean$\pm$standard error of the mean (s.e.mean). Concentration–response curves were analysed by a least squares curve-fitting computer programme (GraphPAD Prism, San Diego, CA, U.S.A.). Statistical significance of the difference between means was determined by Student's *t*-test.

Materials

[^{35}S]-GTPγS (1306 Ci mmol^{-1}), [2,8-^{3}H]-adenine (28.8 Ci mmol^{-1}) and [8-^{14}C]-cyclic AMP (45.1 mCi mmol^{-1}) were obtained from New England Nuclear (Bad Homburg, Germany). [^{3}H]-NMS (83 Ci mmol^{-1}) and myo-[^{3}H]-inositol with PT6-271 stabilizer (99 Ci mmol^{-1}) were purchased from Amersham (U.K.). GTPγS was from Calbiochem (La Jolla, CA, U.S.A.). PACAP 38 was from Peninsula Laboratories (Merseyside, U.K.). MT-7 was purified from the venom of *Dendroaspis angusticeps* by gel filtration followed by ion-exchange and reverse phase-high performance liquid cromatographies (Adem & Karlsson, unpublished; Vandermeers *et al.*,

1995). ACh chloride, CCh chloride, atropine sulphate and the other reagents used were from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Results

Effects of MT-7 on [^{35}S]-GTPγS binding to CHO/M_1-M_4 cell membranes

In membranes of CHO/M_1 cells, MT-7 caused a concentration-dependent reduction of the maximal stimulation of [^{35}S]-GTPγS binding elicited by ACh (Figure 1). At 1.0, 10 and 30 nM, MT-7 inhibited the maximum of agonist response by 15.7±1.5 ($P<0.01$), 50.5±3.1 and 72.8±1.7% ($P<0.001$), respectively. The EC_{50} values of ACh were 6.0±0.8, 6.8±0.5, 13.0±1.2 and 7.3±1.0 μM in the absence and in the presence of 1.0, 10 and 30 nM MT-7, respectively. Conversely, in membranes of CHO/M_2, M_3 and M_4 cells MT-7 failed to affect the ACh- induced stimulation of [^{35}S]-GTPγS binding (Figure 1).

Effect of MT-7 on CCh-stimulated [^{3}H]-IPs accumulation in N1E-115 cells

In N1E-115 mouse neuroblastoma cells, CCh increased [^{3}H]-IPs accumulation by approximately 5 fold with an EC_{50} value of 33.8±4.0 μM (Figure 2A). The addition of MT-7 at 0.3, 1.0 and 3.0 nM progressively reduced the maximal agonist stimulation by 35.1±3.5, 69.3±2.1 and 84.9±1.3% ($P<0.001$), respectively. The agonist EC_{50} values were 54.0±6.0, 100±9.0 and 89.9±7.0 μM at 0.3, 1.0 and 3.0 nM MT-7, respectively.

Effect of MT-7 on CCh inhibition of [^{3}H]-cyclic AMP accumulation in N1E-115 cells

CCh caused a concentration-dependent inhibition of PACAP 38 (10 nM)-stimulated [^{3}H]-cyclic AMP accumulation with a maximal effect corresponding to a 35.5±2.1% ($n=5$, $P<0.001$) reduction and an EC_{50} value of 5.6±0.8 μM. The addition of MT-7 (5 nM) failed to affect the CCh inhibitory effect (Figure 2B).

Effect of MT-7 on [^{3}H]-NMS binding to CHO/M_1 cell membranes

In competition experiments, MT-7 caused a concentration-dependent reduction of specific [^{3}H]-NMS binding at different radioligand concentrations (Figure 3). However, the toxin consistently failed to cause a complete inhibition of [^{3}H]-NMS binding. This behaviour was particularly evident at 3 and 15 nM [^{3}H]-NMS, where the maximal inhibitions elicited by 25 nM MT-7 corresponded to ~93 and 82% of total specific binding. The toxin IC_{50} values were 0.26±0.02, 0.27±0.03 and 0.48±0.05 nM at 0.05, 3 and 15 nM [^{3}H]-NMS, respectively.

