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Binding of the labelled muscarinic toxin ^{125}I-MT1 to rat brain muscarinic M_1 receptors

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Abstract

The green mamba (*Dendroaspis angusticeps*) 'muscarinic toxin', MT1, was radioiodinated by the chloramine T method. ^{125}I-MT1 labelled the muscarinic M_1 receptor subtype with a very good selectivity in rat brain. It had no preference for the receptor states with high or low affinity for agonists, and was not affected by Gpp(NH)p addition to the incubation medium. The ^{125}I-MT1 binding was reversible, with a half life of 45 min at 25°C. The effect of competitive and allosteric muscarinic antagonists on ^{125}I-MT1 binding and dissociation can be rationalized by assuming that the radioiodinated toxin is able to label the muscarinic (acetylcholine) binding site.

Keywords: Muscarinic receptor subtype; Muscarinic toxin; Allosteric site

1. Introduction

Snake venoms contain many potent toxins, which have been useful in many instances for the study of the nervous system. The venoms of *Dendroaspis* (mamba) snakes contain several neurotoxins, some of which are pharmacologically different from the toxins found in other snake venoms. Recently, several toxins from the *Dendroaspis angusticeps* venom have been demonstrated to recognize one or several muscarinic receptor subtypes (for review: Karlsson et al., 1994; Jerusalinsky and Harvey, 1994).

Five muscarinic receptor subtypes have been cloned; four of these subtypes are widely expressed throughout the central nervous system (for reviews: Bonner, 1989; Hulme et al., 1990; Caulfield, 1993). The green mamba toxin called MT1 or MTX-1 was originally described as inhibiting only partially the binding of [^{3}H]quinuclidinyl benzilate to the brain (Adem et al., 1988). All subsequent studies agree that MT1 recognizes preferentially the muscarinic M_1 receptors. Its exact selectivity pattern is, however, a matter of discussion: some authors observed a $M_1 > M_3 \gg M_2$, M_4 binding pattern (Jerusalinsky and Harvey, 1994), while others found a $M_1 \gg M_4 \gg M_2$, M_3 binding pattern (Jolkkonen et al., 1995). Its peptide sequence was determined recently (Jolkkonen et al., 1995).

Radioiodination of a peptide on tyrosine residues is quite easy, and has been used very often to prepare a 'tracer' for identification of peptide receptors. In some cases, however, the radioiodinated peptide may have a very different (higher or lower) affinity as compared to the native peptide.

To assess the effect of radioiodination on the MT1 binding profile, we identified the muscarinic receptor subtype which is labelled by ^{125}I-MT1 with well characterized selective muscarinic antagonists. We also attempted to identify the actual binding site which is labelled by the toxin. Indeed, it has been suggested that muscarinic toxins might interact with the allosteric site rather than with the acetylcholine binding site of muscarinic receptors (Max et al., 1993).

2. Materials and methods

2.1. Purification of the toxin

The muscarinic toxin MT1 was purified in our laboratory by sequential chromatographies on a Sephadex G50 (gel filtration) column followed by ion-exchange and re-

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verse-phase high performance liquid chromatography (HPLC) columns as described elsewhere for MT 4 (Vandermeers et al., 1995). The active fractions were identified by inhibition of 1-[*N*-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]*N*-methylscopolamine) binding to rat brain homogenate. The purity of the isolated toxin was (> 99%) tested by analytical reverse phase HPLC and by capillary electrophoresis. The identity of the toxin was checked by protein sequencing, as described elsewhere for MT 4 (Vandermeers et al., 1995).

2.2. Radiolabelling

5 μg of the MT1 toxin were radioiodinated with ^{125}I by the chloramine T method, as previously described for other peptides (Christophe et al., 1976) with some modifications. Following the addition of metabisulfite (to stop the reaction), the reaction mixture was diluted 6 fold with sodium phosphate buffer (100 mM, pH 7.4) and applied to a C18 Sep-Pak cartridge. The unreacted iodine was washed with 4.5 ml of 10% acetonitrile-0.1% trifluoroacetic acid, then the radioiodinated toxin was eluted with 50% acetonitrile-0.1% trifluoroacetic acid, flushed, and diluted in a 10 mM sodium phosphate buffer enriched with 150 mM NaCl, 1 g/l sodium azide and 500 μl/l of Tween 20, and stored at −20°C.

