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Purification and Properties of m1-Toxin, a Specific Antagonist of m1 Muscarinic Receptors

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The venom of the Eastern green mamba from Africa, *Dendroaspis angusticeps*, was found to block the binding of ^{3}H-quinuclidinyl benzilate to pure m1 and m4 muscarinic ACh receptors expressed in Chinese hamster ovary cells. The principal toxin in the venom with anti-m1 muscarinic activity was purified by gel filtration and reversed-phase HPLC. This toxin has 64 amino acids, a molecular mass of 7361 Da, and an isoelectric point of 7.04. Its cysteine residues are homologous with those in curare-mimetic α-neurotoxins, and with those in fasciculin, which inhibits AChE. At low concentrations the toxin blocked m1 receptors fully and pseudoirreversibly while having no antagonist activity on m2–m5 receptors; the toxin is therefore named "m1-toxin." At higher concentrations m1-toxin interacted reversibly with m4 receptors, and half of the toxin dissociated in 20 min at 25°C. The affinity of m1-toxin is therefore much higher for m1 than for m4 receptors. By comparison with m1-toxin, pirenzepine has sixfold higher affinity for m1 than for m4 receptors. Autoradiographs of muscarinic receptors in the rat brain demonstrated that m1-toxin blocked the binding of 2 nM ^{3}H-pirenzepine only in regions known to bind m1-specific antibodies. Thus, m1-toxin is a much more selective ligand than pirenzepine for functional and binding studies of m1 muscarinic receptors.

[Key words: muscarinic receptor, snake toxin, ACh, antagonist, venom]

Studies of the functions of genetically defined m1–m5 muscarinic receptors (Bonner, 1989) have been hampered by the lack of specific antagonists (Buckley et al., 1989; Dörje et al., 1991b) and agonists for each receptor. The most useful antagonist for m1 receptors to date has been pirenzepine (Hammer et al., 1980), which pharmacologically defines a subgroup of "M_1" receptors (Birdsall et al., 1989). However, pirenzepine binds to pure human m1 receptors with only 6-, 14-, 22-, and 35-fold higher affinity than to m4, m5, m3, and m2 receptors, respectively (Dörje et al., 1991b). It has been particularly difficult to distinguish m1 and m4 receptors; the best available ligand is himbacine, which shows a 10-fold higher affinity for m4 than for m1 receptors (Dörje et al., 1991b). In order to obtain more specific ligands, several groups of investigators have prepared antibodies to distinct intracellular portions of m1–m5 receptors. These antibodies have proved useful for localizing muscarinic receptors in tissue sections, and for assaying solubilized receptors (e.g., Levey et al., 1991; Li et al., 1991; Wall et al., 1991a,b). However, specific and readily diffusible ligands are still needed for functional studies of m1–m5 receptors, and for other assays and experiments concerning the extracellular portions of these receptors.

In 1988 Adem et al. reported the isolation of two anti-muscarinic toxins, MTX1 and MTX2, from the venom of *Dendroaspis angusticeps*, by gel filtration on Sephadex G-50, cation-exchange chromatography, and reversed-phase HPLC. They found that each toxin blocked the binding of ^{3}H-quinuclidinyl benzilate (^{3}H-QNB) to synaptosomes prepared from the rat cerebral cortex, by about 54%. They therefore suggested that the toxins might be specific for one subtype of receptor. Subsequent reports established the amino acid sequence of MTX2 (Karlsson et al., 1991) and its cDNA (Ducancel et al., 1991), and it was suggested that the secondary structures of anti-muscarinic, anti-nicotinic, and anti-esterase toxins are homologous. Jerusalinsky et al. (1992) have repeated the work of Adem et al. (1988). They confirmed that both isolated toxins blocked the binding of ^{3}H-QNB to cortical synaptosomes by about 54%. Their data indicated further that the toxins acted competitively to block the binding of pirenzepine to M_1 receptors in the cortex, and to antagonize the binding of other ligands, including agonists, to muscarinic receptors in the heart and brainstem. Since the latter tissues are believed to express almost pure m2 receptors (Dörje et al., 1991a,b; Levey et al., 1991), the results of Jerusalinsky et al. (1992) suggested that MTX1 and MTX2 are not fully selective for one receptor subtype.

Here we demonstrate that the venom of *D. angusticeps* contains components that antagonize m1 and m4 muscarinic receptors. The principal anti-m1 toxin in the venom was isolated and found to be capable of blocking m1 receptors selectively. The amino acid sequence of this "m1-toxin" is different from that of MTX2, and its amino acid composition is different from that of MTX1. m1-Toxin should prove very useful for anatomical, physiological, biochemical, and pharmacological studies of m1 receptors. A toxin that shows high specificity for m4 receptors has also been isolated; its purification and properties will be described elsewhere.

Materials and Methods

Lyophilized venom from *D. angusticeps* was purchased from the Miami Serpentarium Labs (Punta Gorda, FL).

^{3}H-QNB (L-[benzylic-4,4′-^{3}H(N)]-quinuclidinyl benzilate; 45 Ci/

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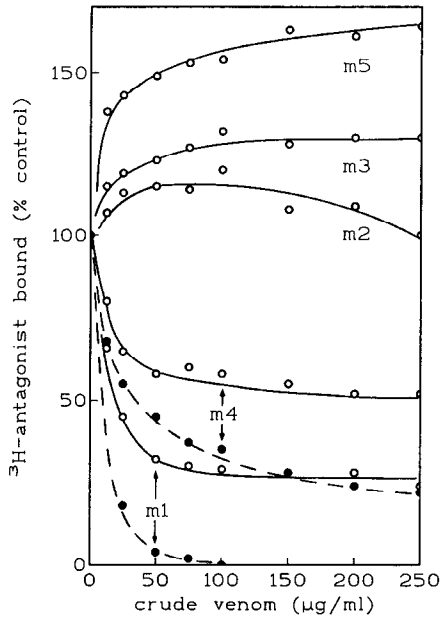

Figure 1. Effect of incubating membranes from CHO cells with venom from *D. angusticeps,* followed by sextuplicate assays of residual m1–m5 muscarinic receptors with 1.0 nM ^{3}H-QNB (*solid lines*) or 0.1 nM ^{3}H-NMS (*dashed lines*). The results show that venom components blocked both m1 and m4 receptors, indicating the presence of anti-m1 and anti-m4 toxins in the venom.

mmol), [*N*-methyl-^{3}H]-methscopolamine (^{3}H-NMS; 75 Ci/mmol), and [*N*-methyl-^{3}H]-pirenzepine (70 Ci/mmol) were purchased from Dupont–New England Nuclear Products (Boston, MA).

