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# m2-Toxin: A Selective Ligand for M2 Muscarinic Receptors

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## ABSTRACT

Selective ligands are needed for distinguishing the functional roles of M2 receptors in tissues containing several muscarinic receptor subtypes. Because the venom of the green mamba *Dendroaspis angusticeps* contains the most specific antagonists known for M1 and M4 receptors (m1-toxin and m4-toxin), it was screened for toxins that inhibit the binding of [$^3$H]*N*-methylscopolamine ([$^3$H]NMS) to cloned M2 receptors. Desalted venom had as much anti-M2 as anti-M4 activity. The most active anti-M2 toxin in the venom was isolated by gel filtration, cation-exchange chromatography, and reversed-phase HPLC, and called m2-toxin. Spectrometry yielded a mass of 7095 Da, and N-terminal sequencing of 53 amino acids showed RICHSQMSSQPPTTTFCRVNSCYRRTLRDPHDPRGT-IIVRGCGCPRMKPGTKL. This sequence is more homologous to antinicotinic than antimuscarinic toxins, but it lacks three almost invariant residues of antinicotinic toxins required for their activity. m2-Toxin fully blocked the binding of [$^3$H]NMS and [$^3$H]oxotremorine-M to M2 receptors with Hill coefficients near 1, and blocked 77% of the binding sites for 0.1 nM [$^3$H]NMS in the rat brainstem ($K_i$ = 11 nM). Concentrations that fully blocked cloned M2 receptors had no effect on M4 receptors, but slightly increased [$^3$H]NMS binding to M1 receptors, an allosteric effect. In accord with these results, light microscopic autoradiography of the rat brain showed that m2-toxin decreased [$^3$H]NMS binding in regions rich in M2 receptors and increased binding in regions rich in M1 receptors. Thus m2-toxin is a novel M2-selective, short-chain neurotoxin that may prove useful for binding and functional studies.





Experiments with subtype-selective antibodies and cDNA probes have partially mapped the distribution of M2 muscarinic receptors in the brain and peripheral tissues (Levey et al., 1991; Levey, 1993). These receptors are prevalent in cholinergic nerves and at many postsynaptic sites. Brain regions and tissues with relatively high concentrations include the nuclei of the facial and trigeminal nerves; the superior and inferior colliculi; the anteroventral nucleus of the thalamus; cardiac muscle; and smooth muscles.

Although subtype-selective antibodies are useful for elucidating the distribution and approximate levels of M2 receptors in tissues, they are not useful for studies of the functional roles of M2 receptors. The most M2-selective antagonists currently available, gallamine and AF-DX 116, show only 30- and 15-fold higher affinity for M2 than for M1 receptors (Dong et al., 1995). Similarly, there are no truly selective agonists for M2 receptors (Lazareno et al., 1993). Therefore, it is important to find new ligands for M2 receptors. Because the venom of the east African green mamba has been shown to contain toxins that bind with very high selectivity to M1 and M4 receptors (m1-toxin and m4-toxin) (Max et al., 1993a,c; Liang et al., 1996; Carsi and Potter, 1999), we screened this venom for toxins with anti-M2 activity. This article describes the isolation and partial characterization of the first toxin that binds with high selectivity to M2 receptors.

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## Materials and Methods

**Materials.** [*N*-methyl-$^3$H]-scopolamine ([$^3$H]NMS; 84 Ci/mmol) and [$^3$H]-oxotremorine-M ([$^3$H]oxo-M; 85 Ci/mmol) were obtained from Dupont–New England Nuclear Products (Boston, MA).

