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# Rat striatal muscarinic receptors coupled to the inhibition of adenylyl cyclase activity: potent block by the selective $m_4$ ligand muscarinic toxin 3 (MT3)

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**1** In rat striatal membranes, muscarinic toxin 3 (MT3), a selective ligand of the cloned m4 receptor subtype, antagonized the acetylcholine (ACh) inhibition of forskolin-and dopamine $D_1$ receptor-stimulated adenylyl cyclase activities with $pA_2$ values of 8.09 and 8.15, respectively.

**2** In radioligand binding experiments, MT3 increased the $K_d$ but did not change the $B_{max}$ value of [$^3$H]-N-methylscopolamine ([$^3$H]-NMS) binding to rat striatal muscarinic receptors. The toxin displaced the major portion of the [$^3$H]-NMS binding sites with a $K_i$ of 8.0 nM.

**3** In rat myocardium, MT3 antagonized the ACh inhibition of adenylyl cyclase with a $K_i$ value of 860 nM.

**4** In rat cerebral cortical membranes prelabelled with [$^3$H]-*myo*-inositol, MT3 counteracted the methacholine stimulation of [$^3$H]-inositol phosphates formation with a $K_i$ value of 113 nM.

**5** The present study shows that MT3 is a potent antagonist of the striatal muscarinic receptors coupled to inhibition of adenylyl cyclase activity. This finding provides strong evidence for the classification of these receptors as pharmacologically equivalent to the m4 gene product ($M_4$). On the other hand, the weaker potencies of MT3 in antagonizing the muscarinic responses in cerebral cortex and in the heart are consistent with the reported lower affinities of the toxin for the cloned m1 and m2 receptor subtypes, respectively.

**Keywords:** *Dendroaspis angusticeps* toxin; muscarinic receptor subtypes; adenylyl cyclase; phosphoinositide hydrolysis; [$^3$H]-N-methylscopolamine binding; rat striatum; rat heart; rat cortex

## Introduction

In rat striatum, activation of muscarinic cholinoceptors inhibits adenylyl cyclase activity (Olianas *et al.*, 1983a, b). A number of studies have investigated the pharmacological properties of this response to identify the muscarinic receptor subtype(s) involved. Gil & Wolfe (1985) initially found that the $M_1$ antagonist, pirenzepine, antagonized the muscarinic inhibition of striatal adenylyl cyclase with a lower potency than the stimulation of phosphoinositide hydrolysis, suggesting that the inhibitory response was mediated by the $M_2$ receptor subtype. Keen & Nahorski (1988) noted that the striatal and cardiac muscarinic receptor linked to inhibition of adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation could be distinguished on the basis of agonist efficacy. The development of a series of subtype selective drugs and the identification in the brain of distinct molecular forms of the muscarinic receptor allowed a further characterization of the striatal adenylyl cyclase inhibiting muscarinic receptors. Thus Ehlert *et al.* (1989) and McKinney *et al.* (1989) found that these receptors displayed a sensitivity to $M_1$, $M_2$ and $M_3$ selective antagonists different from that of cardiac $M_2$ receptors. Also on the basis of the knowledge that the mRNA for the m4 receptor gene is abundantly expressed in rat striatum (Brann *et al.*, 1988; Buckley *et al.*, 1988) and that the m4 gene product is linked to inhibition of cyclic AMP formation (Peralta *et al.*, 1988), these investigators postulated that the receptor involved was neither $M_1$ nor $M_2$, but probably $M_4$ (the receptor subtype pharmacologically equivalent to the m4 gene product cf. Lazareno *et al.* 1990). The possibility that the $M_4$ subtype mediates the muscarinic inhibition of striatal adenylyl cyclase was also supported by the studies of Olianas & Onali (1991) and Onali *et al.* (1994). However, the lack of selective $M_4$ antagonists and the presence of multiple muscarinic receptor subtypes in the striatum (Waelbroeck *et al.*, 1990; Hersch *et al.*, 1994) made this conclusion largely hypothetical.

Very recently, the isolation of a new peptide toxin from green mamba venom, named muscarinic toxin 3 (MT3), was reported (Karlsson *et al.*, 1994). MT3 displayed high selectivity for the cloned m4 receptor. Specifically, in radioligand binding studies using CHO cells separately expressing the five cloned muscarinic receptors, the toxin showed a high affinity for the m4 ($pK_i = 8.70$), a lower affinity for the m1 ($pK_i = 7.11$) and a very low affinity for the m2, m3 or m5 subtype ($pK_i$ values $> 6$) (Jolkkonen *et al.*, 1994). The ability of the toxin to discriminate the m4 from the other receptor subtypes is much higher than that displayed by compounds, such as himbacine, methoctramine and tropicamide, used to characterize pharmacologically the $M_4$ receptors in tissues and cell lines (Lazareno *et al.*, 1990). Thus, MT3 appears to be a useful tool to characterize putative $M_4$ receptor-mediated responses.