CHO-K1 cells containing human m1–m5 receptors (Dörje et al., 1991b) were grown in humidified air containing 5% CO_2, 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and geneticin (0.1 mM; GIBCO Labs, Grand Island, NY). Cells were scraped into 50 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA ("phosphate-EDTA buffer"), and sedimented (Potter et al., 1984).

Membranes were prepared from whole rat cerebral cortex or from packed CHO cells, and were resuspended either in phosphate-EDTA buffer or in 20 mM Tris-HCl buffer, pH 7.4, containing 1 mM $MnCl_2$ ("Tris-Mn buffer"), as described by Potter et al. (1984). Phosphate-EDTA buffer was used for receptor assays when the radioligand was

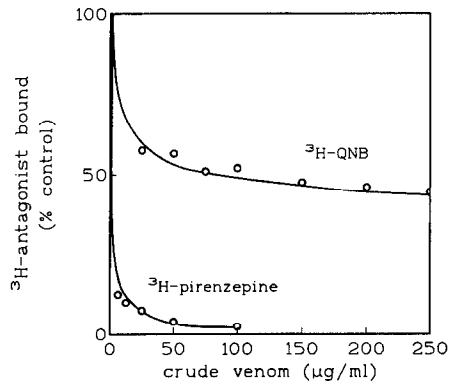

Figure 2. Effect of venom on the binding of antagonists to muscarinic receptors in membranes from the rat cerebral cortex. Points are mean values from sextuplicate assays. Venom components could fully antagonize the binding of 1.0 nM ^{3}H-pirenzepine, which is primarily to m1 receptors in this tissue. The partial blockade of binding of 1.0 nM ^{3}H-QNB may be interpreted as due to blockade of m1 and m4 receptors but not m2, m3, or m5 receptors.

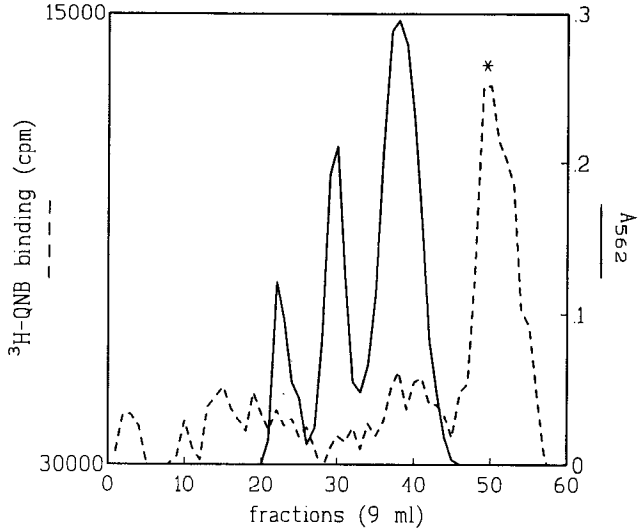

Figure 3. Gel filtration of 1 gm of venom protein on Sephadex G-50 at 4°C. This first step in the purification of m1-toxin yielded three major peaks of protein, followed by a single broad peak of anti-muscarinic activity (marked with an *asterisk*). The active peak was collected and lyophilized for HPLC.

QNB or NMS, and Tris-Mn buffer was used when pirenzepine was the ligand.

The anti-muscarinic activity of venom components was assessed as follows. Resuspended membranes from 5 mg of tissue or cells (0.1 ml) were incubated with diluted toxin (0.1 ml) for 20 min at 25°C, so as to allow maximum toxin binding. The usual amount of m1 receptors was about 0.2 pmol, whereas the amount of m2–m5 receptors varied from 0.05 to 0.4 pmol. A low concentration of ^{3}H-NMS (9.8 ml of 0.1 nM), ^{3}H-QNB (9.8 ml of 1.0 nM), or ^{3}H-pirenzepine (0.8 ml of 1.25 nM) was then added and incubation was continued for 45 min to label free muscarinic receptors. Membranes were collected by filtration and radioactivity was counted as described (Potter et al., 1984). Nonspecific binding was measured in the presence of 1 μM (±)-QNB, and has been subtracted from the data presented. One unit of anti-muscarinic activity is defined as the minimum amount of venom protein necessary to block the binding of ^{3}H-NMS to m1 receptors by at least 95% under standard assay conditions (see Results).

To assess the rate of dissociation of m1-toxin from m1 receptors, rat cortical membranes were first treated with purified m1-toxin, as noted above, and then sedimented by centrifugation at 38,000 × g_{max} for 10 min. The membranes were then resuspended in Tris-Mn buffer (10 ml for membranes from each 5 mg of tissue) containing 1 nM ^{3}H-pirenzepine, incubated at 25°C, and sampled at hourly intervals for 8 hr to measure the reappearance of any muscarinic receptors capable of binding ^{3}H-pirenzepine. A similar assay was used to measure the rate of dissociation of m1-toxin from m4 receptors, except that toxin-treated membranes from CHO cells were resuspended in phosphate-EDTA buffer containing 1.0 nM ^{3}H-NMS, and were sampled at 10 min intervals.

