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Site-Directed Mutagenesis of m1-Toxin1: Two Amino Acids Responsible for Stable Toxin Binding to M_1 Muscarinic Receptors

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ABSTRACT

m1-Toxin1 binds specifically and irreversibly to M_1 muscarinic receptors and can slow the dissociation of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) from these receptors. Yet only 7 of its 65 amino acids are not conserved in six other mamba toxins that bind reversibly to M_2-M_5 muscarinic receptors. Two of these seven residues (Phe^{38}, Lys^{65}) were mutated to corresponding residues of the other toxins (Ile^{38}, Glu^{65}), to evaluate amino acids in m1-toxin1 that confer its remarkable affinity and specificity. The cDNA for m1-toxin1 was cloned from venom gland mRNA using polymerase chain reaction (PCR)-based techniques. Its nucleotide sequence is remarkably similar to those of other short-chain neurotoxins. The cDNAs for mutant toxins Phe^{38} to Ile^{38} (F38I) and Lys^{65} to Glu^{65} (K65E) were constructed by PCR-based techniques. Each cDNA was expressed in yeast, and the toxins were purified from yeast media by cation-exchange and reversed phase chromatography. Recoveries were 40 to 152 μg/l. Recombinant m1-toxin1 was identical to the native toxin (observed mass: 7471 Da; irreversible blockade of [^{3}H]NMS binding to cloned M_1 receptors at 25°C; no blockade of M_2-M_5 receptors; 6-fold slowing of [^{3}H]NMS dissociation at 37°C). F38I also bound specifically to M_1 receptors, but reversibly and without effect on NMS dissociation. Thus, Phe^{38} contributes to the stability of toxin-receptor complexes, but not to M_1-selectivity. K65E bound selectively and irreversibly to unliganded M_1 receptors but did not slow NMS dissociation. It is suggested that the C-terminal Lys^{65} of m1-toxin1 may contact an outer loop of the M_1 receptor.

The venom of the green mamba, *Dendroaspis angusticeps*, contains a number of trace isotoxins of m1-toxin (Max et al., 1993a,b,c), of which m1-toxin1 (Fig. 1; the renamed original m1-toxin) is the most prevalent (Carsi and Potter, 2000). m1-Toxin1 is a remarkably useful ligand for studying genetically defined M_1 muscarinic receptors. It binds specifically to otherwise unliganded M_1 receptors in vitro and in vivo (Max et al., 1993a,c; Liang et al., 1999; Carsi and Potter, 2000). Binding of the toxin completely blocks the subsequent binding of the antagonist [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) for several hours at 4–37°C (Max et al., 1993b; Carsi and Potter, 2000), and prevents the action of muscarinic agonists on M_1 receptors (Max et al., 1993c; Cuevas et al., 1997; Marino et al., 1998; Rouse et al., 1999, 2000). When applied after [^{3}H]NMS, the toxin forms toxin-NMS-receptor complexes that are quite stable in membranes and completely stable in digitonin-solution (Max et al., 1993b). The toxin must therefore bind allosterically to the unique extracellular loops and/or N terminus of M_1 receptors, which are located outside the receptor pocket that contains the binding site for NMS. ^{125}I-m1-Toxin1 dissociates slowly from toxin-receptor complexes at 37°C, indicating that toxin binding is not covalent (Potter, 2001).

The binding properties of m1-toxin1 indicate an exceptionally good molecular fit between the loops and/or ends of the toxin, and the extracellular loops and/or N terminus of M_1 receptors (Max et al., 1993b). The present studies are an initial step toward characterizing this fit, using site-directed mutants of m1-toxin1 to establish toxin residues that are essential for toxin binding. Future studies of interactions between mutants of both proteins should reveal a detailed picture of the toxin-receptor complex, and a mirror image of the receptor may then emerge from knowledge of the structure of the toxin. Several lines of information facilitate this approach. First, m1-toxin1 belongs to a large family of relatively rigid spoon-shaped short-chain toxins. NMR studies have shown that muscarinic toxin MT2, like other members of this family, has a dense core containing four disulfide

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ABBREVIATIONS: [^{3}H]NMS, [^{3}H]*N*-methylscopolamine; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; MG, minimal glycerol media; MM, minimal methanol media; BSA, bovine serum albumin; CHO, Chinese hamster ovary; HPLC, high-performance liquid chromatography.

bonds and four loops (Figs. 1 and 2; Ségalas et al., 1995). Most of the sequence diversity among short-chain toxins resides in their three larger loops (Fig. 1). Several residues in the corresponding loops of the similar toxin erabutoxin a have been mutated, yielding information about residues that perform structural and functional roles (Pillet et al., 1993; Tremeau et al., 1995). Second, the toxins that bind to muscarinic receptors differ only modestly in their sequences (Fig. 1), despite large differences in their binding characteristics. Only seven of the amino acids of m1-toxin1 are not conserved between other toxins that bind to muscarinic receptors, although only the m1-isotoxins bind specifically, irreversibly, and allosterically to M_1 receptors (Max et al., 1993a,b,c; Carsi and Potter, 2000). Two of these seven residues (Phe^{38}, Lys^{65}) were mutated to the corresponding residue that is conserved in the other muscarinic toxins (Ile^{38}, Glu^{65}; Fig. 1). The structural ramifications of making F38I and K65E were predicted from computer modeling of the atomic coordinates of MT2 (Ségalas et al., 1995). Ile^{38} exhibits multiple interactions with surrounding residues in the middle loop of MT2 (Fig. 2), so F38I is likely to have a slightly different conformation than m1-toxin1, but still be a stable molecule. The C-terminal Glu^{65} is exposed on the convex face of MT2 and interacts minimally with surrounding residues. So K65E should have a protruding C terminus with an opposite charge to that of the native toxin, yet no change in conformation. Finally, only 13 of the 77 extramembranous amino acids of M_1 receptors are not conserved in M_2-M_5 receptors, so the most likely sites in M_1 receptors for the specific binding of m1-toxin1 are limited (Hulme et al., 1990; Max et al., 1993b).