In the present study we show that in rat striatum, MT3 binds with high affinity to a high percentage of muscarinic receptors and behaves as a potent antagonist of the muscarinic inhibition of both forskolin- and dopamine $D_1$ receptor-stimulated adenylyl cyclase activities. On the other hand, MT3 is much less potent in antagonizing the muscarinic stimulation of phosphoinositide hydrolysis in cerebral cortex and the muscarinic inhibition of adenylyl cyclase in myocardium.

## Methods

Male Sprague-Dawley rats (Charles River, Italy) weighing 150–200 g were used. The animals were killed by decapitation and the organs rapidly dissected and used immediately for the biochemical assays.

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*Adenylyl cyclase assay*

Striata were homogenized in 10 volumes of an ice-cold isotonic buffer containing 0.32 M sucrose, 10 mM HEPES/NaOH, 1 mM EGTA and 1 mM $MgCl_2$ (pH 7.4). The homogenate was diluted twofold and centrifuged at 1,000 *g* for 10 min at 4°C. The supernatant was aspirated and centrifuged at 11,000 *g* for 20 min at 4°C. The pellet (P2 fraction) was resuspended by repeated aspiration through a plastic pipette in 50 volumes of ice-cold hypotonic buffer containing all the constituents of the homogenization buffer except sucrose. For measurements of dopamine $D_1$ receptor-stimulated adenylyl cyclase activity, this tissue preparation was incubated for 20 min in the hypotonic buffer at ice-bath temperature to favour synaptosomal lysis, and was then used for the enzyme assay. When forskolin-stimulated enzyme activity was measured, the P2 fraction lysed in the hypotonic buffer was centrifuged at 27,000 *g* for 20 min at 4°C and the final pellet was resuspended in the same buffer. For both tissue preparations the final protein concentration was 1.0–1.3 mg $ml^{-1}$. Hearts were dissected and homogenized as previously described (Olianas & Onali, 1991) with the exception that dithiothreitol was omitted from the homogenization buffer.

Acetylcholine (ACh) inhibition of forskolin-stimulated adenylyl cyclase activity was determined as previously described (Onali *et al.*, 1994). The reaction mixture (final volume: 100 μl) contained: HEPES/NaOH 50 mM (pH 7.4), $MgCl_2$ 2.3 mM, EGTA 0.3 mM, [α-$^{32}$P] adenosine-5'-triphosphate (ATP) 0.05 mM (50–70 c.p.m. $pmol^{-1}$), [$^3$H]-cyclic AMP 1 mM (80 c.p.m. $nmol^{-1}$), GTP 100 μM, 3-isobutyl-1-methylxanthine 1 mM, phosphocreatine 5 mM, creatine kinase 50 u $ml^{-1}$, bovine serum albumin 50 μg, bacitracin 10 μg, aprotinin 10 kallikrein inhibitor units (KIU) and physostigmine 10 μM. On occasion, forskolin (10 μM) was added. When the ACh inhibition of dopamine $D_1$ receptor-stimulated enzyme activity was assayed, the selective $D_1$ receptor agonist, (±)-chloro-APB HBr was used at the maximally effective concentration of 1 μM. The concentrations of [α-$^{32}$P]-ATP, GTP and $MgCl_2$ were 0.1, 0.01 and 0.8 mM, respectively. The incubation was started by adding the tissue preparation (30–40 μg of protein) and was carried out at 30°C for 10 min. [$^{32}$P]-cyclic AMP was isolated according to Salomon *et al.* (1974). Assays were performed in duplicate.

For investigation of reversibility of MT3 antagonism, the lysed P2 fraction of rat striatum was resuspended in 10 mM HEPES/NaOH (pH 7.4) buffer containing EGTA 1 mM, $MgCl_2$ 1 mM, bacitracin 100 μg $ml^{-1}$ and aprotinin 100 KIU $ml^{-1}$ preincubated with and without (control) MT3 150 nM for 10 min at 30°C. The samples were then diluted 10 fold and centrifuged at 32,500 *g* for 20 min at 4°C. The supernatants were carefully removed and the pellets were resuspended in the original volume of buffer and centrifuged as above. The final pellets were resuspended to a tissue protein concentration of 1 mg $ml^{-1}$ and tested for the ACh inhibition of adenylyl cyclase activity.