**Venom.** Lyophilized venom from the green mamba was obtained from the Miami Serpentarium (Punta Gorda, FL). To remove acetylcholine (ACh), which constitutes 2 to 3% of the venom, 0.5 g of dry venom was dissolved in 10 ml of ice-cold 30 mM ammonium acetate buffer at pH 7, and centrifuged at 27,000$g_{max}$ for 15 min to remove insoluble material. The fluid was centrifuged at 3000$g_{max}$ in a Centriprep-3 concentrator (Amicon/Millipore, Bedford, MA) with a molecular weight cutoff of 3000 Da until its unfiltered volume was 1 ml. This residual milliliter was diluted 10-fold with fresh buffer, and centrifugation and dilution were repeated twice to remove >99% of the ACh and other small molecules in the venom sample.

**Gel Filtration of Venom Proteins.** Gel filtration was performed using Sephadex G-50 as described previously (Max et al., 1993a). Fractions of the eluate were screened for anti-M2 activity, pooled as required, and lyophilized.

**Cation-Exchange Chromatography.** Fractions obtained by gel filtration were reconstituted with 10 ml of 30 mM ammonium acetate buffer (pH 6.5) and applied to a 100-ml column of Bio-Rex 70 (Bio-Rad, Hercules, CA) at 4°C. Proteins were eluted with a linear gra-

**ABBREVIATIONS:** NMS, *N*-methylscopolamine; oxo-M, oxotremorine-M; ACh, acetylcholine; TFA, trifluoracetic acid; CHO, Chinese hamster ovary.

dient formed by mixing 500 ml of 0.1 M ammonium acetate and 500 ml of 1.0 M ammonium acetate (both pH 6.5). Ninety 8.0-ml fractions were collected and tested for anti-M2 activity. Fractions with anti-M2 activity were pooled and lyophilized.

**Reversed-Phase HPLC.** The most active anti-M2 component retained from the Bio-Rex 70 column was reconstituted in 1 ml of 15% acetonitrile in 0.1% trifluoracetic acid (TFA) and applied to a 10 × 100 mm reversed-phase column (Aquapore C18; Applied Biosystems, San Jose, CA). Proteins were eluted at 23°C with a linear gradient of 15 to 30% acetonitrile in 0.1% TFA, during 70 min, at a flow rate of 1.5 ml/min. Protein peaks were monitored at 280 nm, collected manually, and assayed for anti-M2 activity. The active peak was lyophilized, reconstituted in 1 ml of 15% acetonitrile in 0.085% TFA, and applied to an analytical $C_{18}$ reversed-phase column (4.6 × 250 mm; Vydac, Separations Group, Hesperia, CA). m2-Toxin was eluted at 23°C with a linear gradient of 15 to 25% acetonitrile in 0.085% TFA, at a flow rate of 1.5 ml/min, during 90 min. Protein peaks were detected at 220 nm and tested for antimuscarinic activity, and the pure toxin was lyophilized.

**Assays of the Ability of Toxins to Inhibit Binding of [$^3$H]NMS.** Membranes from the rat brainstem (Potter et al., 1984) and Chinese hamster ovary (CHO) cells containing cloned M2 receptors (Max et al., 1993a) were prepared as described previously. Membranes from 1 mg of cells or tissue were incubated with aliquots of toxin and 0.1 nM [$^3$H]NMS in 1.0 ml of 50 mM sodium phosphate buffer containing 1.0 mM EDTA (pH 7.4) (phosphate–EDTA buffer) for 2 h at 25°C. Membranes were collected on glass fiber filters; the filters were dried and radioactivity was counted at an efficiency of 49 to 51% (Potter et al., 1984). Inhibition curves with analyzed with a computer program (GraphPad Prism; San Jose, CA). Cloned M2 receptors were used during the purification of m2-toxin.

**Selectivity of m2-Toxin for M1, M2, and M4 Receptors.** Membranes from 1 mg of CHO cells expressing M1, M2, or M4 muscarinic receptors were incubated with aliquots of toxin and 1.0 nM [$^3$H]NMS in 0.15 ml of phosphate–EDTA buffer for 2 h at 25°C. Membranes were collected by filtration, dried, and counted for radioactivity.