Recombinant m1-toxin1 and F38I and K65E were tested for four properties: 1) the ability of each toxin to block the binding of [^{3}H]NMS to cloned M_1 receptors in membranes, 2) the selectivity of the toxin for M_1 versus M_2-M_5 receptors, 3) the reversibility of M_1-blockade, and 4) the ability of the toxin to slow the dissociation of [^{3}H]NMS from M_1 receptors.

Experimental Procedures

Materials. An adult *Dendroaspis angusticeps* was obtained from Natural Selections (Miami, FL), guanidinium isothiocyanate and primers and a TA cloning kit from from Invitrogen (Carlsbad, CA), [^{3}H]*N*-methylscopolamine (82 Ci/mmol) and [^{32}P]ATP (3000 Ci/mmol) from PerkinElmer Life Science Products (Boston, MA), and restriction enzymes from New England Biolabs (Beverly, MA). Unless specified, all other reagents were from Sigma (St. Louis, MO). DNA sequencing was performed using a Thermal Cycling Sequencing kit (PerkinElmer). Sequences were aligned by the Clustal method (DNASTAR, Madison, WI). Protein assays were performed using a Micro-BCA kit from Pierce (Rockford, IL). Antiserum to m1-toxin1 was generated to a synthetic peptide (PKAEYRDVIN) by Tana Labs (Houston, TX).

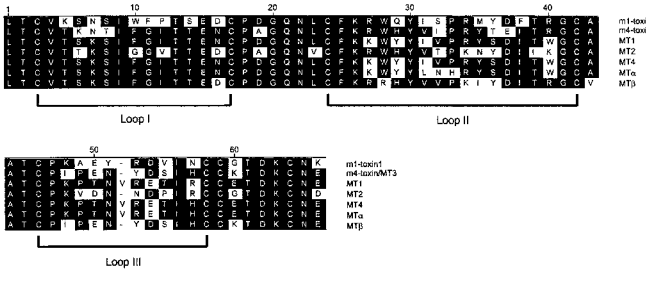

Fig. 1. Amino acid alignment of seven toxins from *D. angusticeps* and *D. polyepsis* that bind to muscarinic receptors. Black shading shows consensus homologies, and the C–C bonds that form the three larger loops in each toxin are indicated. The sequence diversity among these toxins is primarily in these loops. m1-Toxin1 binds specifically and irreversibly to M_1 receptors (Carsi and Potter, 2000) and m4-toxin binds with 102-fold higher affinity to M_4 than M_1 receptors (IC_{50} values of 0.95 and 95 nM; Liang et al., 1996). The other toxins bind reversibly and with nearly equal affinity to at least two receptor subtypes (Jolkkonen, 1996). MTα is unusual because it binds with 3 to 5 nM affinity to M_3-M_5 receptors. Other differences between these toxins include the fact that m1-toxin1 and m4-toxin are antagonists (Max et al., 1993a; Cuevas et al., 1997; Cuevas and Adams, 1997; Marino et al., 1998), whereas MT1 behaves as an agonist (Jerusalinsky et al., 1995).

Cloning the cDNA for m1-Toxin1. An adult *D. angusticeps* was chilled in a freezer and decapitated. Its venom glands were homogenized in 4.0 M guanidinium isothiocyanate, the homogenate was centrifuged over 5.7 M cesium chloride for 16 h at 214,000g_{max}, and the RNA pellet was resuspended in water treated with diethylpyrocarbamate (Chirgwin et al., 1979). Messenger RNA was isolated from total RNA using a PolyATract mRNA isolation kit (Promega, Madison, WI). Two degenerate oligonucleotides were designed to amino acid sequences unique to m1-isotoxins (Max et al., 1993a; Carsi and Potter, 2000). Reverse transcription was performed using a downstream primer, DEG2 (5′CAYTTRTCIGTICCRCARCARTTIATIAC3′), and 250 ng of venom gland mRNA. The resultant first strand cDNA was then used as a template for PCR, using an upstream primer, DEG1 (5′GARGAYTGYCCIGAYGGICARAAY3′), in combination with DEG2. PCR was performed with 25 cycles of 1 min at 95°C, 1 min at 65°C, and 1 min at 72°C. An adapter-ligated cDNA library was prepared from 1 μg of venom gland mRNA using a Marathon RACE kit (CLONTECH, Palo Alto, CA). RACE was initiated using 10 μl of a 1:25 dilution of the adaptor-ligated cDNA mix. The 50-μl reaction mixture contained 2.0 mM Mg, 0.3 mM dNTP, 0.5 μM each of primer AP1 (CLONTECH) and GSP-1 (5′AACAGTTAATGACGTCACGGTATTCC3′), and 2.5 units of Primezyme (Biometra, Inc., Tampa, FL). PCR was performed with 30 cycles of 30 s at 94°C, 2.5 min at 68°C, and a final 15-min incubation at 68°C. Iden-