To assess the affinity of m1-toxin for m4 receptors, membranes from 5 mg of CHO cells expressing m4 receptors were incubated with varying amounts of m1-toxin and with 1.0 nM ^{3}H-NMS in 1.0 ml of phosphate-EDTA buffer for 2 hr at 25°C. Membranes with bound ^{3}H-NMS were recovered by filtration for counting as described (Potter et al., 1984). For comparative purposes, the same assay parameters were used to study membranes from CHO cells having m1 receptors; in these assays equilibrium between m1-toxin and NMS was not approached because of the pseudoirreversible nature of the binding of m1-toxin.

Protein was assayed with the Pierce bicinchoninic acid reagent (Pierce, Rockford, IL), using BSA as the standard. The enhanced assay protocol was necessary for all toxin samples after HPLC.

Gel filtration was performed at 4°C with a 500 ml column of Sephadex G-50-80 (Sigma Corp., St. Louis, MO) in 0.1 M ammonium acetate buffer at pH 6.8. One gram of dried venom was dissolved in 10 ml of buffer containing 10 mM EDTA and 0.1 mM freshly prepared phenylmethylsulfonyl fluoride. The fluid was centrifuged at 12,800 × *g* for 10

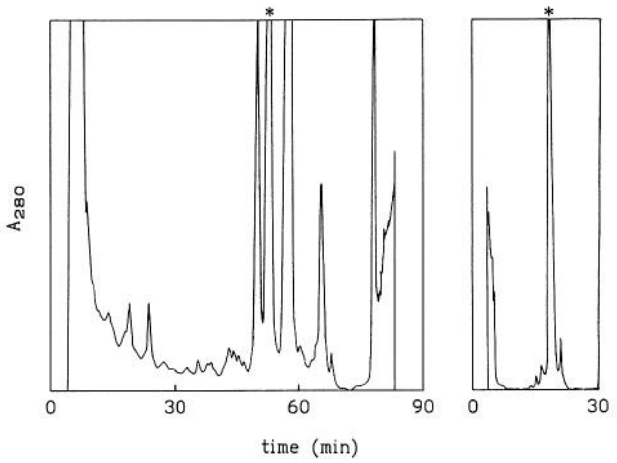

Figure 4. The *left panel* shows HPLC of the active material eluted from Sephadex G-50 on a preparative reversed-phase C-18 column. This second step in the purification of m1-toxin yielded several protein peaks and one peak of anti-muscarinic activity (marked with an *asterisk*). The *right panel* shows rechromatography of the active peak on an analytical reversed-phase C-18 column. m1-Toxin was eluted from both columns in approximately 32% acetonitrile.

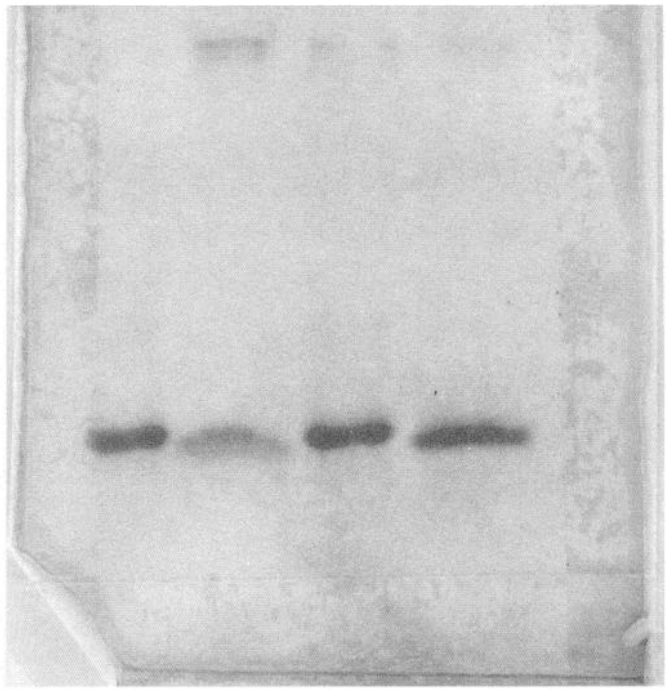

Figure 5. Electrophoresis of m1-toxin in 15% polyacrylamide in SDS. The four lanes show 1.5 μg α-bungarotoxin, 0.75 μg m1-toxin, 1.5 μg α-bungarotoxin, and 1.0 μg m1-toxin, *left to right,* respectively. The results suggest a mass of about 7000 Da for the toxin.

min and the supernatant fluid was applied to the column. Nine-milliliter fractions were collected; 0.1 ml samples were assayed for protein, and 0.002 ml samples were assayed for anti-muscarinic activity. Peak fractions with anti-muscarinic activity were pooled, lyophilized, and redissolved in 0.75 ml of 0.1% trifluoroacetic acid (TFA) for HPLC.

HPLC was carried out with a preparative reversed-phase C-18 column (Applied Biosystems, Santa Clara, CA) equilibrated with the sample mobile phase at 22°C. After sample application the column was eluted with a 135 ml linear gradient of 10–50% acetonitrile in 0.1% TFA. Protein was monitored at OD 280 nm, and 0.002 ml samples of eluate were assayed for anti-muscarinic activity. The purity of the m1-toxin peak was established by rechromatography on an analytical reversed-phase C-18 column (25 cm Dynamax-300A, Rainin Instrument Co., Emeryville, CA) in 25–55% acetonitrile in 0.1% TFA.