*Assay of [$^3$H]-inositol phosphates ([$^3$H]-IPs) formation in cerebral cortical membranes*

The assay was carried out essentially as described by Claro *et al.* (1992) in membranes prelabelled with [$^3$H]-*myo*-inositol. Cerebral cortex was homogenized in 10 volumes (w/v) of an ice cold buffer containing Tris/HCl 20 mM (pH 7.0) and EGTA 1 mM in a motor driven tissue grinder. The homogenate was diluted twofold, centrifuged at 32,500 *g* for 20 min at 4°C and the pellet resuspended in the original volume of homogenization buffer. This procedure was repeated twice and the final tissue pellet was stored at −70°C for no more than 2 weeks. On the day of the experiment, the tissue pellet was resuspended in ice-cold 20 mM Tris/HCl (pH 7.0) buffer containing EGTA 1 mM, $MgCl_2$ 6 mM, cytidine monophosphate 1 mM and [$^3$H]-*myo*-inositol 0.1 μM. The tissue was incubated for 45 min at 37°C. The labelling of the membranes was stopped by 5 fold dilution with ice-cold buffer and centrifugation at 32,500 *g* for 20 min at 4°C. The supernatant was removed and the pellet was resuspended in 25 mM Tris/maleate buffer (pH 6.8) containing EGTA 1 mM. Aliquots of the membrane suspension were incubated in a reaction mixture (final volume: 100 μl) containing Tris/maleate 25 mM (pH 6.8), sodium deoxycholate 1 mM, $MgCl_2$ 6 mM, LiCl 10 mM, ATP 2 mM, EGTA 1 mM, guanosine-5'-(3-O-thio) triphosphate (GTPγS) 1 μM, aprotinin 10 KIU, physostigmine 10 μM and sufficient $CaCl_2$ to yield a free $Ca^{2+}$ concentration of 0.8 μM. The reaction was carried out at 37°C for 15 min. The incubation was stopped by adding 1.2 ml of chloroform/methanol (1 : 2, v/v) with rapid vortexing. Then, 0.5 ml each of 0.25 M HCl and chloroform were added followed by vigorous shaking and centrifugation at 1,000 *g* for 10 min at 4°C. An aliquot (1 ml) of the upper aqueous phase was neutralized with 1.5 M $NH_4OH$, diluted with 4 ml of distilled water and applied to a column of Dowex 1X8 in the formate form. After washing the column with 3 × 4 ml of distilled water, 3 × 4 ml of 5 mM inositol and 2 × 4 ml of 60 mM sodium formate/0.1 M formic acid, [$^3$H]-IPs were eluted with 3 ml of 1 M ammonium formate/0.1 M formic acid. The radioactivity present in the eluate and in the organic phase was determined by liquid scintillation counting in Aquasol 2 (Du Pont NEN) as scintillant. In the presence of 1 μM GTPγS, the percentage of the total radioactivity incorporated that was converted to [$^3$H]-IPs (percentage of conversion) was 3.12 ± 0.4 ($n = 6$). In the absence of GTPγS, no significant stimulation of [$^3$H]-IPs formation was observed. In samples prelabelled with [$^3$H]-inositol but extracted with chloroform/methanol before the incubation at 37°C, the percentage of conversion was routinely 10 fold lower than the basal value. Assays were performed in triplicate. The concentration of free $Ca^{2+}$ in the reaction mixture was calculated with the computer programme EQCAL (Biosoft), using the stability constants for metal-chelate and metal-nucleotide complexes reported by Martell & Smith (1975).