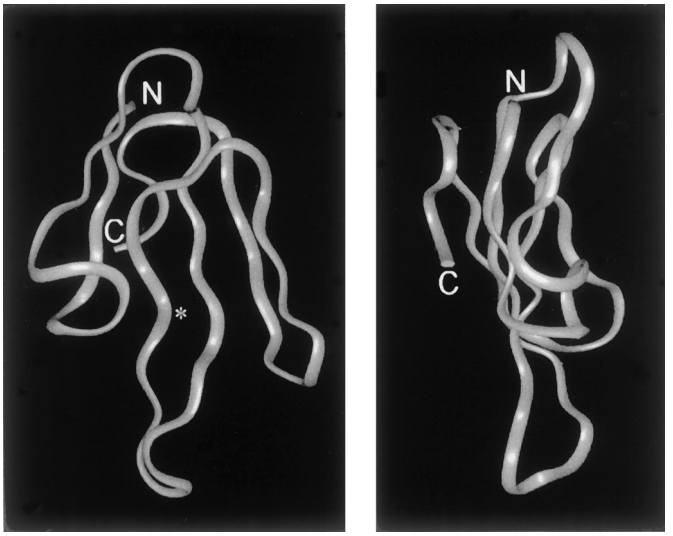

Fig. 2. Solution NMR structure of MT2. Atomic coordinates were provided by Ségalas et al. (1995). In the picture on the left, the molecule has been rotated to show the "three-fingered" folding pattern found in all short-chain neurotoxins. The position of Ile^{38} in the ascending portion of the middle loop is indicated with an asterisk. This region is stabilized by four C-C bonds in the core, by a double-stranded antiparallel β-sheet in the left loop shown here, and by a triple-stranded antiparallel β-sheet formed by the middle loop and the ascending portion of the right loop. In the picture on the right, the molecule has been rotated to show that the C-terminal region is exposed on the convex face of the toxin. Mutations of the C-terminal residue (Lys^{65} in m1-toxin1) are obviously much less likely to modify the conformation of the whole molecule than mutations near Ile^{38} (Phe^{38} in m1-toxin1).

tical conditions were used for 3′-RACE, except that GSP-2 (5′TTCTCCAAGAATGTATGACTTCACCAGG3′) replaced GSP-1. Both RACE products were purified on a 1% agarose gel, ligated into pCRII (Invitrogen) and used to transform DH5α cells (Invitrogen). Plasmids were purified using a Wizard Mini-Prep kit (Promega). Both strands were sequenced to check their fidelity. Restriction mapping of both PCR products identified a unique AatII site present in the region of overlap between the 5′ and 3′ RACE clones. This site was used to ligate the RACE products into pCRII to reconstruct the full-length cDNA. Two primers were designed to remove the untranslated region and to add restriction sites, 5′*Bam*HI (5′GCGGATCCGCCACCATGAAAACTCTGC3′) and 3′*Eco*RI (5′CGCGCGAATTCGAGCTATTTGTTGC3′); the underlined regions indicate the restriction sites. These facilitated cloning of the cDNA into a *Pichia pastoris* expression vector pPIC3.5 (Invitrogen), resulting in the m1-toxin1 expression plasmid pm1tx1. The GenBank accession number for m1-toxin1 cDNA is AF241871.

Site-Directed Mutagenesis. Site-directed mutants of m1-toxin1 were prepared by primer mismatch PCR. The primers for F38I were F38Iup (5′ACATCCCCTGGTGATGTCATACATTCTTGGAGAAATGTACTG3′) and F38Idown (5′AGAATGTATGACATCACCAGGGGATGTGCTGC3′); the underlined portions include the mismatch. Because K65 is a C-terminal amino acid, only one primer was designed for K65E, (5′TATATATGAATTCTGGAGCTATTCGTTGCATTTGTCTGTTCCGC3′). F38I was created using two separate PCR reactions and an outside primer, in this case the AOX1 primer to the pPIC3.5 vector, F38Iup for the 5′ side of the cDNA, and F38Idown for the 3′ side of the cDNA. The reaction conditions were a 2-min incubation at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, and 30 s at 72°C. Each PCR product was purified and used as a template in a third PCR reaction with only the AOX1 primers. The conditions for the PCR extension were a 2-min incubation at 94°C, followed by five cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. Then the PCR reaction incubation was changed to 25 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C. The PCR product was subcloned into the pPIC3.5 vector and sequenced to ensure PCR fidelity. The expression vector with the F38I mutant cDNA cloned downstream of the AOX1 promoter was called pF38I. K65E was generated by primer mismatch. Using the upstream primer AOX1 and the K65E primer, the PCR product was generated with the following protocol: 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C with a 10-min extension at 72°C. The K65E PCR product was subcloned into the pPIC3.5 vector downstream the AOX1 promoter, and named pK65E.

Pichia Growth Media. Minimal media contained 1.5% yeast nitrogen base, 75 mM ammonium sulfate, and 0.005% *d*-biotin. Minimal glycerol media (MG) was minimal media plus 5% glycerol. Minimal methanol media (MM) was minimal media plus 0.5% methanol. Buffered minimal glycerol media was MG plus 100 mM potassium phosphate at pH 6.0. Buffered glycerol complex media was buffered minimal glycerol media plus 2% peptone and 1% yeast extract. Buffered minimal methanol media was MM plus 0.5% methanol and 100 mM potassium phosphate at pH 6.0.