The amino acid sequence of m1-toxin was determined as follows. Purified toxin was reduced and carboxymethylated (Crestfield et al., 1963), prior to cleavage with trypsin (treated with *N*-tosyl-L-phenylalanine chloromethyl ketone) or α-chymotrypsin. Fragments were acidified with formic acid and separated by HPLC on a reversed-phase C-18 column in 0–60% acetonitrile in 0.1% TFA. Edman degradations were performed with an automatic gas-phase sequencer (Applied Biosystems, Santa Clara, CA).

Autoradiography was performed as described by Mash and Potter (1986), with the following modifications. Alternate 20 μm sections of the rat brain were treated with 0 or 10 U/ml of m1-toxin in Tris-Mn buffer for 30 min at 25°C, before incubation of the sections with 2 nM ^{3}H-pirenzepine for 1 hr. Sections were then washed with fresh ice-cold buffer (3 × 5 min), dried, and apposed to LKB ^{3}H-Ultrofilm (Leica Inc., Wheeling, IL) for 1 month at room temperature. One micromolar (±)-QNB was included during some incubations to assess nonspecific binding.

Results

Figure 1 shows the effects of incubating membranes from CHO cells with diluted venom, followed by assays of residual m1–m5 receptors with ^{3}H-QNB, and in some cases, with ^{3}H-NMS. Venom components decreased the binding of ^{3}H-antagonist to m1 and m4 receptors, and appeared to increase binding to m2, m3, and m5 receptors. Venom components decreased the binding of the quaternary amine, ^{3}H-NMS, more than the binding of the tertiary ligand, ^{3}H-QNB, suggesting that the anti-muscarinic components of venom do not cross membranes to reach intracellular receptors.

The amount of venom protein required to block the binding of ^{3}H-NMS to about 0.2 pmol of pure m1 receptors was about 10 μg per assay (Fig. 1); the concentration during preincubation was 50 μg/ml. One gram of dried venom therefore contains approximately 100,000 units of anti-m1 receptor activity, and can block approximately 0.02 μmol of m1 receptors. If it is assumed that anti-m1 toxins have a molecular mass of 7000–7400 Da and that one molecule of toxin blocks one molecule of receptor, then it may be calculated that a gram of venom has about 145 μg of anti-m1 toxins.

Membranes from the rat cerebral cortex were used for receptor assays during the purification of m1-toxin, since this tissue is a convenient source of m1 receptors. [The proportions of different muscarinic receptors in the rat cortex have been estimated from the immunoprecipitation of solubilized receptors with subtype-specific antibodies, as 40% m1, 37% m2, 15% m4, and 0% m3 and m5 (Levey et al., 1991), and as 34% m1, 20% m2, and 10% m3 (Li et al., 1991; Wall et al., 1991a,b).] Figure 2 shows the effect of diluted venom on the binding of ^{3}H-pirenzepine and ^{3}H-NMS to cortical receptors. Ninety-five percent of the binding sites for 1 nM ^{3}H-pirenzepine were blocked by 50 μg/ml of venom, in keeping with the results in Figure 1 for m1 receptors. Fifty-six percent of the binding sites for ^{3}H-NMS were blocked by 250 μg/ml venom, in keeping with the antagonist action of venom on both m1 and m4 receptors, and the combined percentage of these receptors in the rat cortex.

Figure 3 shows the results of gel filtration of one gram of venom protein on Sephadex G-50 at 4°C. Three major peaks of protein were eluted, followed by a well-separated, although broad, peak of anti-muscarinic activity. The most active anti-muscarinic fractions were combined, assayed for recovery of activity, and lyophilized for HPLC. Typically the recovery of protein in this anti-muscarinic peak was 0.3–1.0% of that in the

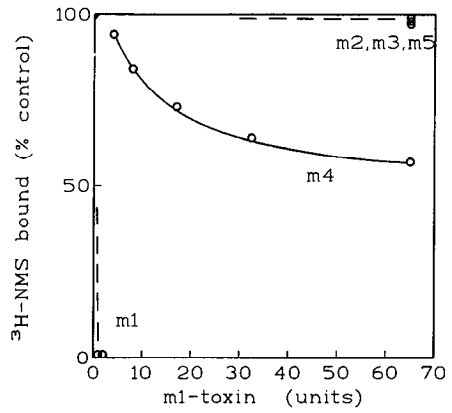

Figure 6. Specificity of binding of m1-toxin. Membranes from CHO cells with m1–m5 receptors were incubated first with m1-toxin and then with 0.1 nM ^{3}H-NMS. Points are mean values from sextuplicate assays. It is evident that m1-toxin can produce a complete blockade of m1 receptors at concentrations that have no effect on m2–m5 receptors.

venom, and the recovery of anti-m1 muscarinic activity was about 80%. The protein peaks shown in Figure 3 are virtually identical to those found by Adem et al. (1988), who used the same resin and buffer at 20°C. However, in their studies, and in those of Jerusalinsky et al. (1992), all anti-muscarinic activity was assumed to be associated with the third protein peak, which is known to contain toxins with molecular masses of about 7000 Da.

Figure 4 shows HPLC of the anti-muscarinic material purified by gel filtration, on a reversed-phase C-18 column. Anti-m1 receptor activity was eluted in about 32% acetonitrile. Rechromatography indicated satisfactory purity. Recovery of protein at this stage was about 100 μg, but the recovery of anti-m1 activity was typically less than 10% of that in the venom.

PAGE in SDS (Fig. 5) established that the purified protein ran very slightly faster than α-bungarotoxin, which has a molecular mass of about 8000 Da. The purified toxin thus has a size similar to that of MTX2 from the same venom (formula weight of 7040; Karlsson et al., 1991).