In dissociation experiments, MT-7 (3 nM) markedly decreased the rate of atropine-induced dissociation of [^{3}H]-NMS from M_1 receptors (Figure 4). The estimated dissociation rate constants were 0.05 and 0.01 min^{-1} in control and toxin-treated membranes, respectively.

To investigate the reversibility of MT-7 binding, CHO/M_1 cell membranes were pretreated with either vehicle or MT-7 (100 nM), washed by centrifugation and resuspension and assayed for [^{3}H]-NMS binding. As shown in Figure 5, in membranes pretreated with MT-7 there was a reduction in radioligand binding, which remained constant for at least 8 h.

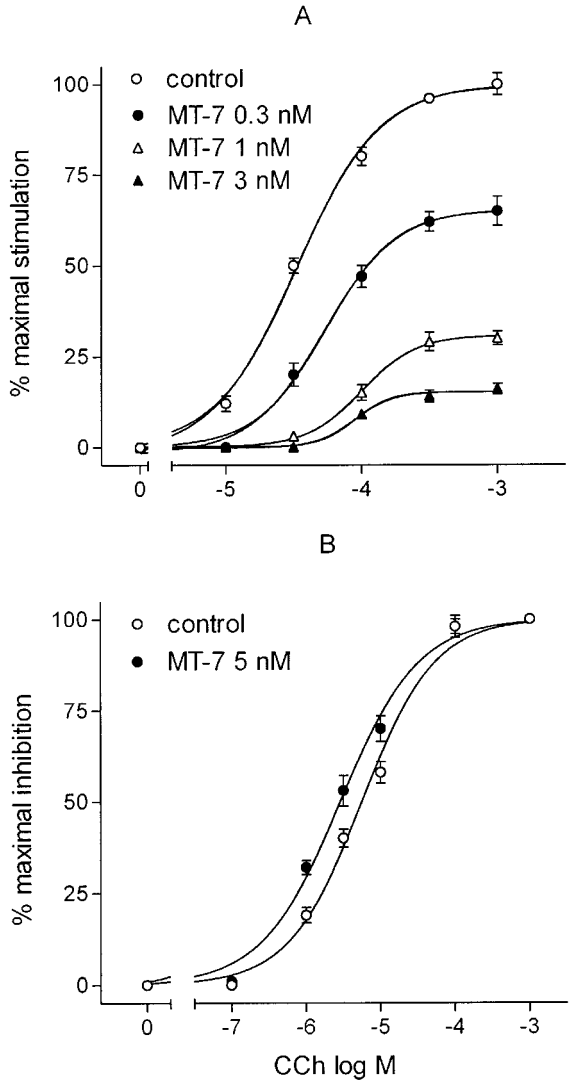

Figure 2 Effects of MT-7 on CCh-stimulated [^{3}H]-IPs accumulation (A) and CCh-induced inhibition of PACAP38-stimulated cyclic AMP accumulation (B) in N1E-115 neuroblastoma cells. Cells were pretreated with the indicated concentrations of MT-7 for 30 min at 37°C and then exposed to the indicated concentrations of CCh. In (B), PACAP 38 was also added at the final concentrations of 10 nM. Data are the mean±s.e.mean of three experiments for both assays.

Discussion

MT-7 has recently been reported as a selective ligand for the muscarinic M_1 receptor (Adem & Karlsson, 1997). In the present study, we have characterized the pharmacological activity of the toxin at different muscarinic receptor subtypes by using either CHO cells expressing the cloned human M_1–M_4 receptors or mouse N1E-115 neuroblastoma cells containing the native M_1 and M_4 receptor subtypes. In CHO/M_1–M_4 cells, we found that, at nanomolar concentrations MT-7 significantly inhibited the stimulation of [^{35}S]-GTPγS binding elicited by ACh at M_1 receptors but did not affect the response to the agonist when M_2, M_3 and M_4 receptors were stimulated. At each receptor subtype, the toxin failed to stimulate basal [^{35}S]-GTPγS binding, indicating a lack of agonist activity. Similarly, in N1E-115 neuroblastoma cells, MT-7 potently inhibited the CCh stimulation of [^{3}H]-IPs accumulation, a response previously proposed to be mediated by endogenous M_1 receptors (Kamba *et al*., 1990). In these cells, the toxin did not affect the CCh inhibition of PACAP 38-stimulated cyclic