Expression and Purification of Recombinant m1-Toxin1. Ten micrograms of the expression vector with the cDNA for m1-toxin1 was linearized with *Sac*I and used to transform *P. pastoris* GS115 cells, using the Easy-Comp kit (Invitrogen). Recombinant cells were screened for their ability to grow on histidine-deficient media. An immunoblot method was used for the initial screening of recombinant toxin production (Wung and Gascoigne, 1996). Recombinant cells were grown on a nitrocellulose disk placed on MG agar plates for 2 days at 30°C. The disk was transferred to a fresh plate containing MM agar and incubated at 30°C. Fresh methanol was added to the lid every 24 h to replenish that lost by metabolism and evaporation. After 48 h a fresh nitrocellulose disk was placed on top of the cells for 3 h at 30°C. The top disk was removed and the cells were washed off with phosphate-buffered saline. The nitrocellulose was blocked with 5% dry milk in 0.1% Tween 20 for 1 h, treated with rabbit antiserum to m1-toxin1 (1:100), and then exposed to a secondary anti-rabbit antibody coupled to horseradish peroxidase (Sigma) at a dilution of 1:20,000. Detection of recombinant expression was performed with an ECL-Plus kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Recombinant *P. pastoris* clones were grown in buffered glycerol complex media overnight at 30°C at 300 rpm to an A_{600} of 2.0 to 6.0. Cells were pelleted at $4000g_{max}$ for 10 min at room temperature, resuspended in buffered minimal methanol media to an A_{600} of 2.0, and incubated at 30°C and 300 rpm. The level of expression was assessed after 24 to 72 h. Media from expressing cells were clarified by centrifugation at $10{,}000g_{max}$ for 10 min. The media were then filtered through a 0.45-μm cellulose acetate filter (Corning, Palo Alto, CA) and diluted 10-fold with sodium acetate buffer at pH 4.5 (buffer A) to a final concentration of 25 mM. The sample was pumped onto a 5.0-ml cation-exchange column (SP Hi-Trap; Amersham Pharmacia Biotech) at a flow rate of 5.0 ml/min. The column was washed with buffer A and then eluted with 100 mM sodium chloride. The anti-M_1 activity of fractions was determined with a radioligand binding assay (see below). The most active fractions were pooled, the pH was adjusted to 6.0 with NaOH, BSA was added to 0.5%, and the sample was lyophilized. The dry sample was dissolved in 10% acetonitrile-0.1% trifluroacetic acid and loaded on a C_{18} reversed phase column (10 × 250 mm; Vydac). The toxin was eluted with a gradient of 10 to 90% acetonitrile at 5.0 ml/min over 80 min, and fractions were assayed for activity. The active peak was lyophilized with 0.5% BSA at pH 6.0. The sample was resuspended in buffer A plus 20% acetonitrile, loaded on a cation-exchange HPLC column (7.5 by 50 mm; Vydac) and eluted with 0–500 mM NaCl at 1.0 ml/min during one hour. The pure toxin was desalted on a reversed-phase column (4.5 × 250 mm, Vydac), and samples of the single peak of toxin protein were taken for a Western immunoblot and for mass spectrometry. The remainder was lyophilized with 0.5% BSA at pH 6.0, resuspended in 50 mM sodium phosphate-1.0 mM EDTA at pH 7.41, and stored at 4°C.

Western Blot of Recombinant m1-Toxin1. One hundred nanograms of native and recombinant m1-toxin1 was loaded on a 10 to 20% Tricine gel and subjected to electrophoresis under denaturing conditions. The proteins were transferred to nitrocellulose, probed with antiserum to m1-toxin1, and detected using the ECL-Plus kit (Amersham Pharmacia Biotech).

Mass Spectrometry of Recombinant m1-Toxin1. Matrix-assisted laser desorption ionization/time of flight mass spectral analysis was performed at the Macromolecular Resources Center at the University of Colorado.

Expression and Purification of F38I and K65E. Ten micrograms of pF38I and pK65E was linearized with *Sac*I and used to transfect competent *P. pastoris* GS115 cells. Colonies were screened for mutant toxin production using the same immunoblot method as for wild-type toxin. The clones that secreted the most toxin were chosen for further studies. The purification procedures for F38I and K65E were identical to those for m1-toxin1, except that the sodium acetate buffer used for cation-exchange steps had a pH of 4.0.

Radioligand Binding Assays. The antimuscarinic activity of recombinant toxins was determined by measuring their ability to block the binding of 1.0 nM [^{3}H]NMS to membranes from CHO cells transfected with the cDNA for human M_1-M_5 receptors (Max et al., 1993a). Membranes were first incubated in 0.2 ml of 50 mM phosphate/1.0 mM EDTA buffer at pH 7.4 (phosphate-EDTA) and various concentrations of each toxin for 30 min at 25°C. [^{3}H]NMS was added to 1.0 nM and a final volume of 1.0 ml (0.25 ml for F38I), and incubation was continued for 1 h. Membranes were collected on filters, dried, and radioactivity was counted. Nonspecific binding was determined by including 1 μM (±)quinuclidinyl benzilate and omitting the toxin during the preincubation step. The amount of membranes used was adjusted so that the amount of specific [^{3}H]NMS binding was 1900 to 2800 cpm in each assay. Binding curves were analyzed with Prism 3.0 (GraphPad, San Diego, CA).

Measurement of Toxin Dissociation. Membranes from CHO cells expressing M_1 receptors were incubated in 0.1 ml of phosphate-EDTA buffer for 30 min at 25°C with sufficient amounts of each toxin to occupy 90% of M_1 receptors, as determined from competition assays. One nanomolar [^{3}H]NMS was added to a final volume of 5.0 ml (50-fold dilution) and incubation was continued for 5 to 180 min. The rate of association of [^{3}H]NMS to M_1 receptors was used to indicate the rate of dissociation of each toxin. Nonlinear regression was analyzed with Prism 3.0. The dissociation time given is the mean from three experiments ± S.E.M.

Allosteric Binding of Toxins. Membranes from CHO cells expressing M_1 receptors were incubated in phosphate-EDTA buffer with 1 nM [^{3}H]NMS for 1 h at 25°C to saturate all M_1 receptors with NMS as determined by competition assays. The rate of dissociation of [^{3}H]NMS from M_1 receptors was then determined after the addition of 1 μM atropine. To measure the effects of toxins on the dissociation of [^{3}H]NMS, 250 to 1000 nM toxin was added with 1 μM atropine. Nonlinear regression was analyzed with Prism 3.0.