The specificity of binding of the purified toxin was assessed using CHO cells expressing m1–m5 receptors (Fig. 6). At low concentrations the toxin blocked only m1 receptors, and it is therefore named "m1-toxin." The amount of m1-toxin necessary to block about 0.2 pmol of m1 receptors (one unit/assay) was about 30 ng. Given a toxin mass of 7361 Da, this amount is about 4 pmol, and the molar toxin : receptor ratio was about 20:1. With 5–65-fold higher concentrations of m1-toxin there was partial blockade of m4 receptors, with no effect on m2, m3, or m5 receptors. The incomplete blockade of m4 receptors at high toxin concentrations suggested that m1-toxin was binding to m4 receptors and then partially dissociating from them during the second step of our binding assay. The nature of this reversible binding was therefore studied further as described below.

The amino acid sequence of m1-toxin is shown in Figure 7, aligned in homology with the sequences of several other toxins. The calculated mass of m1-toxin is 7361 Da, and its calculated isoelectric point is 7.04. The degree of sequence identity between m1-toxin and MTX2 is 63%. The amino acid composition of m1-toxin also differs substantially from that of MTX1 (Jerusalinsky et al., 1992). The probable secondary structure of m1-toxin is shown in Figure 8. Those amino acids that are common to m1-toxin, MTX2, and curare-mimetic neurotoxins (Chiappinelli, 1985) are shown; it is evident that regions of S–S bonding are well preserved. Those amino acids that are different in the two anti-muscarinic toxins from the amino acids in curare-mimetic toxins are identified; some of these amino acids may prove to be essential for anti-muscarinic activity.

The duration of binding of m1-toxin to m1 receptors was examined as shown in Figure 9. Toxin-treated membranes immersed in 1 nM ^{3}H-pirenzepine for up to 8 hr showed no recovery of free receptor sites. The binding of m1-toxin is therefore irreversible for practical purposes. In other experiments we have shown that certain ways of solubilizing toxin-labeled receptors remove the toxin and allow m1 receptors to bind ^{3}H-QNB again (Max, 1992). It may therefore be concluded that m1-toxin does not damage m1 receptors, and that its binding is pseudoirreversible.

The rate of dissociation of m1-toxin from m4 receptors was assessed as shown in Figure 10. Receptors treated with m1-toxin regained their ability to bind ^{3}H-NMS with a half-time of about 20 min at 25°C. The affinity of m1-toxin for m4 receptors was therefore assessed by competition between the toxin and 1.0 nM ^{3}H-NMS as shown in Figure 11. Half-blockade of NMS binding to m4 receptors was achieved with about 45 units of m1-toxin per assay. With the same batch of m1-toxin and conditions, half-blockade of NMS binding to m1 receptors was achieved with about 0.55 units of m1-toxin per assay (Fig. 11). The approach and data shown in Figure 11 are the most practicable way to compare the protective effect of m1-toxin on m1 and m4 receptors, even though one assay was not performed under equilibrium conditions, and the results do not yield the true relative affinities of m1-toxin for these receptors.

The usefulness of m1-toxin for autoradiography is illustrated in Figure 12. The concentration of ^{3}H-pirenzepine used for these studies (2 nM) was chosen so as to occupy 40% of m1 receptors ($K_d \approx 3$ nM; Potter et al., 1988), 12% of m4 receptors (5-fold lower affinity than for m1 receptors in the rat; Buckley et al.,

Figure 7. Amino acid sequences of m1-toxin and related toxins. The 64 amino acids of m1-toxin are aligned in homology with the sequences of the anti-muscarinic toxin MTX2, the anti-nicotinic toxins erabutoxin b and cobrotoxin, the cardiotoxin cytotoxin II, and the anti-cholinesterase fasciculin (Low et al., 1976; Karlsson et al., 1991). The positions of all eight cysteine residues in these toxins are highly conserved, suggesting that the secondary structures of these toxins are also similar. See Figure 8.

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                1        10        20        30        40        50        60
m1-toxin        LTCVKSNSIWFPTSEDCPDGQNLCFKRHWYISPRMYDFTRGCAATCPKAEYRDVINCCGT-DKCN
MTX2            LTCVTTKSIGGVTTEDCPAGQNYCFKRWHYVTPKNYDIIKGCAATCPKVDNNDPIRCCGT-DKCND
erabutoxin      RICYNQHSSQPQTTKTCSPGESSCYHKQWSDF-RGTIIERGC-G-CPTVKPGIKLSCCES-EVCNN
cobrotoxin      LECHNQQSSQTPTTTGCSGGETNCYKKRWRDH-RGYRTERGC-G-CPSVKNGIEINCCTT-DRCNN
cytotoxin       LKC-NKLVPLFYKT--CPAGKNLCYKMYMVATPKVPVK-RGCIDVCPKSSLVLKYVCCNT-DRCN
fasciculin      TMCYSHTTTSRAILTNC--GENSCYRKSRRHPPKMV-LGRGC-G-CPPGDDNLEVKCCTSPDKCNY
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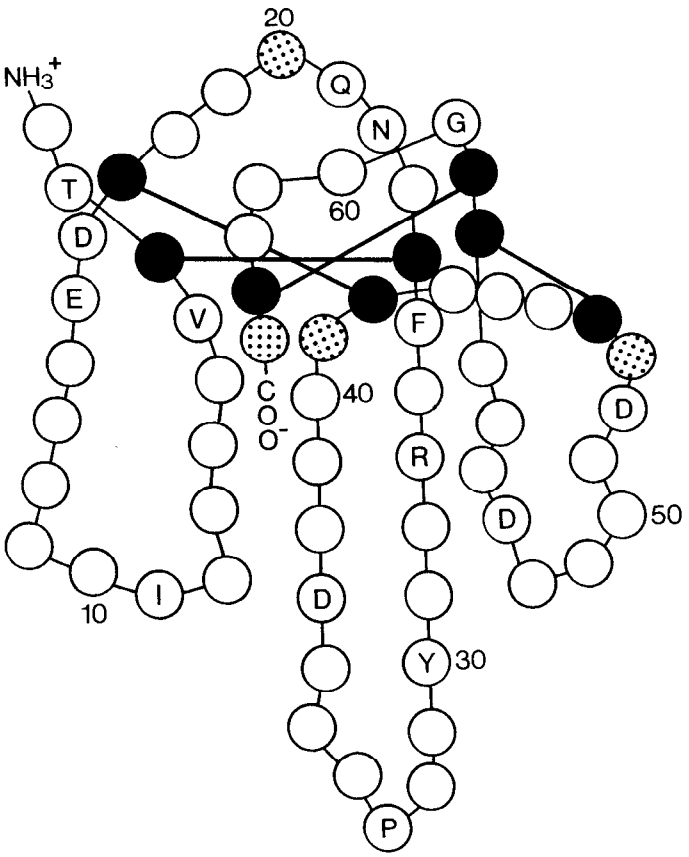