*Assay of [$^3$H]-N-methylscopolamine ([$^3$H]NMS) binding*

The binding of [$^3$H]-NMS to muscarinic receptors was assayed in 20 mM HEPES/NaOH buffer (pH 7.4) containing 4 mM $MgCl_2$ and 0.1% bovine serum albumin using a lysed P2 fraction of rat striatum (25–30 μg of protein). The concentration of [$^3$H]-NMS ranged from 10 pM to 3 nM and the final assay volume was 1 ml. The incubation was carried out at 30°C for 60 min. This incubation time allowed the [$^3$H]-NMS binding to reach a steady state at all the radioligand concentrations used with and without 10 nM MT3. To terminate the incubation 4 ml of ice-cold 10 mM HEPES/NaOH (pH 7.4) buffer containing 1 mM $MgCl_2$ was added to each sample followed by immediate filtration through GF/C glass fibre filters presoaked in 0.05% polyethylenimine. The filters were washed twice with the same buffer, dried and the bound radioactivity was counted by liquid scintillation. Nonspecific binding was determined in the presence of 1 μM atropine and corresponded to less than 3% of the total [$^3$H]-NMS bound. Assays were performed in triplicate and the data were analyzed by the computer programme EBDA. This programme yielded the initial estimates of equilibrium binding parameters by Scatchard, Hill and Eadie-Hofstee analysis. These estimates were used in the non linear curve-fitting computer programme LIGAND (Munson & Rodbard, 1980), which provided the final estimates of the dissociation constant ($K_d$), inhibition constant ($K_i$) and binding capacity ($B_{max}$). The computer programmes were obtained from Biosoft, Cambridge, U.K.

Protein content was determined by the method of Bradford (1976) with bovine serum albumin used as the standard.

*Statistical analysis*

Results are given as mean ± standard error of the mean (s.e.mean). Agonist concentration-response curves were ana-

lyzed by a least squares curve-fitting computer programme (Graph-Pad, ISI, Philadelphia, PA, U.S.A.). The MT3 antagonist effects on ACh inhibition of striatal adenylyl cyclase were examined according to Arunlakshana-Schild analysis (Arunlakshana & Schild, 1959) and the potency was determined from the ratios between the $EC_{50}$ values of the agonist in the absence and in the presence of different concentrations of the toxin. The $pA_2$ values were determined from the x intercepts and calculated by least squares regression analysis of the Schild plots, where the log of the dose ratios (DR) − 1 is plotted as a function of the antagonist concentration. In other experiments where the effect of a single concentration of the toxin was considered, the $K_i$ of the toxin was calculated from the equation:

$$EC_{50b} = EC_{50a}(1 + I/K_i) \quad (1)$$

where $EC_{50a}$ and $EC_{50b}$ are the concentrations of the agonist producing half-maximal effect in the absence and in the presence of the toxin, respectively, and I is the toxin concentration. Statistical significance of the difference between means was determined by Student's $t$ test.

### *Materials*

[$\alpha$-$^{32}$P]-ATP (30–40 Ci mmol$^{-1}$) and [2,8-$^3$H]-cyclic AMP (25 Ci mmol$^{-1}$) were obtained from Du Pont de Nemours (Bad Homburg, Germany). 1-[N-methyl-$^3$H]-scopolamine methyl chloride ([$^3$H-NMS) (83 Ci mmol$^{-1}$) and [$^3$H]-*myo*-inositol (112 Ci mmol$^{-1}$; TRK 912) were purchased from Amersham (U.K.). Forskolin and GTP$\gamma$S were from Calbiochem (La Jolla, CA, U.S.A.), (±)-chloro-APB HBr (SKF 82958 HBr) was from Research Biochemicals International (Natick, MA, U.S.A.). Pirenzepine dihydrochloride was obtained from Dr Karl Thomae GmbH (Biberach an der Riss, Germany). MT3 was purified from the venon of *Dendroaspis angusticeps* according to the procedure of Jolkkonen *et al.* (1994) in the laboratory of A.A.. ACh chloride, metacholine bromide, physostigmine hemisulphate, atropine sulphate and the other reagents used were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

## Results

### *Effect of MT3 on ACh inhibition of forskolin-stimulated adenylyl cyclase activity*

As shown in Figure 1, ACh inhibited forskolin-stimulated adenylyl cyclase activity in a concentration-dependent manner with an $EC_{50}$ value of 1.1±0.3 $\mu$M. Addition of 0.03, 0.1 and 1.0 $\mu$M MT3 shifted the ACh curve by 5.3, 12 and 112 fold, respectively, without affecting the maximal inhibitory effect. Arunlakshana-Schild analysis of the MT3 antagonism yielded a $pA_2$ value of 8.09±0.03 with a slope value of 0.965±0.05 (Figure 1, inset). At the concentrations used, MT3 *per se* failed to affect the forskolin-stimulated enzyme activity (enzyme activities, expressed as nmol cyclic AMP min$^{-1}$ mg$^{-1}$ protein were: control 1.65±0.02; MT3 30 nM 1.66±0.02; MT3 100 nM 1.66±0.01; MT3 1.66±0.02).