NMR Structure of MT2. The atomic coordinates of the solution NMR structure of MT2 (Ségalas et al., 1995) were kindly provided by Isabelle Ségalas (C.E. Saclay, Gif-Sur-Yvette, France). The molecule was modeled using an Insight II program on a Silicon Graphics Indigo^2 computer.

Results

Cloning of cDNA for m1-Toxin1. Reverse transcription-PCR was carried out with mRNA purified from venom glands, using primers degenerate to portions of the m1-toxin1 protein sequence. These experiments yielded a PCR product of 93 base pairs, which was used to design primers specific to m1-toxin1 for use in RACE. The 5′- and 3′-RACE products were ligated together to form the full-length cDNA for m1-toxin1 (Fig. 3). This cDNA has 518 base pairs, of which 258 are the open reading frame. The deduced amino acid sequence of the open reading frame has a 21 amino acid signal peptide upstream of the mature amino acid sequence of m1-toxin1. The coding region of the cDNA for m1-toxin1, including its signal peptide, was subcloned into the expression vector pPIC3.5, placing it under control of the yeast alcohol oxidase 1 promotor.

Expression and Purification of Recombinant m1-Toxin1, F38I, and K65E. Recombinant clones were screened on MG agar deficient in histidine, and screened for toxin production using a modified immunoblot assay. Once a suitable clone was selected, the time course for toxin expression was examined. Maximal expression of m1-toxin1 was achieved in 48 to 60 h (Fig. 4). Similar results were observed for F38I and K65E (data not shown).

Recombinant m1-toxin1 was recovered from yeast culture media in three steps: concentration from the media by cation-exchange, purification on a reversed phase column, and purification on a cation-exchange HPLC column (Fig. 5). Pooled fractions from the peak of protein marked with an asterisk in Fig. 5 were desalted on a reversed phase column, and a single peak of toxin protein was obtained (data not shown). Yields ranged from 67 to 152 μg of toxin/l of media. Recombinant m1-toxin1 showed the same mobility during electrophoresis as the native toxin (Fig. 6) and its linear mass (7470.6 Da; Fig. 7) was essentially identical to the theoretical mass of 7472 Da. Thus, yeast processed and secreted a recombinant toxin identical in size to native m1-toxin1. F38I and K65E were purified with a similar protocol, and their yields ranged from 40 to 110 μg of toxin/liter of media.

Binding of Recombinant m1-toxin1, F38I, and K65E to Muscarinic Receptors. Preincubation of M_1 receptors with recombinant m1-toxin1, F38I, and K65E all blocked the binding of 1.0 nM [^{3}H]NMS to cloned M_1 receptors (Fig. 8). Similar amounts of m1-toxin1 and K65E were required to block similar amounts of M_1 receptors, indicating the presence of similar amounts of the active toxins. Nonetheless, the molar ratio of each toxin to receptor protein far exceeded a stoichiometric ratio (see *Discussion*). No blockade of binding was observed with M_2-M_5 receptors at toxin concentrations up to 2.2 μM for m1-toxin1 and K65E, and up to 5.0 μM for F38I (data not shown).

Duration of Binding of m1-Toxin1, K65E, and F38I to M_1 Receptors. The stability of M_1 receptor-toxin complexes was examined by observing the ability of [^{3}H]NMS (1.0 nM) to bind to receptors preincubated with a saturating amount of each toxin. Figure 9 shows that M_1 receptors preincubated with m1-toxin1 or K65E did not bind [^{3}H]NMS in 3 h at 25°C. However, M_1 receptors preincubated with F38I showed a rapid increase in [^{3}H]NMS binding sites (Fig. 9). Thus, recombinant m1-toxin1 and K65E bound irreversibly, whereas

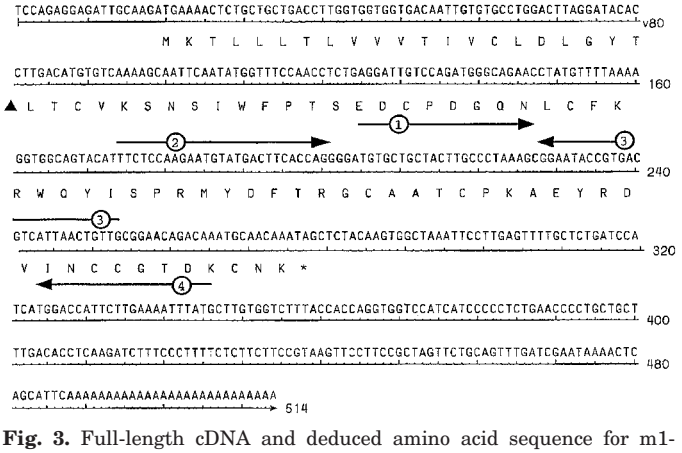

Fig. 3. Full-length cDNA and deduced amino acid sequence for m1-toxin1. The predicted site of signal peptide cleavage is indicated by the triangle (▲). Amino acid sequences that were used to design the primers DEG1 and DEG2 are numbered 1 and 4, and the gene specific primers GSP1 and GSP2 are numbered 3 and 2, respectively. The asterisk denotes the stop codon.

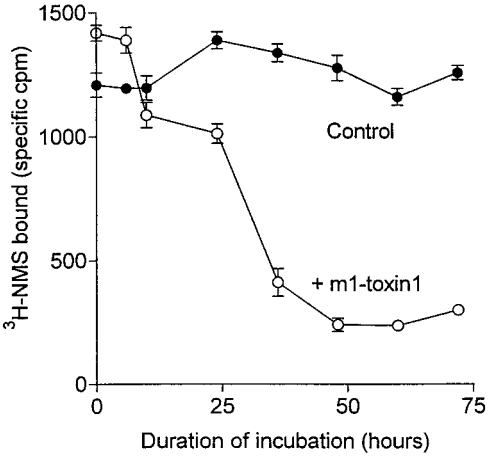

Fig. 4. Time course of expression of recombinant m1-toxin1. Aliquots of yeast media (100 μl) were assayed for their ability to block the binding of 1.0 nM [^{3}H]NMS to cloned M_1 receptors (n = 3). Media from cells transfected with parent plasmid pPIC3.5 served as controls (n = 3). Maximal expression was at 48 to 60 h.