2.3. Preparation of the crude synaptic membranes

Wistar rats were killed by decapitation, and the brain was dissected and homogenized in 10 ml of 0.3 M sucrose, filtered on two layers of medical gauze, and then centrifuged at 360 × *g* for 10 min. The pellet was discarded, and supernatant centrifuged at 32 000 × *g* for 20 min. The pellet was rehomogenized in distilled water, diluted 2 fold with a Tris/HCl buffer (25 mM, pH 7.5) enriched with 5 mM $MgCl_2$, and recentrifuged at 32 000 × *g* for 20 min. This pellet was then homogenized in 0.3 M sucrose, and stored in liquid nitrogen until use.

2.4. Binding studies

Unless otherwise indicated, the ^{125}I-MT1 binding was measured by incubating 100 000 cpm/assay ^{125}I-MT1 (approximately 0.03 nM) with crude synaptic membranes (200–300 μg protein/ assay), or Chinese hamster ovary (CHO) cell homogenates (see below) in 1.2 ml of 20 mM Tris/HCl buffer (pH 7.5) enriched with 4 mM $MgCl_2$, and the indicated unlabelled drug or Gpp(NH)p (guanyl-(β-imidodiphosphate), 1–100 μM) concentrations for 2 h at 25°C. Bound and free tracers were separated by filtration on Whatman GFC filter, presoaked overnight in 0.1% polyethyleneimine. The filters were rinsed 4 times with ice-cold 50 mM Tris/HCl buffer (pH 7.5). [^{3}H]Telenzepine binding was assayed in the same buffer by filtration, after 4 h of incubation, using a tracer concentration of 0.2 nM and a crude synaptic membrane concentration of 30–50 μg protein per assay.

2.5. Dissociation kinetics

To ensure a high initial labelling of the membranes, we preincubated ^{125}I-MT1 with synaptic membranes (1.0 mg/ml) in a 20 mM Tris/HCl (pH 7.5) enriched with 4 mM $MgCl_2$ for 1 h at 25°C. The reaction mixture was then diluted 2.5 fold with the same ice-cold Tris/Mg^{2+} buffer and centrifuged for 20 min at 24 000 × *g*. The supernatant was discarded and the pellet rinsed superficially in ice-cold Tris/Mg^{2+} buffer, before resuspension of the membranes in the same buffer, in the absence or presence of the indicated unlabelled drug concentrations. Duplicate samples were filtered at the indicated time intervals after resuspension. Tracer reassociation to the membranes was avoided by the low residual tracer concentration (10 000 cpm/assay) and residual membrane concentration (20 μg protein per assay) during the dilution experiment, as compared to our normal binding conditions.

2.6. Determination of the unlabelled toxin MT1 binding profile

CHO cells expressing each recombinant human receptor (a generous gift from Dr N. Buckley, London, UK) were maintained in Dulbecco's Modified Essential Medium, enriched with 10% foetal calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Geneticin (0.2 mg/ml) was further added to the culture medium of the stock culture only. For binding studies, the cells were harvested with a rubber policeman, centrifuged and rinsed with an ice-cold phosphate buffered saline, containing 1 mM ethylene diamine tetraacetic acid (EDTA), then homogenized in a 20 mM Tris-HCl buffer (pH 7.5) enriched with 250 mM sucrose, and frozen in liquid nitrogen.

CHO cell homogenates, expressing each receptor subtype, were incubated at 25°C in the presence of increasing concentrations of MT1 and of 0.2 nM [^{3}H]*N*-methylscopolamine, in 1.2 ml of 20 mM Tris-HCl buffer (pH 7.5) enriched with 4 mM $MgCl_2$. (This incubation buffer gave the highest ^{125}I-MT1 binding: see below). The homogenate concentration was adjusted to give a final concentration of 80–150 fmol muscarinic receptor per assay. The incubation was terminated by filtration as previously described (Waelbroeck et al., 1990) after 2 h (CHO cells expressing hm 1, hm 2 or hm 4 muscarinic receptors) or 4 h (CHO cells expressing hm 3, hm 5 muscarinic receptors) of incubation.

2.7. Chemicals

Carrier free ^{125}I was obtained from Dupont de Nemours (Haren, Belgium); [^{3}H]telenzepine (86 Ci/mmol) and [^{3}H]*N*-methylscopolamine (85 Ci/mmol) were obtained from Amersham International (Bucks, UK).