The MT3 antagonism was completely reversible as in membranes preincubated with 150 mM MT3 and subsequently washed, ACh inhibited the forskolin-stimulated enzyme activity with a potency and an efficacy similar to those displayed in control membranes (the ACh $EC_{50}$ values were: control membranes, 1.9±0.5 $\mu$M; MT3-treated membranes, 1.6±0.4 $\mu$M, $P>0.05$, $n=3$).

### *Effect of MT3 on ACh inhibition of dopamine $D_1$ receptor-stimulated adenylyl cyclase activity*

Striatal adenylyl cyclase activity stimulated by the dopamine $D_1$ receptor agonist, (±)-chloro ABP HBr (1 $\mu$M) was inhibited by ACh with an $EC_{50}$ value of 0.51±0.08 $\mu$M (Figure 2). MT3, at concentrations ranging from 15 to 500 nM, antagonized the ACh inhibition with a $pA_2$ value of 8.15±0.02. The slope of the Schild plot (Figure 3, inset) was 1.14±0.09. MT3 did not affect the (±)-chloro APB HBr-stimulated enzyme activity (enzyme activities, expressed as pmol cyclic AMP min$^{-1}$ mg$^{-1}$ protein, were: control 125.1±7.1; MT3 15 nM 127.2±5.6; MT3 50 nM 128.7±8.2; MT3 150 nM 127.7±4.9; MT3 500 nM 125.3±6.8).

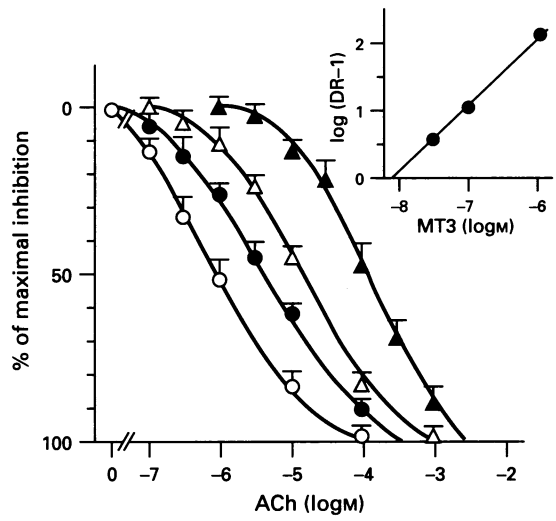


**Figure 1** Antagonism of acetylcholine (ACh)-induced inhibition of forskolin-stimulated adenylyl cyclase activity in rat striatum. The enzyme activity was assayed at the indicated concentrations of ACh in the absence (○) and in the presence of 30 (●), 100 (△) and 1000 (▲) nM MT3. The concentration of forskolin was 10 $\mu$M. Inset: Schild plot of the antagonism. Data are the mean±s.e.mean of three experiments. Enzyme activities (expressed as nmol cyclic AMP min$^{-1}$ mg$^{-1}$ protein±s.e.mean) were: control±1.65±0.02; ACh (100 $\mu$M) 1.20±0.01 ($P<0.01$).

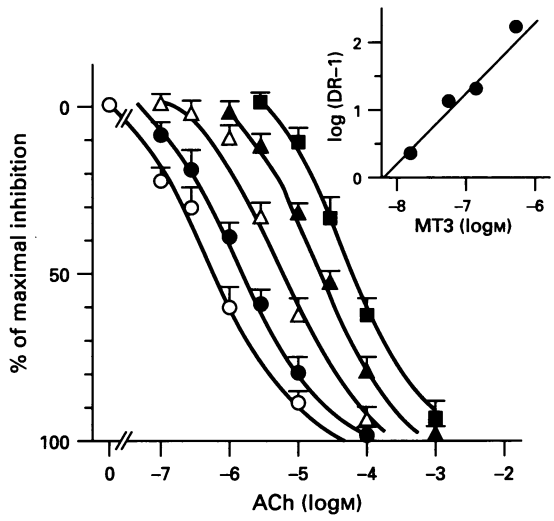


**Figure 2** Antagonism of acetylcholine (ACh)-induced inhibition of dopamine $D_1$ receptor-stimulated adenylyl cyclase activity by MT3. The enzyme activity was assayed at the indicated concentrations of ACh in the absence (○) and in the presence of 15 (●), 50 (△), 150 (▲) and 500 (■) nM MT3. The concentration of the $D_1$ receptor agonist, (±) chloro APB HBr was 1 $\mu$M. Inset: Schild plot of the antagonism. Data are the mean±s.e. of three experiments. Enzyme activities (expressed as pmol cyclic AMP min$^{-1}$ mg$^{-1}$ protein±s.e.-mean) were: control 125.1±7.1; ACh (100 $\mu$M) 81.2±3.4 ($P<0.001$).