F38I bound reversibly. Because F38I bound reversibly, its affinity for M_1 receptors must be lower than that of m1-toxin1 and K65E.

Allosteric Binding of Toxins to M_1 Receptors. The ability of recombinant toxins to stabilize the binding of [^{3}H]NMS to M_1 receptors was tested by measuring the rate of dissociation of [^{3}H]NMS from toxin-NMS-receptor complexes. [^{3}H]NMS had a half-life of 2.74 ± 0.14 min with no toxin (Fig. 10). In the presence of m1-toxin1 the half-life increased 6-fold to 15.98 ± 0.31 min, in keeping with prior results for the native toxin (Max et al., 1993b; Carsi and Potter, 2000). In contrast, neither F38I nor K65E had an observable effect on the dissociation of NMS (Fig. 10). These results indicate that K65E and F38I do not bind to NMS-M_1 receptor complexes with the same affinity as m1-toxin1.

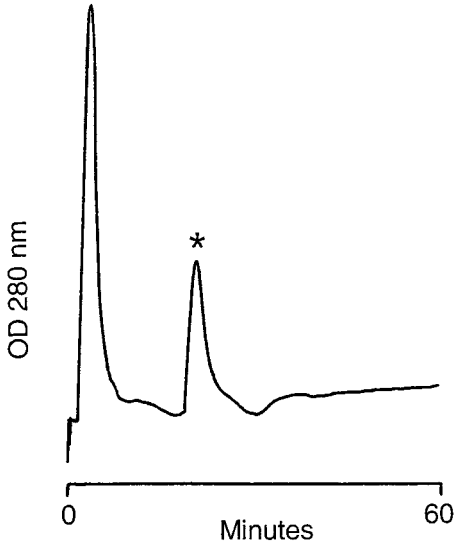

Fig. 5. Cation-exchange purification of recombinant m1-toxin1. This figure shows the third step in the purification of recombinant m1-toxin1. The lyophilized sample obtained after reversed phase purification was resuspended in buffer A plus 20% acetonitrile, loaded on a cation-exchange HPLC column and eluted with 0 to 500 mM NaCl at 1.0 ml/min during 1 h. The peak fractions of the peak of protein marked with an asterisk were used for all further studies (Western blot, mass spectrometry, desalting by reversed phase HPLC).

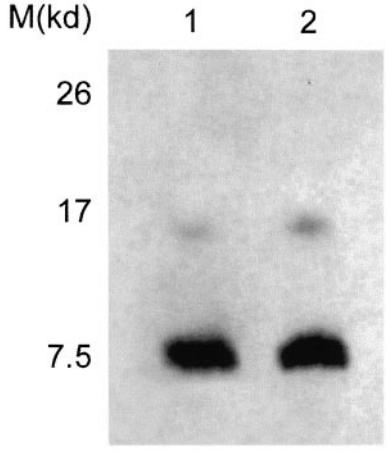

Fig. 6. Immunoblot of recombinant m1-toxin1. Aliquots of recombinant m1-toxin1 (lane 1) and m1-toxin1 purified from venom (lane 2) were loaded on a 10 to 20% Tricine gel and subjected to electrophoresis under denaturing conditions. The protein was transferred to nitrocellulose and probed with antisera to m1-toxin1. The recombinant toxin migrated at the same rate as the native toxin.

Discussion

The amino acid sequence of m1-toxin1 is highly homologous to those of cloned cDNAs for more than 100 short-chain toxins from members of families *Elapidae* and *Hydrophiidae* (GenBank), despite the fact that the snakes live in environments as diverse as trees and oceans, and the toxins bind to targets as diverse as nicotinic and muscarinic receptors, acetylcholinesterase, calcium channels, and voltage-gated potassium channels (Harvey, 1991). The signal peptide sequences of these toxins are 100% conserved at the amino acid level and 98% identical at the nucleotide level, implying a common ancestral origin for their genes (Ohno et al., 1998). The coding sequence of cloned m1-toxin1 established the correct full sequence of this toxin for the first time (Potter et al., 1996), because the original sequence for m1-toxin contained two errors (His^{28} and Trp^{29}; Max et al., 1993a). Trp^{28} and Gln^{29} have since been found in all the m1-isotoxins (Carsi and Potter, 2000). We did not obtain any full-length cDNAs cor-

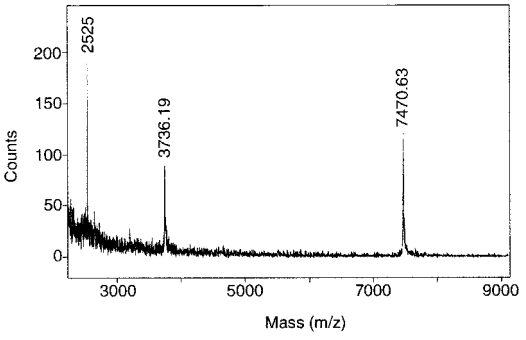

Fig. 7. Mass spectrometry of recombinant m1-toxin1. The parent peak measured 7470.6 Da, in agreement with a theoretical mass for m1-toxin1 of 7472 Da. The lower mass peaks are double and triple mass species, respectively.