Atropine, Gpp(NH)p (guanyl-(β-imidodiphosphate)) and polyethyleneimine were obtained from Sigma Chemicals Co. (St Louis, MO, USA). All other chemicals were of the highest grade available. The following drugs were gifts: pirenzepine and 11-({2-[(diethylamino)methyl]-1-piperidinyl}acetyl)-5,11-dihydro-6*H*-pyrido(2,3-*b*)(1,4)-benzodiazepin-6-one (AF-DX 116) from Boehringer Ingelheim (Ingelheim, FRG); guanylpirenzepine, from Dr H. Ladinski (Instituto de Angeli, Milan, Italy) and himbacine, from Dr W.C. Taylor (University of Sydney, Australia).

3. Results

3.1. Choice of assay buffer

We compared the total and non-specific binding of ^{125}I-MT1 in a 20 mM Tris/HCl buffer (pH 7.5) enriched or not with $MgCl_2$ (4 mM) or NaCl (100 mM) and in a 50 mM sodium phosphate buffer enriched with 2 mM $MgCl_2$.

The highest specific binding was observed after incubation in the Tris-Mg^{2+} buffer (100%), followed by the Tris buffer (77%), Tris-NaCl buffer (23%) and the phosphate-Mg^{2+} buffer (46%). The non-specific binding in these experiments was completely accounted for by tracer binding to the filters.

In view of these results, we performed all the subsequent experiments in the Tris-Mg^{2+} buffer.

3.2. Association and dissociation kinetics

^{125}I-MT1 binding was slow (Fig. 1A), and its dissociation from the synaptic membranes monoexponential, with a half life of 45 min (Fig. 1B).

3.3. Competition curves: muscarinic antagonists

Atropine inhibited completely ^{125}I-MT1 binding at low concentrations. The rather steep competition curve is fully explained by the high muscarinic receptor concentration in the assay (approximately 0.2 nM).

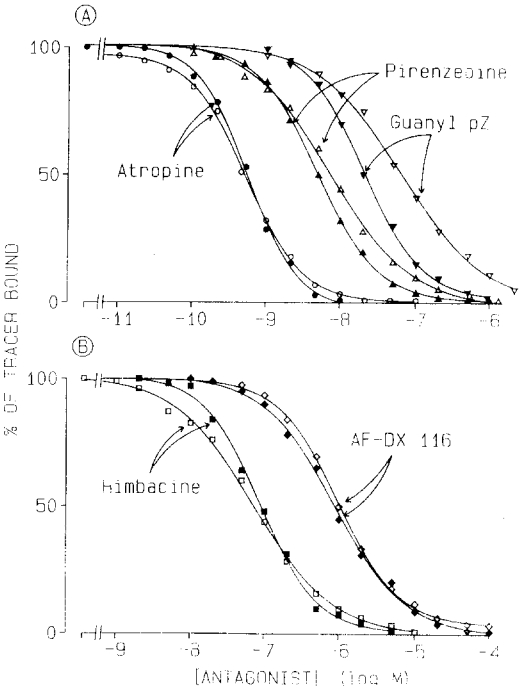

Fig. 2. Inhibition of ^{125}I-MT1 (full symbols) and [^{3}H]telenzepine (open symbols) binding by atropine (circles) and the selective muscarinic antagonists: pirenzepine (triangles), guanylpirenzepine (inverted triangles), AF-DX 116 (diamonds) and himbacine (squares). Average of 3 experiments in duplicate.

The competition curves obtained with the selective antagonists: pirenzepine, guanylpirenzepine, himbacine and AF-DX 116 (Fig. 2) suggest that ^{125}I-MT1 labelled only muscarinic M_1 receptors in the brain synaptic membranes (the Hill coefficients n_H of the competition curves were between 0.95 and 1.05 ± 0.1; the pK_i values of these antagonists are summarized in Table 1). This is in contrast with [^{3}H]telenzepine, since we were able to demonstrate that this tracer, even at the low concentration used in these