**Effect of m2-Toxin on the Binding of [$^3$H]oxo-M.** Membranes from 1 mg of rat brainstem were incubated with various concentrations of m2-toxin and 5.0 nM [$^3$H]oxo-M in 0.15 ml of 20 mM Tris buffer containing 1.0 mM $MnCl_2$ (pH 7.4) for 90 min at 25°C. Membranes were collected by filtration and dried, and their radioactivity was counted.

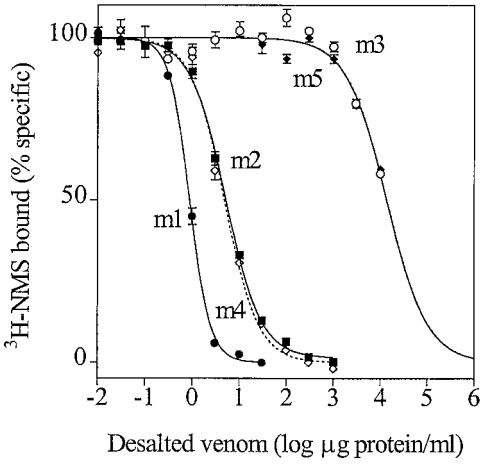


**Fig. 1.** Effect of desalted venom on the binding of [$^3$H]NMS to cloned human M1 to M5 receptors in CHO cell membranes. The points are mean values from triplicate assays ± S.E. Specific counts per minute for these assays with M1 to M5 receptors were 2084, 4240, 2056, 3060, and 2906 cpm, respectively. Lines are computer fits to a one-site binding model, except for the curve for M1 receptors, which had a Hill coefficient of 2.0. Note the presence of as much anti-M2 as anti-M4 activity, and the fact that the venom contains components that can block fully the binding of [$^3$H]NMS to M1, M2, and M4 receptors.

**Autoradiography.** The procedure was modified slightly from Mash and Potter (1986). Parasagittal sections of rat brain, 18-µm thick, were incubated on slides with 0 or 10 µg/ml of m2-toxin in phosphate–EDTA buffer for 30 min at 25°C. [$^3$H]NMS was added to a concentration of 0.1 nM, and incubation was continued for 1 h. Sections were rinsed three times for 10 min with ice-cold buffer containing 1.0 µM (±)-quinuclidinyl benzilate, dried, and apposed to Hyperfilm (Amersham, Arlington Heights, IL) for 7 weeks at room temperature.

**Amino Acid Sequencing.** Ten micrograms of m2-toxin was sequenced by Edman degradation on a Procise liquid/gas phase sequencer (Applied Biosystems, Foster City, CA). Sequences were aligned with a computer program (DNASTAR, Madison, WI).

## Results

Figure 1 shows the effect of desalted mamba venom on the binding of [$^3$H]NMS to cloned M1 to M5 receptors in CHO cell membranes. It is evident that the venom contains toxins that prevent the binding of [$^3$H]NMS to M1, M2, and M4 receptors, and that there is as much anti-M2 as anti-M4 activity. Negligible anti-M3 or anti-M5 activity was found.

Figure 2 shows gel filtration of crude mamba venom on Sephadex G-50. Anti-M2 activity was prevalent in the third protein peak, which contains monomeric toxins (Harvey et al., 1984). These fractions were pooled and lyophilized.

Figure 3 shows chromatography of the anti-M2 material retained from the Sephadex G-50 eluate on the cation exchange resin Bio-Rex 70. Two peaks of anti-M2 activity were obtained. The second peak showed higher total activity and less protein and was lyophilized.

Figure 4 shows reversed-phase chromatography of the anti-M2 material retained from the previous step, on a preparative C18 column. A single peak of anti-M2 activity was eluted in 18% acetonitrile, pooled, lyophilized, and run again on an analytical C18 column. By comparison, m1-toxins 1–4 and m4-toxin elute from the same column in 25 to 27% acetonitrile (Liang et al., 1996; Carsi and Potter, 1999). Examination of the purified toxin by mass spectrometry showed a linear mass of 7095 Da (Carsi-Gabrenas, 1997).