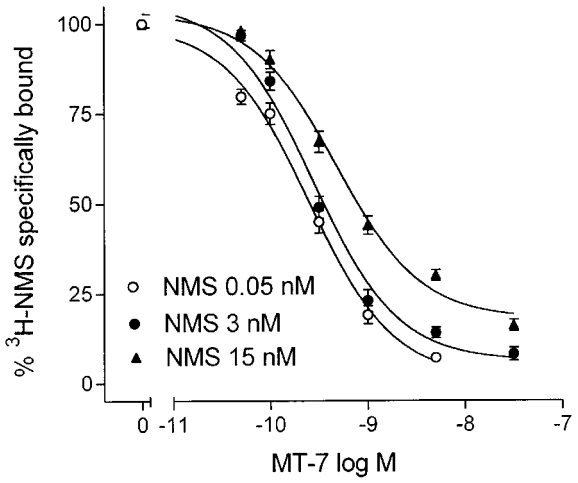

Figure 3 Inhibition of specific [^{3}H]-NMS binding to CHO/M_1 cell membranes by MT-7. Binding assays were performed at three concentrations of the radioligand in the presence of the indicated concentrations of MT-7. Data are expressed as per cent binding in the absence of MT-7 and are the mean ± s.e.mean of three experiments.

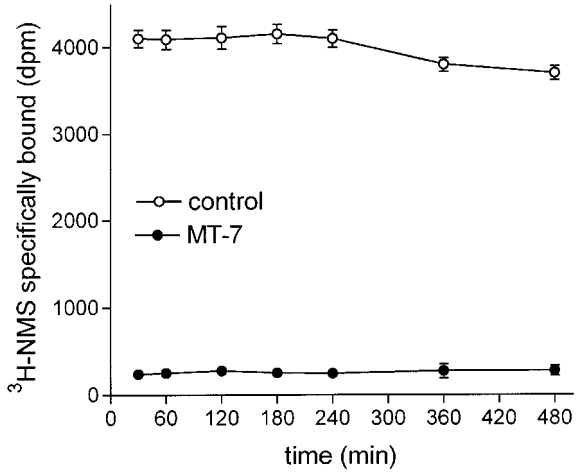

Figure 5 Stable inhibition of [^{3}H]-NMS binding to M_1 receptors by MT-7. CHO/M_1 cell membranes were pretreated with either vehicle (control) or 100 nM MT-7, centrifuged and resuspended in fresh buffer. Aliquots of the membrane suspension were incubated in the presence of 1.5 nM [^{3}H]-NMS at 30°C for the time periods indicated in abscissa. Data are the mean ± s.e.mean of three experiments.

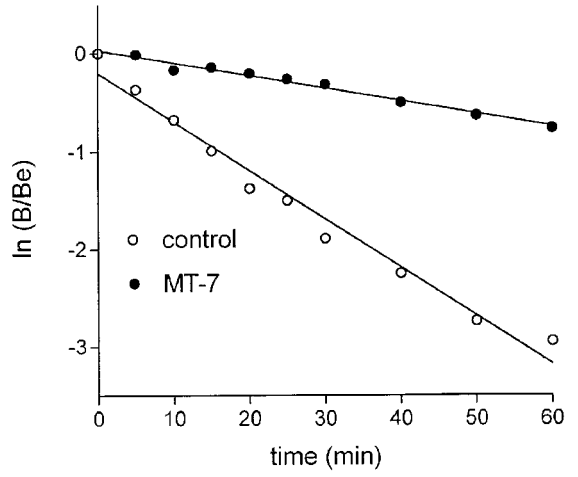

Figure 4 Effects of MT-7 on atropine-induced [^{3}H]-NMS dissociation from M_1 receptors. CHO/M_1 cell membranes were incubated at 30°C first with 1 nM [^{3}H]-NMS to equilibrium for 60 min and then with either vehicle (control) or 3 nM MT-7 for 20 min. The dissociation [^{3}H]-NMS was started by the addition of 10 μM atropine and the incubation was stopped at the indicated time points. [^{3}H]-NMS binding data are expressed as ln B/Be, where B is the amount of [^{3}H]-NMS specifically bound at the indicated time and Be is the specific binding determined at zero time. Lines represent the least-squares linear regressions of the data. Values are the mean of three experiments.