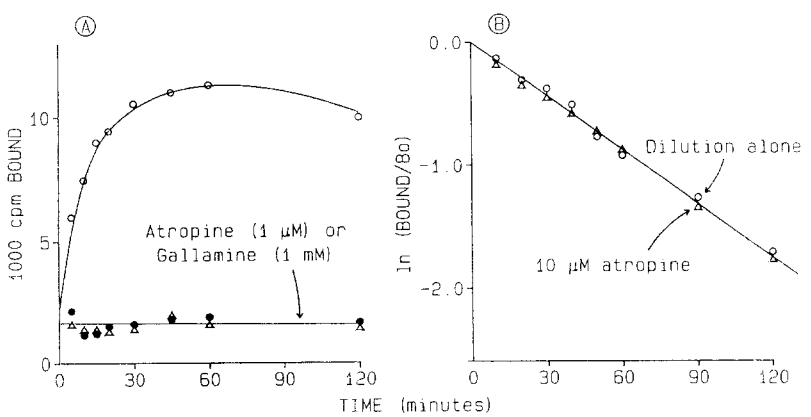

Fig. 1. Left panel: association kinetic of ^{125}I-MT1 to crude brain synaptic membranes, in the absence (○) or presence of 1 μM atropine (●) or 1 mM gallamine (△). Right panel: dissociation kinetic of ^{125}I-MT1 after chemical dilution (○), or dilution in the presence of 10 μM atropine (△). Representative of 2 experiments in duplicate.

Table 1
pIC_{50} ($-\log IC_{50}$) of selective antagonists, inhibiting ^{125}I-MT1 binding to crude brain synaptic membranes, and pK_i values of selective antagonists receptors, at four muscarinic receptor subtypes [a]

	pIC_{50}	pK_i			
		M_1	M_4	M_2	M_3
Pirenzepine	8.4	8.3	7.3	6.5	6.9
Guanylpirenzepine	7.8	7.6	6.2	5.3	6.2
Himbacine	7.1	7.1	8.1	8.0	6.5
AF-DX 116	6.3	6.5	6.5	7.3	5.8

[a] From: Waelbroeck et al., 1990 and unpublished results. The S.D. of the pIC_{50} and pK_i values were always below 0.1 log unit).

experiments (0.2 nM) labelled some muscarinic M_4 receptors (see the pirenzepine (n_H 0.8 ± 0.1), guanylpirenzepine (n_H 0.75 ± 0.1) and himbacine (n_H 0.85 ± 0.1) competition curves in Fig. 2).

3.4. Competition curves: muscarinic agonists and Gpp(NH)p

We compared the competition curves obtained with 3 muscarinic agonists, using either ^{125}I-MT1 or [^{3}H]telenzepine as tracer. As shown in Fig. 3, the competition curves obtained with both tracers were shallow and very similar (the Hill coefficients of the competition curves using ^{125}I-MT1 and [^{3}H]telenzepine were, respectively, 0.56 and 0.53 (carbamylcholine), 0.77 and 0.74 (oxotremorine) and 0.87 and 0.79 (pilocarpine)).

In contrast with [^{3}H]oxotremorine-M binding (Gillard et al., 1995), Gpp(NH)p at concentrations up to 100 μM did not affect ^{125}I-MT1 binding.

3.5. Competition curves: allosteric drugs

Gallamine and *d*-tubocurarine inhibited completely ^{125}I-MT1 binding. The gallamine competition curve was compatible with competitive inhibition of tracer binding (n_H 1.00 ± 0.05). The *d*-tubocurarine competition curve, in contrast, was slightly 'too steep' (n_H 1.10 ± 0.05) (Fig. 4A).

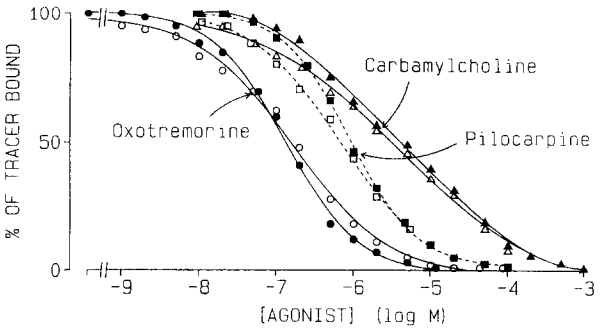

Fig. 3. Agonist competition curves obtained using ^{125}I-MT1 (closed symbols) or [^{3}H]telenzepine (open symbols) as tracer, and oxotremorine (circles), pilocarpine (squares) or carbamylcholine (triangles) as competitor. Representative of 2 experiments in duplicate.