Figure 5 shows the ability of the purified toxin to inhibit the binding of [$^3$H]NMS to muscarinic receptors in mem-

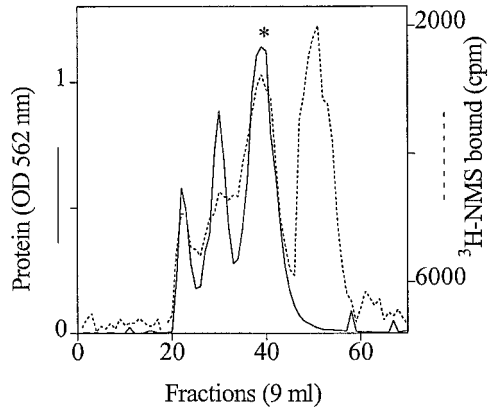


**Fig. 2.** Gel filtration of green mamba venom on a 600 ml column of Sephadex G-50. Three major protein peaks were obtained, as in previous studies (Max et al., 1993a). Anti-M2 activity was prominent with the third protein peak (*), which was pooled and lyophilized for the next step. The next peak of anti-M2 activity is absent when the sample is desalted venom, and it appears to be attributable to ACh, although most small molecules elute near fraction 67 (J. S. Liang and L. T. Potter, unpublished observations).

branes from the rat brainstem. The inhibition curve had a Hill coefficient close to 1.0, indicating that the toxin excludes NMS from M2 receptors. The calculated $K_i$ was 11 nM, assuming a toxin mass of 7095 Da and a $K_d$ of 0.125 nM for [$^3$H]NMS under the conditions used (Potter et al., 1991). The toxin blocked 77% of the total [$^3$H]NMS sites, in accord with evidence that 70 to 84% of brainstem muscarinic receptors precipitate with M2-selective antibodies (Levey, 1993).

The selectivity of the toxin for M2 muscarinic receptors was tested on CHO cells expressing cloned human M1, M2, and M4 receptors (Fig. 6). The toxin inhibited the binding of [$^3$H]NMS only to M2 receptors, and therefore was named m2-toxin. However, concentrations sufficient to fully inhibit [$^3$H]NMS binding to M2 receptors slightly increased binding to M1 receptors. Receptors of the M3 and M5 subtypes were not tested, because they were unaffected by desalted venom.

The ability of m2-toxin to block the binding of [$^3$H]NMS was examined further by light microscopic autoradiography (Fig. 7). In the presence of m2-toxin, there was a marked decrease of [$^3$H]NMS binding in regions of the rat brain that show high levels of M2 receptors, including the trigeminal and facial motor nuclei, the superior and inferior colliculi, and pontine nuclei. At the same time, [$^3$H]NMS binding was enhanced in the cortex, striatum, and hippocampus, where M1 receptors are known to be prevalent.

The effect of m2-toxin on the binding of [$^3$H]oxo-M to M2 receptors is shown in Fig. 8. The toxin inhibited the binding

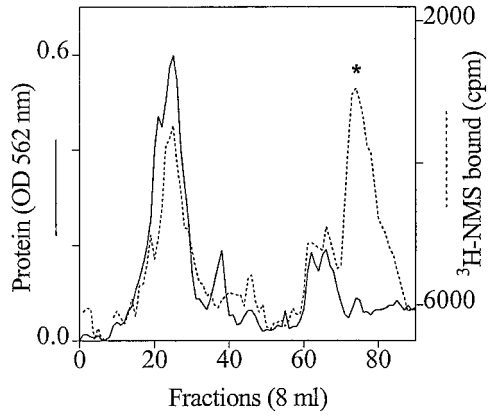


**Fig. 3.** Cation-exchange chromatography of the anti-M2 material retained from Sephadex G-50 on Bio-Rex 70. Two major peaks of anti-M2 activity were obtained. The second was pooled and lyophilized.