AMP accumulation, an effect mediated by M_4 receptors (McKinney *et al*., 1991; Olianas *et al*., 1999). Collectively, these functional data indicate that MT-7 is an antagonist of the M_1 receptor and confirm the subtype selectivity previously observed in radioligand binding studies (Adem & Karlsson, 1997).

In both CHO/M_1 and N1E-115 cells the inhibitory effect of MT-7 consisted in a depression of the maximal agonist effect with minor changes in the agonist potency. This type of inhibition indicated that the toxin behaved as a noncompetitive antagonist. To further characterize the mode of toxin action on M_1 receptors, radioligand binding studies were conducted in CHO/M_1 cell membranes.

In competition experiments with [^{3}H]-NMS, it was found that the toxin was not able to inhibit completely the binding of the radioligand and the fraction of [^{3}H]-NMS not displaced by the toxin increased with increasing concentrations of the radioligand. In addition, the IC_{50} values of the toxin were little affected by large increases in the concentration of the radiolabelled ligand. These data are inconsistent with an action of the toxin as a competitive antagonist but rather indicate that it behaves as an allosteric antagonist which binds to a secondary allosteric site to decrease the affinity of the radioligand in a negative cooperative manner.

An important feature of allosteric ligands of muscarinic receptors is the ability to decelerate the dissociation of [^{3}H]-NMS from the receptors (Nedoma *et al*., 1986; Waelbroeck, 1994; Lazareno & Birdsall, 1995). When this property was investigated, it was found that MT-7 markedly decreased the atropine-induced dissociation of [^{3}H]-NMS from the muscarinic M_1 receptor. At 3 nM, MT-7 decreased the [^{3}H]-NMS dissociation rate by about 5 fold. This finding indicates that MT-7 is capable of binding to the M_1 receptor also when the primary binding site is occupied by a competitive ligand and, by acting on the secondary allosteric site, can sterically regulate the accessibility of the primary binding site to competitive ligands.

Radioligand binding experiments also showed that MT-7 binds stably to the muscarinic M_1 receptor. The binding withstood washing and resuspension of the membranes and was not reversible for at least 8 h at 30°C, as judged by the lack of recovery of [^{3}H]-NMS binding to control values. A stable binding to muscarinic receptors has previously been observed for different muscarinic toxins. Thus, a large fraction of the binding of MT-1 and MT-2 to the cloned M_1 receptor and to brain muscarinic receptors was found to be irreversible (Jerusalinsky & Harvey, 1994), whereas m1-toxin has been reported to bind pseudo-irreversibly to the M_1 and reversibly to the M_4 receptor (Max *et al*., 1993a). On the other hand, the blockade of the M_4 receptor elicited by MT-3 appeared to be completely reversible (Olianas *et al*., 1996).

Several properties of MT-7 closely resemble those of m-1 toxin (Potter *et al*., 1993). Both toxins show high affinity for the m1 receptor subtype, act as allosteric modulators and bind tightly to the receptor. The two toxins display a high sequence

homology, as they only differ by the fact that in position 28–29 MT-7 has a dipeptide Trp-Gln whereas m-1 toxin has His-Trp and lacks Lys in position 65 (Adem & Karlsson, 1997). Despite these similarities, MT-7 shows a higher selectivity for the M_1 receptor than m-1 toxin. In fact, m-1 toxin also binds to the M_4 receptor subtype at 5–65 fold higher concentrations (Max *et al*., 1993a), whereas MT-7 failed to affect the M_4 receptor-induced [^{35}S]-GTPγS binding at a concentration 100 fold higher than that significantly blocking the M_1 receptor activity.

In conclusion, the present study shows that MT-7 behaves as a selective and noncompetitive antagonist of the cloned and native muscarinic M_1 receptor subtype. These properties make MT-7 an unmatched tool for the identification and characterization of M_1 receptors in different biological systems.

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