3.6. Effect of competitive and allosteric drugs on ^{125}I-MT1 dissociation

At 1 mM, gallamine and *d*-tubocurarine increased the dissociation of ^{125}I-MT1 as compared to dilution alone, dilution +1 μM atropine or dilution +10 μM atropine. The ^{125}I-MT1 dissociation rate remained monophasic in the presence of these drugs (Fig. 4B).

3.7. Binding properties of unlabelled toxin

In agreement with the results of Jolkkonen et al. (1995) and of Kornisiuk et al. (1995), MT1 inhibited [^{3}H]*N*-methylscopolamine binding to muscarinic Hm1 receptors (cloned CHO cells) in the Tris-$MgCl_2$ buffer, with a K_i of 50–100 nM. It had a 5 to 10 fold lower affinity for muscarinic Hm4 and Hm5 receptors, a very weak affinity for muscarinic Hm3 receptors (K_i > 500 nM), and, at concentrations up to 1 μM, did not inhibit [^{3}H]*N*-methylscopolamine binding to muscarinic Hm2 receptors.

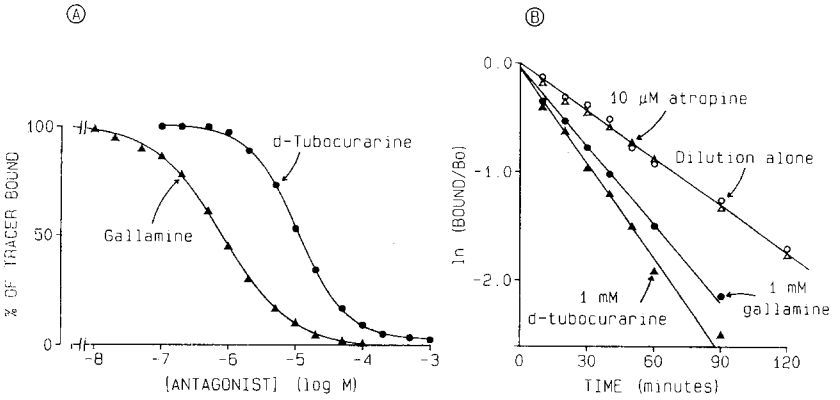

Fig. 4. Left panel: inhibition of ^{125}I-MT1 binding by gallamine (closed triangles) or *d*-tubocurarine (closed circles). Right panel: dissociation of ^{125}I-MT1, in the absence (circles) or presence of 10 μM atropine (triangles), 1 mM gallamine (closed circles) or 1 mM *d*-tubocurarine (closed triangles). Average of 3 experiments in duplicate.

We attempted to demonstrate recognition of the allosteric site by MT1, by comparing the residual [^{3}H]*N*-methylscopolamine binding 30 min after the addition of atropine (1 μM) alone and after the simultaneous addition of atropine (1 μM) and 10 μM MT1. The results suggested that, at concentrations up to 10 μM, MT1 did not recognize the allosteric state on the [^{3}H]*N*-methylscopolamine-receptor complex.

4. Discussion

4.1. Receptor subtype labelled by ^{125}I-MT1?

We estimated, using competition curves with selective antagonists, that the rat brain synaptic membranes used in this work contain approximately 40% muscarinic M_1 and 20% muscarinic M_2, M_3 and M_4 receptors (not shown). The pK_i values of the selective antagonists used are summarized in Table 1.

We separated ^{125}I-MT1 from the radioactive iodine, but did not attempt to purify the different peptide isoforms present in the reaction mixture: our 'tracer' probably was a mixture of unlabelled peptide, of monoiodinated MT1 labelled on the tyrosine residues 29, 30 or 35, and very little diiodinated peptide. Our results suggest that only one of the iodopeptides was able to recognize the muscarinic receptors: indeed, the dissociation kinetics of ^{125}I-MT1 were monoexponential, suggesting that a single type of tracer-receptor complex had been formed before dissociation (Fig. 1). The competition curves obtained with every selective antagonist were also compatible with competitive inhibition to a single receptor subtype (Fig. 2).