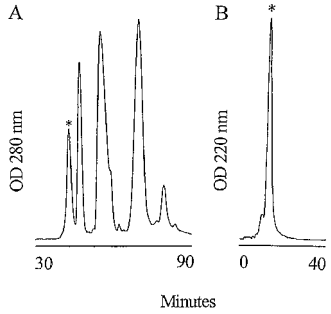


**Fig. 4.** Reversed-phase HPLC of the anti-M2 material retained from Bio-Rex 70 on (A) preparative and (B) analytical C18 columns. In each run, anti-M2 activity eluted in 18% acetonitrile.

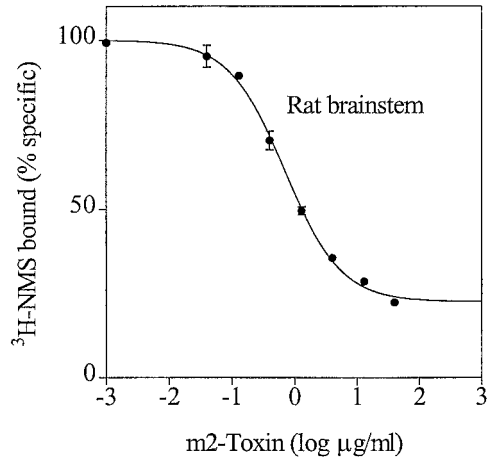


**Fig. 5.** Ability of m2-toxin to inhibit the binding of 0.1 nM [$^3$H]NMS to muscarinic receptors in membranes from the rat brainstem. The points are mean values from triplicate assays ± S.E. The line is a sigmoid curve with $r^2$ = 0.995, Hill slope = 0.98, and $IC_{50}$ = 0.7 μg/ml. Seventy-seven percent of the total binding sites for [$^3$H]NMS were blocked by m2-toxin.

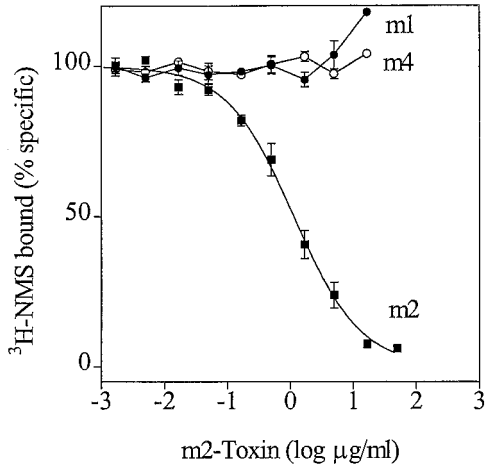


**Fig. 6.** Effect of m2-toxin on the binding of [$^3$H]NMS to cloned human M1, M2, and M4 receptors in CHO cell membranes. Points are mean values from triplicate assays ± S.E. The line for M2 receptors is a sigmoid curve with $r^2$ = 0.984, Hill slope = 0.82, and $IC_{50}$=1.1 μg/ml.

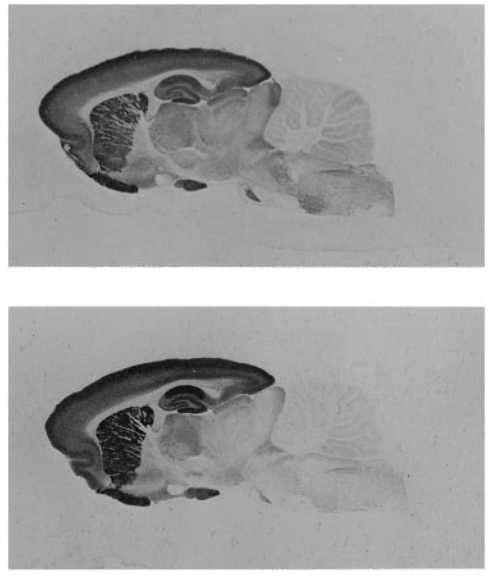

**Fig. 7.** Effect of m2-toxin on the binding of [$^3$H]NMS in parasagittal sections of the rat brain. The top image was obtained with [$^3$H]NMS alone, and the bottom image was obtained in 100 nM m2-toxin.