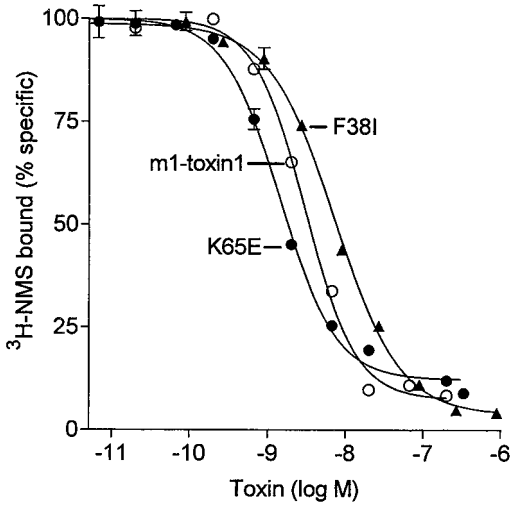

Fig. 8. Binding of recombinant toxins to muscarinic receptors. Membranes containing cloned human M_1 receptors were exposed first to recombinant m1-toxin1, F38I, or K65E, and then to 1.0 nM [^{3}H]NMS (n = 3 for each toxin). The total specific binding in each assay was 1900 to 2800 cpm. IC_{50} values for m1-toxin1 and K65E were calculated on the basis of the volume of buffer used during the preincubation step, because these toxins bound irreversibly (Fig. 9). The IC_{50} value for F38I was calculated on the basis of the volume used during exposure to [^{3}H]NMS, because this toxin bound reversibly. IC_{50} values for m1-toxin1, F38I, and K65E were 3.0 ± 0.2, 7.3 ± 0.26, and 1.7 ± 0.16 nM, respectively. No blockade of binding was observed with M_2-M_5 receptors at toxin concentrations up to 2.2 μM for m1-toxin1 and K65E, and 5.0 μM for F38I (data not shown).

responding to other m1-toxins. The full sequence of MT7 has been determined recently and was found to be the same as that of m1-toxin1 (Näsman et al., 2000).

The cDNAs for m1-toxin1, F38I, and K65E were expressed in *P. pastoris* so that the toxins would be correctly folded, processed, and secreted. The use of methylotropic yeast also meant that each toxin was secreted into media containing only salts and methanol, simplifying the purification of each toxin. Recombinant m1-toxin1 had the same linear mass as native m1-toxin1, and it bound specifically, irreversibly, and allosterically like the native toxin, demonstrating that our yeast expression system was satisfactory for producing m1-toxin1. The yeast also produced useful amounts of F38I and K65E.

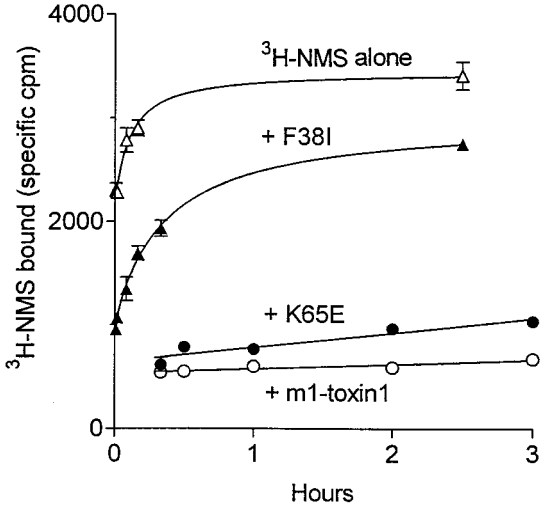

Fig. 9. Stability of toxin-receptor complexes. M_1 receptors were exposed first to a IC_{90} concentration of m1-toxin1 (15 nM), F38I (100 nM), or K65E (11 nM) and then to 1.0 nM [^{3}H]NMS (n = 3 for each toxin). No toxin was used for the controls. The rate of appearance of binding sites for [^{3}H]NMS at 25°C was taken as the rate of dissociation of each toxin. After exposure of M_1 receptors to F38I the binding of [^{3}H]NMS was near control levels at 45 min, indicating that F38I bound reversibly. In contrast, receptors exposed with m1-toxin1 or K65E showed no increase in [^{3}H]NMS binding sites throughout the experiment, indicating that [^{3}H]NMS binding sites were still occluded by these toxins.

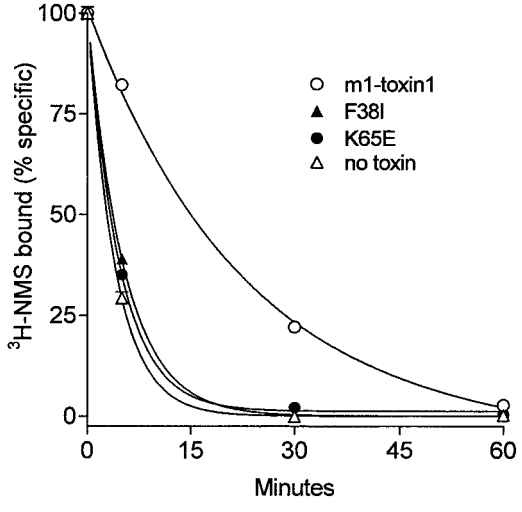

Fig. 10. Stability of toxin-NMS-M_1 receptor complexes. M_1 receptors were incubated with 1.0 nM [^{3}H]NMS at 37°C and then exposed to a saturating concentration of atropine (1.0 μM) with or without m1-toxin1 (50 nM), K65E (250 nM), or F38I (1.0 μM). The rate of dissociation of ^{3}H- NMS from M_1 receptors at 37°C with atropine alone was taken as the half-life for [^{3}H]NMS (2.74 ± 0.14 min). The half-life increased to 15.98 ± 0.31 min in the presence of m1-toxin1, a 6-fold increase ($p < 0.001$). K65E and F38I did not slow the dissociation of [^{3}H]NMS from M_1 receptors, indicating that these toxins were unable to bind to receptors occupied with [^{3}H]NMS (n = 3 for all toxins)..