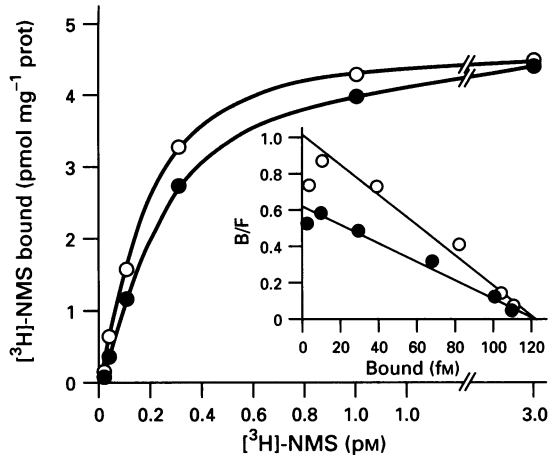


**Figure 3** Effect of MT3 on [$^3$H]-NMS binding to rat striatal membranes. Membranes were incubated with increasing concentrations of [$^3$H]-NMS (from 10 pM to 3 nM) in the absence (○) and in the presence (●) of 10 nM MT3. Specific binding of the radioligand is expressed as pmol mg$^{-1}$ protein. Values are the mean of three experiments with s.e.mean < 3%. The Inset: Scatchard analysis of the saturation binding data.

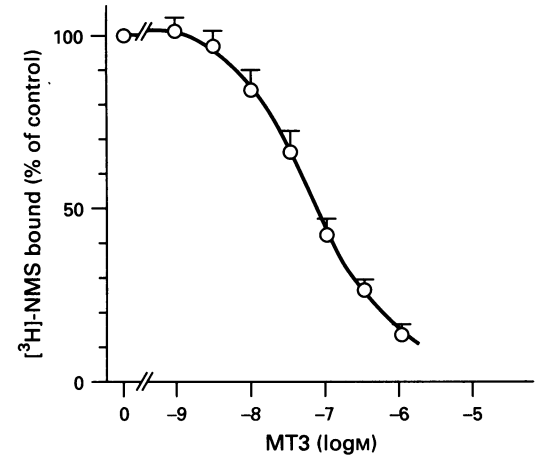


**Figure 4** Concentration-dependent displacement of [$^3$H]-NMS binding by MT3. Membranes were incubated with 0.5 nM [$^3$H]-NMS for 60 min at 30°C in the presence of the indicated concentrations of MT3. Binding is expressed as percentage of control (specific binding determined in the presence of buffer only). Data are the mean ± s.e.mean of three experiments.

### *Effect of MT3 on [$^3$H]-NMS binding to rat striatal muscarinic receptors*

Saturation experiments indicated that [$^3$H]-NMS bound to rat striatal muscarinic receptors with a $K_d$ of 127.5 ± 10.5 pM and a $B_{max}$ of 4.96 ± 0.09 pmol mg$^{-1}$ of protein (Figure 3). The addition of 10 nM MT3 significantly reduced the $K_d$ of [$^3$H]-NMS to 192.5 ± 12.5 pM ($P < 0.05$) without affecting the $B_{max}$ value. The binding of [$^3$H]-NMS was antagonized by MT3 in a concentration-dependent manner (Figure 4). Analysis of the MT3 displacement curve with the programme LIGAND indicated a significantly better fit ($P < 0.02$) of the data to a two site model than to a one site model, with 71% of the sites labelled by [$^3$H]-NMS displaced with a $K_i$ of 8.01 nM and the remaining with a $K_i$ of 150 nM.

### *Effect of MT3 on ACh inhibition of forskolin-stimulated adenylyl cyclase of rat myocardium*

In rat myocardial homogenates, the ACh-induced inhibition of adenylyl cylase activity was not significantly affected by the addition of 50 nM MT3 (Figure 5). A 10 fold higher concentration of the toxin shifted the ACh $EC_{50}$ value from 0.95 ± 0.06 μM to 1.51 ± 0.08 μM. This shift corresponds to a MT3 $K_i$ value, calculated according to equation (1) of Methods, of 860 ± 65 nM. Also in this tissue, MT3 failed to affect the enzyme activity *per se* (enzyme activities, expressed as pmol cyclic AMP min$^{-1}$ mg$^{-1}$ protein, were: control 81.1 ± 3.6; MT3 50 nM 80.9 ± 4,1; MT3 500 nM 80.3 ± 3.5).