The receptors labelled by ^{125}I-MT1 had a high affinity for pirenzepine and guanyl pirenzepine, and low affinities for AF-DX 116 and himbacine as compared to the muscarinic M_2, M_3 or M_4 receptors (Fig. 2 and Table 1). The binding patterns of these four antagonists were exactly as expected for muscarinic M_1 receptors (see Table 1). In this respect, ^{125}I-MT1 was more M_1 selective than the commonly used tracers, [^{3}H]pirenzepine and [^{3}H]telenzepine, since we were able to demonstrate that these two compounds were able to label some muscarinic M_4 receptors (Waelbroeck et al., 1990 and Fig. 2).

We unfortunately were not able to obtain an ^{125}I-MT1 saturation curve to estimate the concentration of the labelled receptors; the tracer's affinity was too low. Assuming that the bound over free ratio is proportional to the total muscarinic M_1 receptor concentration ($B/F = K R_o$), and using the muscarinic M_1 receptor concentration labelled by [^{3}H]*N*-methylscopolamine, we calculated a K_d value of approximately 4 nM for ^{125}I-MT1. This is approximately 10 fold lower than the K_d value of the unlabelled toxin in the same buffer (see also Jerusalinsky and Harvey, 1994), suggesting that radioiodination increased the MT1 affinity for the rat and human muscarinic M_1 receptors. It would be very interesting to identify which iodotyrosine increases the peptide affinity for M_1 receptors. Further experiments are however necessary to answer this question.

4.2. Receptor state labelled by ^{125}I-MT1?

It has been suggested that MT1 and the homologous toxin MT2 behave as muscarinic agonists in functional studies (Jerusalinsky and Harvey, 1994). Our observation that ^{125}I-MT1 binding was enhanced, in a low ionic strength buffer, by the addition of $MgCl_2$, is also reminiscent of the binding properties of muscarinic agonists. We therefore compared the agonist competition curves obtained using ^{125}I-MT1 and [^{3}H]telenzepine (a muscarinic receptor antagonist). We also investigated the effect of Gpp(NH)p on ^{125}I-MT1 binding.

As shown in Fig. 3, the agonist competition curves obtained with ^{125}I-MT1 and [^{3}H]telenzepine were very similar: ^{125}I-MT1 did not label preferentially the muscarinic M_1 receptors with the highest affinity for agonists. It was also insensitive to the addition of (up to 100 μM) Gpp(NH)p (not shown). These two results are in marked contrast with the binding properties generally observed with labelled muscarinic agonists (see for example Gillard et al., 1987), and suggest that ^{125}I-MT1 is capable of labelling all muscarinic M_1 receptors, irrespective of their state of association with G proteins.

4.3. Receptor site labelled by MT1?

At least two binding sites have been identified on muscarinic receptors: the acetylcholine binding site, shared by muscarinic agonists and antagonists, and an allosteric site, recognized inter alia by neuromuscular blocking agents (Ellis and Seidenberg, 1992; Waelbroeck, 1994; Proska and Tucek, 1994). We therefore decided to study the effect of two allosteric compounds, gallamine and *d*-tubocurarine, on the equilibrium binding and dissociation rate of ^{125}I-MT1.

Like atropine and the other selective muscarinic antagonists, gallamine and *d*-tubocurarine inhibited ^{125}I-MT1 binding with a Hill coefficient close to 1 (Fig. 4A). We did not observe any non-displaceable binding: at maximally effective concentrations, both muscarinic allosteric compounds prevented completely ^{125}I-MT1 binding to the membranes (Fig. 1A and Fig. 4A and results not shown).

We therefore decided to investigate the effect of the different compounds on the ^{125}I-MT1 dissociation rate, in comparison with the dissociation rate observed after the removal of the tracer. Atropine, at 1–10 μM, did not affect the ^{125}I-MT1 dissociation rate (Fig. 4B). In contrast, at 1 mM, gallamine and d-tubocurarine increased slightly the tracer's k_{off} value (Fig. 4B). In the same concentration range, they decreased the [^{3}H]telenzepine dissociation (not shown). This indicated that the two neuromuscular block-

ing agents were able to recognize the ^{125}I-MT1-receptor complex, and suggested that ^{125}I-MT1 does not label the allosteric site (Matsui et al., 1995).