Figure 8. Probable secondary structure of m1-toxin, based on the known crystal structure of erabutoxin b (Low et al., 1976; Tsernoglou and Petsko, 1976). Cysteine residues are shown in *black*, and four other amino acids that are invariant in anti-muscarinic and anti-nicotinic toxins are *stippled*. *Letters* identify those amino acids that are highly conserved in m1-toxin and MTX2 but not anti-nicotinic toxins.

1989), 6% of m3 receptors (11-fold lower affinity), and a trivial number of m2 receptors (57-fold lower affinity). As expected, m1-toxin greatly diminished the binding of ^{3}H-pirenzepine in the dentate gyrus and CA1 region of the hippocampus, where

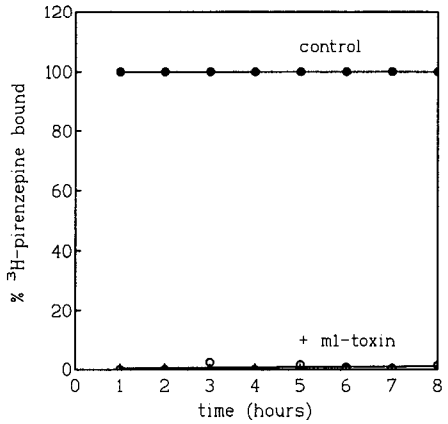

Figure 9. Duration of binding of m1-toxin to m1 receptors. Half of one batch of rat cortical membranes was exposed to m1-toxin, and all were then immersed in 1.0 nM ^{3}H-pirenzepine to label m1 receptors selectively. The membranes not treated with toxin showed 4700 cpm of pirenzepine; points are mean values from sextuplicate assays. The membranes treated with m1-toxin did not recover any free binding sites for pirenzepine in 8 hr at 25°C, indicating stable binding of the toxin.

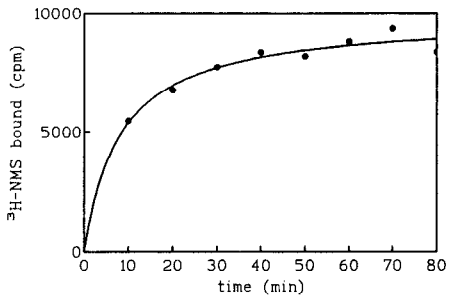

Figure 10. Rate of dissociation of m1-toxin from m4 receptors. Membranes from CHO cells having m4 receptors were treated with m1-toxin, sedimented, and resuspended in 1.0 nM ^{3}H-NMS. Points are mean values from quadruplicate assays. The curve is computer fitted to show that the toxin dissociated from membranes, allowing the increased binding of NMS, with a half-time of 19.5 min. The dissociation rate constant is therefore 0.0036 min^{-1}.

m1 receptors are the predominant muscarinic receptor subtype. The pattern of toxin-spared binding sites in the whole brain was virtually identical to the distribution of m4 receptors found with specific anti-m4 antibodies (Levey et al., 1991). Parallel studies using ^{3}H-QNB as the ligand showed that m1-toxin had no effect on the binding of this ligand in the brainstem, superior colliculus, and other regions known to contain virtually pure m2 receptors (not shown).

In other studies we have demonstrated that m1-toxin blocks the activation of m1 receptors in intact tissues, and that the specific binding of m1-toxin to m1 receptors is retained after receptor solubilization in digitonin. The toxin does not block the binding of ^{3}H-L-nicotine to nicotinic receptors in the CNS (Max, 1992).

Discussion

Toxins have a rich and growing history as ligands for proteins that are difficult to identify, notably receptors and ion channels. Quantitative work with receptor molecules began with the use of ^{125}I-α-bungarotoxin to study nicotinic ACh receptors in intact

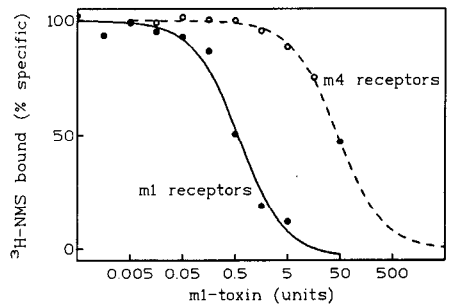

Figure 11. Protective effect of m1-toxin on m1 and m4 receptors, after the co-incubation of toxin, receptors, and 1.0 nM ^{3}H-NMS in 1.0 ml of phosphate-EDTA buffer for 2 hr at 25°C. The receptors were in membranes from CHO cells; points are mean values from triplicate assays. The *left curve* is a sigmoid curve with a Hill coefficient of 1.31, in keeping with the fact that m1-toxin binds pseudoirreversibly (Fig. 9), and with the fact that m1-toxin can bind to receptors that have already bound NMS (Max, 1992). Half-blockade of m1 receptors occurred with 0.55 units of m1-toxin. The *right curve* is a sigmoid curve with a Hill coefficient of 0.98, suggesting a close approach to equilibrium, and true competition between m1-toxin and NMS for m4 receptors. The indicated IC_{50} value is 45 units of m1-toxin per milliliter. Under these assay conditions the K_d of NMS for m4 receptors was determined by self-competition to be 0.17 nM (not shown).