of this agonist with an $IC_{50}$ of 7.3 $\mu$g/ml. This corresponds to a $K_i$ of 231 nM, assuming a mass of 7095 Da for the toxin and a $K_d$ of 1.45 nM for [$^3$H]oxo-M under the conditions used (Potter et al., 1991).

A partial amino acid sequence for m2-toxin is shown in Fig. 9, and is compared with the sequence of erabutoxin b, a well studied antinicotinic toxin that has a compact core containing four disulfide bonds and three loops (Pillet et al., 1993; Ménez, 1998). m2-Toxin clearly has the structure of a curaremimetic toxin. The initial 53 amino acids of m2-toxin are identical with those of a toxin predicted from a cDNA cloned from the venom glands of the green mamba (gm60; A. Trinidad, W. Strauss, and L. T. Potter, unpublished observations). The calculated mass of gm60 is 7092 Da, assuming four disulfide bonds, a value very similar to the linear mass of m2-toxin (7095). Figure 9 also includes a comparison of m2-toxin/gm60 with five other toxins that bind to muscarinic receptors.

## Discussion

m2-Toxin is the first toxin that has been shown to distinguish M2 receptors from other muscarinic receptors, and it is the most specific ligand known for distinguishing M2 from M1 receptors. By comparison, gallamine binds to M2 receptors with 30-, 112-, 151-, and 363-fold higher affinity than to M1, M4, M5, and M3 receptors, respectively (Dong et al., 1995).

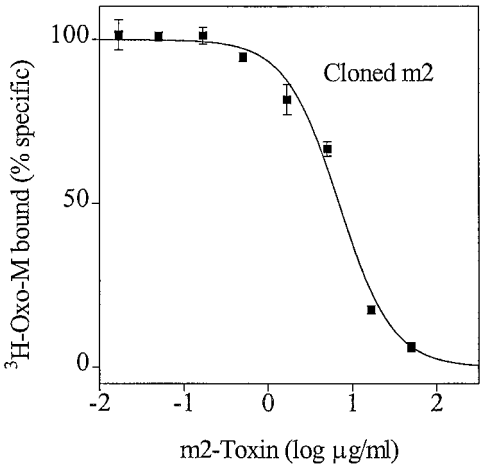


**Fig. 8.** Effect of m2-toxin on the binding of [$^3$H]oxo-M to cloned human M2 receptors. Points are mean values from triplicate assays ± S.E. The line is a sigmoid curve with $r^2$ = 0.981, Hill slope = 1.3, and $IC_{50}$ = 7.1 $\mu$g/ml.

The fact that m2-toxin increased the binding of [$^3$H]NMS to M1 receptors indicates that the toxin binds to M1 receptors allosterically, much as m1-toxin binds to M1 receptors allosterically (Max et al., 1993b). Therefore, m1-toxin can be expected to block the binding of m2-toxin to M1 receptors, and both toxins may be useful whenever it is necessary to distinguish M2 from other muscarinic receptors. Because m2-toxin binds outside the pocket of M1 receptors that holds NMS, it is likely that m2-toxin also binds outside the corresponding pocket of M2 receptors.

m2-toxin is probably identical with gm60 (Fig. 9), because their first 53 amino acid residues are identical and their linear masses were indistinguishable, and m1-toxin behaves like a strongly cationic peptide during cation exchange chromatography, whereas gm60 has a calculated pI of 9.3. The pI of gm60 is significantly higher than the pI values for 11 other toxins that affect muscarinic receptors (6.7–8.7 pI; Carsi and Potter, 1999).