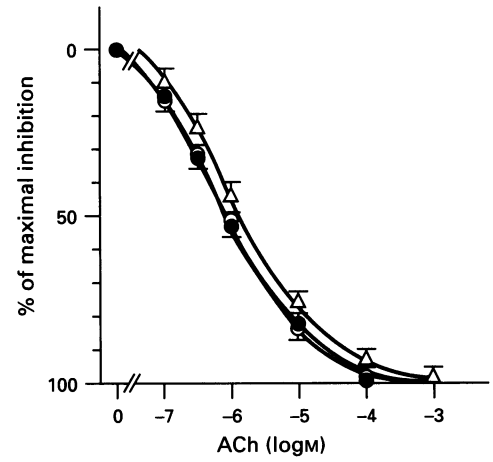


**Figure 5** Antagonism of ACh-induced inhibition of forskolin-stimulated adenylyl cylase activity in rat myocardial membranes. The enzyme activity was assayed at the indicated concentrations of ACh in the absence (○) and in the presence of 50 (●) and 500 (△) nM MT3. The concentration of forskolin was 10 μM. Data are the mean ± s.e.mean of three experiments. Enzyme activities (expressed as pmol cyclic AMP min$^{-1}$ mg$^{-1}$ protein ± s.e.mean) were: control 81.1 ± 3.6; ACh (100 μM) 45.5 ± 2.8 ($P < 0.001$).

### *Effect of MT3 on metacholine-stimulated [$^3$H]-IPs formation in rat cortex*

In cerebral cortical membranes prelabelled with [$^3$H]-*myo*-inositol, metacholine stimulated [$^3$H]-IPs formation in a concentration-dependent manner (Figure 6). The maximal stimulation corresponded to a 53.8 ± 4.9% increase of basal activity ($P < 0.001$) and the $EC_{50}$ value was 1.21 ± 0.08 μM. MT3, tested at 500 nM, failed to affect the basal [$^3$H]-IPs formation but antagonized the methacholine stimulation with an apparent $K_i$ value of 113.2 ± 7.1 nM. Under the same experimental conditions, pirenzepine antagonized the muscarinic stimulation of [$^3$H]-IPs formation with a $K_i$ of 10.9 ± 1.10 nM ($n = 3$).

## Discussion

The present study shows that the MT3 toxin is a potent antagonist of the muscarinic inhibition of adenylyl cyclase activity in rat striatum. The toxin displays similar $pA_2$ values in counteracting either the ACh-induced inhibition of the forskolin-stimulated activity or the inhibition of dopamine $D_1$ receptor-stimulated activity, indicating that the two muscarinic responses are mediated by pharmacologically indistinguishable receptors. The Schild plots of MT3 antagonism show slope values equal or close to 1, within a wide range of concentrations, indicating that the toxin may act as a competitive antagonist. Moreover, in each condition examined the toxin produces a parallel rightward shift of the agonist curve without affecting the maximal response, behaviour consistent with competitive antagonism. The inhibitory effect on the adenylyl cyclase-coupled muscarinic receptors was completely reversible, as when membranes were pretreated with a high

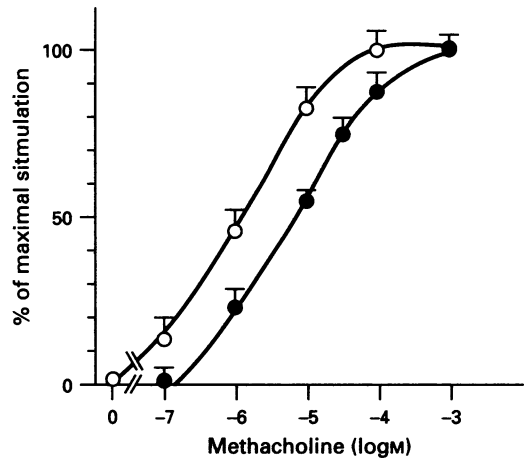


**Figure 6** Antagonism of methacholine-stimulated [$^3$H]-inositol phosphates formation by MT3. Membranes prelabelled with [$^3$H]-*myo*-inositol were incubated in the presence of the indicated concentrations of methacholine in the absence (○) and in the presence (●) of 500 nM MT3. Data are the mean ± s.e.mean of three experiments.