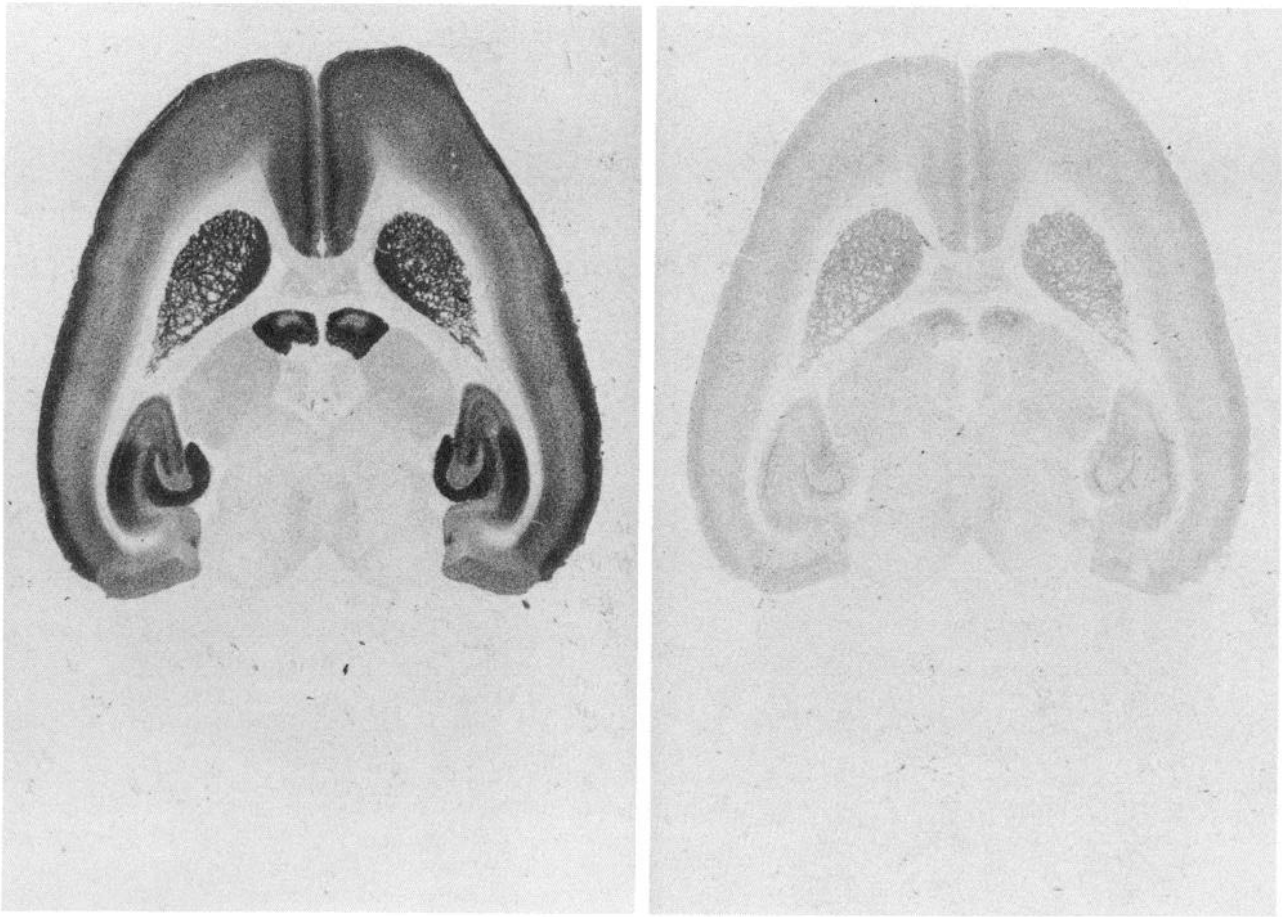

Figure 12. Autoradiography of muscarinic receptors in the rat brain, using a concentration of ^{3}H-pirenzepine (2 nM) that labels many m1 receptors and a few m4 receptors. The horizontal section shown at the *left* was obtained without using m1-toxin, and shows typical dense labeling of m1 receptors overlying the dendritic fields of granule cells in the dentate gyrus, and of pyramidal cells in the CA1 region of the hippocampus. The *right section,* which was treated with m1-toxin before ^{3}H-pirenzepine, shows an almost complete loss of binding over the dendritic fields noted, and clear residual binding over the granule and pyramidal cell layers. The localization of bound pirenzepine in this whole right-hand figure is very similar to the distribution of m4 receptors found by Levey et al. (1991) using m4-selective antibodies. Sections pretreated with nonradioactive QNB were blank.

muscles, in membranes, and in solution (Miledi and Potter, 1971). Adem et al. (1988) were the first to find toxins for muscarinic ACh receptors, and their suggestion that such toxins might be specific for receptor subtypes has proven correct. Only a few toxins have been found that interact with receptors coupled to G-proteins. These include MTX1, MTX2, and m1-toxin from *D. angusticeps,* and two toxins from *Vipera russelli,* which block adrenergic, dopaminergic, 5-HT, and opiate receptors (Freedman and Snyder, 1981; Bevan and Heistand, 1983). The latter toxins are known to have some phospholipase A_2 activity, although enzyme activity is not believed to be necessary for receptor blockade.