At 10 μM, unlabelled MT1 did not reproducibly slow [^{3}H]*N*-methylscopolamine dissociation from muscarinic receptors supporting the hypothesis that, in contrast with the m1 muscarinic toxin (Max et al., 1993), MT1 recognizes the muscarinic rather than the allosteric site on M_1 receptors.

In conclusion: ^{125}I-MT1 labelled the muscarinic M_1 receptor subtype with a very good selectivity. It probably interacted with the muscarinic binding site.

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References

Adem, A., A. Åsblom, G. Johansson, P.M. Mbugua and E. Karlsson, 1988, Toxins from the venom of the green mamba *Dendroaspis angusticeps* that inhibit the binding of quinuclidinyl benzilate to muscarinic acetylcholine receptors, Biochim. Biophys. Acta 968, 340.

Bonner, T.I., 1989, The molecular basis of muscarinic receptor diversity, Trends Neurosci. 12, 148.

Caulfield, M.P., 1993, Muscarinic receptors – characterization, coupling and function, Pharmacol. Ther. 58, 319.

Christophe, J.P., T.P. Conlon and J.D. Gardner, 1976, Interaction of porcine vasoactive intestinal peptide with dispersed pancreatic acinar cells from the guinea pig: binding of radioiodinated peptide, J. Biol. Chem. 251, 4629.

Ellis, J. and M. Seidenberg, 1992, Two allosteric modulators interact at a common site on cardiac muscarinic receptors, Mol. Pharmacol. 42, 638.

Gillard, M., M. Waelbroeck and J. Christophe, 1987, Muscarinic receptor heterogeneity in rat central nervous system. II. Brain receptors labeled by [^{3}H]oxotremorine-M correspond to heterogeneous M2 receptors with very high affinity for agonists, Mol. Pharmacol. 32, 100.

Hulme, E.C., N.J.M. Birdsall and N.J. Buckley, 1990, Muscarinic receptor subtypes, Ann. Rev. Pharmacol. Toxicol. 30, 633.

Jerusalinsky D. and A.L. Harvey, 1994, Toxins from mamba venoms: small proteins with selectivities for different subtypes of muscarinic acetylcholine receptors, Trends Pharmacol. Sci. 15, 424.

Jolkkonen, M., A. Adem, U. Hellman, C. Wernstedt and E. Karlsson, 1995, A snake toxin against muscarinic acetylcholine receptors: amino acid sequence, subtype specificity and effect on guinea-pig ileum, Toxicon 33, 399.

Karlsson, E., M. Jolkkonen, M. Sataypan, A. Adem, E. Kumlin, U. Hellman and C. Wernstedt, 1994, Protein toxins that bind to muscarinic acetylcholine receptors, Ann. NY Acad. Sci. 710, 153.

Kornisiuk, E., D. Jerusalinsky, C. Cerveñansky and A.L. Harvey, 1995, Binding of muscarinic toxins MTx1 and MTx2 from the venom of the green mamba *Dendroaspis angusticeps* to cloned human muscarinic cholinoceptors, Toxicon 33, 11.

Matsui, H., S. Lazareno and N.J.M. Birdsall, 1995, Probing of the location of the allosteric site on m1 muscarinic receptors by site-directed mutagenesis, Mol. Pharmacol. 47, 88.

Max, S.I., J.-S. Liang and L.T. Potter, 1993, Stable allosteric binding of m1-toxin to m1 muscarinic receptors, Mol. Pharmacol. 44, 1171.

Proska, J. and S. Tucek, 1994, Mechanisms of steric and cooperative actions of alcuronium on cardiac muscarinic acetylcholine receptors, Mol. Pharmacol. 45, 709.

Vandermeers, A., M.-C. Vandermeers-Piret, J. Rathé, M. Waelbroeck, M. Jolkkonen, A. Oras and E. Karlsson, 1995, Purification and sequence determination of a new muscarinic toxin (MT4) from the venom of the green mamba (*Dendroaspis angusticeps*), Toxicon 33, 1171.

Waelbroeck, M., 1994, Identification of drugs competing with *d*-tubocurarine for an allosteric site on cardiac muscarinic receptors. Mol. Pharmacol. 46, 685.

Waelbroeck, M., M. Tastenoy, J. Camus and J. Christophe, 1990, Binding of selective antagonists to four muscarinic receptors (M_1 to M_4) in rat forebrain, Mol. Pharmacol. 38, 267.