concentration of the toxin (20 fold the $pA_2$ value) and then washed, ACh inhibited cyclic AMP formation in the same manner as in control membranes. This lessens the possibility that the antagonistic activity results from a covalent interaction of the toxin with either the muscarinic receptor or with some other component of the transduction system. The results of the functional assays agree with those obtained in radioligand binding studies performed in striatal membranes. Thus, MT3 reduces the $K_d$ but not the $B_{max}$ of [$^3$H]-NMS binding, as expected for a competitive antagonist. Analysis of the toxin displacement curve shows that the toxin binds to a high and a low affinity site, the high affinity site corresponding to 71% of the specific [$^3$H]-NMS binding. The estimated $K_i$ for the high affinity site is similar to the toxin $pA_2$ values in antagonizing the muscarinic inhibition of adenylyl cyclase, indicating that the toxin high affinity binding site comprises the muscarinic receptor coupled to inhibition of cyclic AMP formation. In terms of absolute values, both the $K_i$ for the striatal high affinity site and the $pA_2$ values for the antagonism of the ACh inhibition are quite close to the reported affinity constant of the toxin for the m4 receptor subtype ($pK_i = 8.70$) (Jolkkonen *et al.*, 1994). Thus, the data obtained with a selective m4 antagonist corroborate the hypothesis that the striatal muscarinic receptor coupled to inhibition of adenylyl cyclase belongs predominantely, if not exclusively, to the $M_4$ subtype. Importantly, the present study extends this classification to the muscarinic receptor mediating the inhibition of dopamine $D_1$ receptor-stimulated adenylyl cylase activity, a response less well characterized than the muscarinic inhibition of either basal or forskolin-stimulated cyclic AMP formation. The observation of the presence in rat striatal membranes of a large portion of muscarinic receptors displaying high affinity for MT3 is in agreement with the data on the autoradiographic distribution of [$^{125}$I]-MT3 binding sites in rat brain sections, showing a dense labelling of the striatum (Adem *et al.*, 1995). Moreover, the percentage of the high affinity sites for the toxin (71%) is not far from the value reported by Waelbroeck *et al.* (1990) for the proportion of $M_4$ receptors in striatum (56%) estimated by a combined analysis of equilibrium and kinetic binding data.

In contrast to the high potency displayed in striatum, MT3 behaves as a rather weak antagonist of the muscarinic inhibition of adenylyl cyclase activity in rat myocardium, a response probably mediated by the $M_2$ receptor (Ehlert *et al.*, 1989; McKinney *et al*, 1989; Olianas & Onali, 1991). The $K_i$ value of 860 nM obtained in this assay agrees with the low affinity constant of the toxin for the cloned m2 receptor subtype ($pK_i > 6$) (Jolkkonen *et al.*, 1994). On the other hand, in rat cortex, the toxin shows an intermediate potency in antagonizing the methacholine stimulation of phosphoinositide hydrolysis. This response was investigated in a membrane preparation rather than in tissue slices in order to avoid possible differences due to limited tissue penetration of the toxin. Methacholine was also used as muscarinic agonist because it has been reported that it can selectively stimulate phosphoinositide hydrolysis by activating $M_1$ receptors (Forray & El-Fakahany, 1990). The $K_i$ value of 113 nM for the antagonism of methacholine stimulation of [$^3$H]-IPs formation is close to the reported affinity constant of the toxin for the cloned m1 receptor ($pK_i = 7.11$) (Jolkkonen *et al.*, 1994). Thus, the present study shows that MT3 is capable of discriminating functional responses mediated by different muscarinic receptors expressed in native membranes with a selectivity similar to that shown for the binding to the cloned receptor subtypes.

In addition to MT3, the venom of the green mamba has been shown to contain several other peptide toxins capable of binding to muscarinic receptors. Among these, MT-1, MT-2 and m-1 toxin have been well characterized with respect to molecular structure and pharmacological properties. MT-1 and MT-2, initially identificed by Adem *et al.* (1988), have been shown to act as selective and irreversible agonists of the $M_1$ receptor subtypes (Jerusalinski & Harvey, 1994), whereas the m-1 toxin, isolated by Max *et al.* (1993a), is a potent and pseudoirreversible antagonist of the $M_1$ receptor (Max *et al.*, 1993b). As shown in the present study, MT3 appears to behave as a reversible blocker devoid of agonist activity in different functional assays of muscarinic receptors. Considering that these toxins display a high degree of sequence homology (Jerusalinski & Harvery, 1994), it seems likely that subtle differences in their molecular structure confer distinct pharmacological activities.

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