A large fraction of the literature about muscarinic receptors in the last decade has concerned efforts to identify the numbers, locations, and functions of different receptor subtypes. The most reliable data concerning the numbers and locations of m1–m5 receptors have come from the use of specific antibodies to intracellular portions of these receptors (Dörje et al., 1991a; Levey et al., 1991; Wall et al., 1991a,b). Unfortunately these antibodies are not satisfactory for functional studies because of their intracellular sites of action and slow diffusion. Pirenzepine has been the most widely used m1 antagonist, but it is now clear that its selectivity for m1 receptors is limited. Sub-K_d levels of ^{3}H-pirenzepine (1 nM) can be used to label cortical and hippocampal m1 receptors for binding studies with about 95% specificity (Potter et al., 1988; see also Fig. 2), because of the sixfold greater affinity of pirenzepine for m1 than for m4 receptors (Buckley et al., 1989; Dörje et al., 1991b) and the nearly threefold greater number of m1 receptors in these tissues (Levey et al., 1991). However, pirenzepine cannot be used to block m1 receptors selectively so as to identify the coupling mechanisms and responses of activating these receptors, or to study remaining receptors accurately. Our autoradiographs (Fig. 12) illustrate the problem with using pirenzepine as a "selective" m1 antagonist. Toxin-blockable m1 receptors in the CA1 region of the hippocampus are primarily in the dendritic fields of bipolar pyramidal cells. In contrast, toxin-spared ^{3}H-pirenzepine sites (presumptive m4 receptors) are localized close to the somata of these cells. Since many of the receptors near these cells bind low concentrations of pirenzepine but are not m1 receptors, pirenzepine cannot be used as a reliable m1-selective antagonist near these cells. It is not surprising, therefore, that Dutar and Nicoll (1988) were unable to utilize pirenzepine to differentiate the receptors responsible for the different responses of hippo-

campal cells to carbachol. Physiological studies of this kind need to be repeated with m1-toxin.

The negligible dissociation of m1-toxin after its binding to m1 receptors is an advantage for some studies and a disadvantage for others. For anatomical work m1-toxin has the attractive features that it can diffuse to receptors in intact tissue, thereby labeling functional sites rather than sites of receptor synthesis and passage. The fact that the toxin binds almost irreversibly means that the free toxin can be fully removed by prolonged washing. The toxin can also be fully fixed on receptors for histological and electron microscopic studies at high resolution. For biochemical and biophysical studies m1-toxin can be used to identify and stabilize m1 receptors long after their solubilization (Max, 1992); however, the affinity between the toxin and receptors appears too great for the toxin to be used, at least without modification, for affinity chromatography. For physiological and pharmacological studies it is generally an advantage to have a specific but reversible ligand, so that studies can be carried out at equilibrium with competing ligands. Such studies are not now possible with m1-toxin. Nonetheless, m1-toxin should prove very useful for identifying which functional responses are due to m1 receptors. We have also found the toxin useful for blocking m1 receptors in the striatum so as to study the plentiful m4 receptors that remain (Purkerson et al., 1991, 1992). In sum, it appears likely that m1-toxin will prove to be the antagonist of choice for a variety of kinds of studies of m1 receptors.

It is fascinating that snakes have been able to evolve and select toxins that presumably have a common genetic ancestry, so as to block the activity of three entirely different families of ACh-binding proteins: nicotinic receptors, muscarinic receptors, and esterases. The features of MTX2 and m1-toxin that are different from those in anti-nicotinic toxins (Fig. 9) offer some ideas as to which amino acids may be important for anti-muscarinic activity. Future comparisons of the primary structures and nuclear magnetic resonance spectra of anti-muscarinic toxins that have different receptor specificities should offer a better picture of the features that confer m1 and m4 selectivity. At least in theory, the tertiary structure of m1-toxin and of m1 receptors, in complexes, can also be determined by x-ray crystallography.

While it is easy to determine how much m1-toxin is necessary to block m1 receptors selectively (Figs. 6, 11), most other quantitative measurements of anti-muscarinic activity are complicated. The fact that m1-toxin is a trace component of the venom limits the number of accurate assays of toxin protein that can be performed, and limits the volumes of media that can be used. Recovery of m1-toxin during its purification is difficult to estimate because of the presence of at least three toxins in the venom with anti-m1 activity, and because of the possibility that crude venom fractions contain factors that increase the binding of ^{3}H-antagonists to m1 as well as m2, m3, and m5 receptors (Fig. 1). While both of our purification steps would be expected to yield most of the m1-toxin present in the active fractions, and while we have recovered about 100 of about 145 expected micrograms of anti-m1 toxin from a gram of dry venom, our recovery of anti-m1 activity has been variable and generally below 10% of the activity in the venom. We suspect that the acid conditions and/or organic solvents used during HPLC have altered the tertiary structure of m1-toxin, and inactivated about 95%. Therefore, milder conditions for HPLC are being explored. Denaturation of m1-toxin is the most likely explanation for our observation that full blockade of m1 receptors has generally required a molar ratio of toxin to receptor of about 20 (Fig. 6). In theory one molecule of toxin should be sufficient to block one molecule of receptor, and the toxin concentration should be irrelevant as long as there is sufficient total toxin to block all m1 receptors and sufficient time to achieve full blockade. If it is assumed that 5% of our purified toxin is active, then the affinity of m1-toxin for m4 receptors may be estimated from the data in Figure 11 and the Cheng-Prusoff equation as 8 nM. The fact that m1-toxin binds pseudoirreversibly to m1 receptors has so far precluded accurate measurements of its affinity for m1 receptors, and thus precluded accurate statements about its true selectivity for m1 over m4 receptors. An additional factor that complicates assays of the activity and selectivity of m1-toxin is the fact that the toxin can bind allosterically to receptors that have already bound a ^{3}H-antagonist (Max, 1992). For practical purposes, m1-toxin at low concentrations needs to be applied before other ligands in order to demonstrate its activity. In contrast, m1-toxin competes with NMS for m4 receptors, and the concentration of toxin used during assays does determine the results obtained.

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