m2-Toxin is the first toxin that binds to muscarinic receptors that has a sequence like that of antinicotinic toxins (Fig. 9). A search of Genbank nonredundant sequences comparable with m2-toxin showed 89 toxins with 59 to 83 residues, almost all of which are antinicotinic. The closest relative showed 60% identity and 67% homology. Antinicotinic toxins have six very highly conserved residues that are important for their functional activity: K-27, W-29, D-31, R-33, E-38, and K-47 (numbered as in erabutoxin b; Ménez, 1998). Because m2-toxin lacks residues corresponding to K-27, W-29, and E-38, it is not likely to have substantial antinicotinic activity.

Although m2-toxin is intriguing because of its similarity to antinicotinic toxins, it probably will prove more important for clues as to the structural features of antimuscarinic toxins that account for their binding selectivity. Figures 9 and 10 compare the sequences of six toxins that bind to M1 to M5 receptors. Fifteen residues are identical, including a strongly basic residue (R-34) near the tip of the center loop; P-33 also is identical in all but MT$\alpha$. Most of these residues are common among all short-chain neurotoxins. The diversity among the toxins that bind to M1 to M5 receptors is in their loops. m2-Toxin is distinctive for its long center loop, and it has a number of nonconserved residues in each loop. H-31 is of particular interest. Jolkkonen (1996) noted that MT$\alpha$ and MT4 are identical except for residues 31 to 33 (LNH versus

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m2-toxin        RICHSQMSSQPPTTTFCRV--NSCYRRTLRDPHDPRGTIIVRGCGCPRMKPGTKL
gm60            RICHSQMSSQPPTTTFCRV--NSCYRRTLRDPHDPRGTIIVRGCGCPRMKPGTKLECCTSDKCNV
erabutoxin b    RICFNHQSSQPQTTKTCSPGESSCYHKQWS---DFRGTIIERGCGCPTVKPGIKLSCCESEVCNN

m2-toxin/gm60   RICHSQMSSQPPTTTFCRVNS--CYRRTLRDPHDPRGTIIVRGCG--CPRMKPG-TKLECCTSDKCNV
m1-toxin1       LTCVKSNSIWFPTSEDCPDGQNLCFKR--WQYISPRMYDFTRGCAATCPKAE-YRDVINCCGTDKCNK
m4-toxin        LTCVTKNTIFGITTENCPAGQNLCFKR--WHYVIPRYTEITRGCAATCPIPENY-DSIHCCKTDKCNE
MT1             LTCVTSKSIFGITTENCPDGQNLCFKK--WYYIVPRYSDITWGCAATCPKTNVRETIRCCETDKCNE
MT4             LTCVTSKSIFGITTENCPDGQNLCFKK--WYYIVPRYSDITWGCAATCPKTNVRETIHCCETDKCNE
MTα             LTCVTSKSIFGITTENCPDGQNLCFKK--WYYLNHRYSDITWGCAATCPKTNVRETIHCCETDKCNE
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**Fig. 9.** Amino acid sequences. The top sequences compare m2-toxin and gm60 (see text) to a typical antinicotinic toxin, erabutoxin b (Pillet et al., 1993). The bottom sequences compare six toxins that bind to muscarinic receptors; the line identifies the three-loop regions of each toxin (see Fig. 10). m1-toxin binds specifically and irreversibly to M1 receptors (Carsi and Potter, 1999); m4-toxin binds with 102-fold higher affinity to M4 ($K_i$ = 0.96 nM) than to M1 receptors (Liang et al., 1996); MT1 and MT4 bind to M1 and M4 receptors with $K_i$ values of 49 to 87 nM (Jolkkonen, 1996); MT$\alpha$ binds to M1 and M4 receptors with $K_i$ values of 23 to 44 nM, and to M3 to M5 receptors with $K_i$ values of 3 to 8 nM (Jolkkonen, 1996). Note that all of these toxins (including m2-toxin) have some affinity for M1 receptors.

IVP), yet only MT$\alpha$ binds with high affinity to M3 to M5 receptors. Therefore, residues 31 to 33 must confer the different specificities of these two toxins, and the corresponding HDP (and especially the H-31) of m2-toxin may help confer its M2-selectivity. We are expressing mutant forms of m1-toxin with residues 31 to 33 characteristic of the other subtype-selective toxins to test the idea that this region is important for binding specificity (Krajewski et al., 1999).

Antinicotinic toxins labeled with radioiodine have proved extremely useful for quantitative studies of nicotinic receptors (since Miledi and Potter, 1971); thus, experiments with $^{125}$I-labeled m2-toxin are planned. It may be noted that radioactive gallamine has not been available for binding studies; thus, a labeled M2-selective antagonist would be very helpful for research purposes.

As expected, m2-toxin blocked the binding of [$^3$H]oxo-M to M2 receptors, but the $K_d$ for blocking the binding of this agonist was 20-fold higher than the corresponding $K_d$ for [$^3$H]NMS (231 versus 11 nM). Some of the difference may arise from the different buffers used; however, we suspect that different allosteric effects in toxin-antagonist-receptor and toxin-agonist-receptor complexes also may be important. More accurate affinity data without allosteric effects can be expected from direct measurements with $^{125}$I-labeled m2-toxin. In preliminary studies, 30 nM m2-toxin had no effect on the rate of beating of isolated rat atria during exposure at room temperature for 5 min or the ability of 1.0 $\mu$M carbachol to reduce the resting rate by 80 to 100%. This is a long incubation time for a short-chain neurotoxin, which should diffuse well in tissues; thus, we do not understand why there was no effect of the toxin. Higher concentrations of m2-toxin probably are required to establish whether it is an agonist or antagonist, and we have not had enough toxin to perform these studies. In fact, the amounts of m2-toxin available from venom are so limited that useful quantities probably will need to be obtained by expressing the cDNA for m2-toxin or by direct peptide synthesis.

The present results showed at least one other m2-toxin in green mamba venom. Work is in progress to see whether it has a structure like that of m2-toxin or one like other antimuscarinic toxins. The venom did not show significant anti-M3 or anti-M5 activity (Fig. 1). Thus far, the only toxin that binds with high affinity to M3 and M5 receptors is MT$\alpha$ (Jolkkonen et al., 1995).

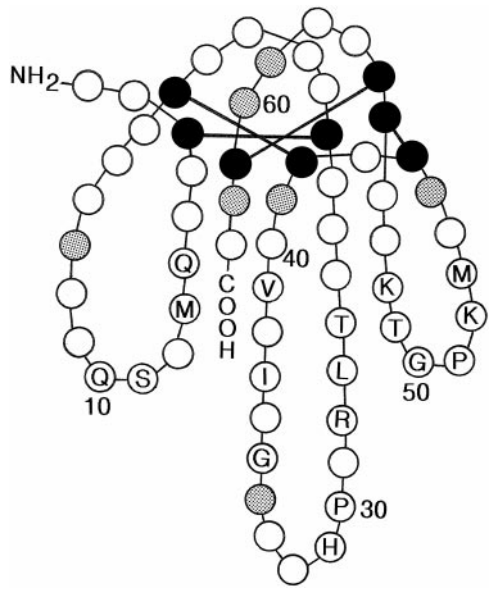


**Fig. 10.** Probable secondary structure of m2-toxin, based on the crystal structure of erabutoxin b (Low et al., 1976), the NMR structure of MT2 (Ségalas et al., 1995), and the assumption that m2-toxin is identical with gm60. Cysteine residues are shown in black, other identical residues of the antimuscarinic toxins in Fig. 9 are stippled, and letters identify nonconserved amino acids in the three loops of m2-toxin that may help to confer its M2-selectivity.

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