Given the remarkable selectivity of m1-toxin1 for M_1 receptors, it was anticipated that any mutation of this toxin would alter its selectivity for M_1-M_5 receptors. To our surprise, we found that recombinant m1-toxin1, F38I, and K65E were all selective for M_1 versus M_2-M_5 receptors. So, positions 38 and 65 do not play a critical role in determining selective binding, and further experiments are necessary to determine which amino acids do confer subtype selectivity. Jolkkonen (1996) suggested that the amino acids at the tip of loop 2 might be critical, because MT4 and MTα are identical except for three amino acids at the tip of this loop (Fig. 1), and these two toxins differ dramatically in their specificity (Fig. 1). But the corresponding three amino acids in m1-toxin1 and m4-toxin differ relatively little, despite the markedly different specificities of these toxins.

m1-Toxin1 is also unique because of its irreversible binding to M_1 receptors (Max et al., 1993a,b,c; Carsi and Potter, 2000; Näsman et al., 2000; Olianas et al., 2000). Recombinant m1-toxin1 bound irreversibly as expected. K65E also bound irreversibly, showing that position 65 is not critical for irreversible binding. Because recombinant m1-toxin1 and K65E each bound irreversibly to unoccupied M_1 receptors, it is not possible to calculate an affinity for either toxin. F38I dissociated rapidly from M_1 receptors, so the Phe^{38} of m1-toxin1 must be important for irreversible binding. F38I may prove useful for functional studies when reversible M_1-blockade is desirable. We cannot calculate a reliable K_d for F38I at this time because of evidence of major losses of toxins during their lyophilization (see last paragraph).

Some inferences can be made about the role of Phe^{38} in m1-toxin1, based on the present results with F38I and the solution NMR structure of MT2 (Ségalas et al., 1995). The homologous residue to Phe^{38} in MT2 is Ile^{38}. Ile^{38} is in the middle of a β-sheet in loop 2 of MT2 (Fig. 2), and it has the second highest number of contacts with adjacent residues of any amino acid in the entire MT2 sequence. Assuming that m1-toxin1 and MT2 have a similar overall structure, mutation of Phe^{38} to Ile^{38} in m1-toxin1 should significantly alter the structure of the toxin in loop 2. The altered binding characteristics of F38I thus indicate that the structure of loop 2 is important for a tight molecular fit between m1-toxin1 and M_1 receptors.

Both native m1-toxin1 (Max et al., 1993b) and recombinant m1-toxin1 slow the dissociation of [^{3}H]NMS from M_1 receptors 6-fold. F38I did not slow the dissociation of [^{3}H]NMS, which is not surprising given its reversible binding to unliganded M_1 receptors. However, K65E also did not slow the dissociation of [^{3}H]NMS, even though K65E bound irreversibly to unliganded M_1 receptors. Thus, the positive C-terminal Lys^{65} of m1-toxin1 seems to be critical for the formation of ternary complexes of toxin, [^{3}H]NMS and M_1 receptors. Again, some inferences can be made about the role of Lys^{65} in m1-toxin1 for its binding to M_1 receptors, based on the solution structure of MT2. The homologous C-terminal residue to Lys^{65} in m1-toxin1 is Glu^{65} in MT2. Glu^{65} protrudes from the convex face of MT2 and makes few contacts with its adjacent residues (Fig. 2). The C-terminal regions of all the toxins

shown in Fig. 1 are nearly identical, so it is unlikely that the C-terminal residue of any of these toxins (including Lys^{65} in m1-toxin1) is important for their structural integrity. Nonetheless, and despite its irreversible binding to unliganded M_1 receptors, K65E did not block the dissociation of [^{3}H]NMS from M_1 receptors. Our interpretation of this result is that there is an interaction between Lys^{65} on the convex face of m1-toxin1, and an extracellular part of M_1 receptors. Because the only difference between m1-toxin1 and K65E is a negative C-terminal, it is possible that the highly basic C-terminal Lys of m1-toxin1 interacts with an acidic residue in one of the outer loops or the N terminus of M_1 receptors. Although any of the acidic residues on M_1 receptors might be involved, Glu^{170} in the middle of outer loop 2, Asp^{393} in the middle of loop 3, and Glu^{401} are of particular interest, because they are not found in M_2-M_5 receptors (Hulme et al., 1990). Glu^{401} is especially intriguing because it lies just outside the membrane surface next to Trp^{400}, which is essential for the allosteric effect of gallamine, and Glu^{401} just overlies Tyr^{404} and Tyr^{408} in transmembrane segment 7, which are important for the binding of NMS (Matsui et al., 1995). Site-directed mutagenesis of Glu^{401} in the M_1 receptor is therefore in progress, to evaluate whether Lys^{65} on the toxin, and Glu^{401} on the receptor, may interact.

It is well known that native m1-toxin1 (Max et al., 1993b) and synthetic MT7 (synthetic m1-toxin1; Olianas et al., 2000) do not block the specific binding of [^{3}H]NMS to M_1 receptors completely, and recombinant m1-toxin1 showed the same effect (Fig. 8). There are a number of possible explanations for this effect, including negative cooperativity between the binding of toxin and NMS at different sites (Olianas et al., 2000), reversible blockade of NMS by partly denatured toxin, and different access of NMS and toxin to intravesicular sites in membrane preparations.

We experienced two significant problems with the amounts of recombinant toxins that were recovered after expression. The first was that the levels of expression were inadequate for structural studies by NMR or circular dichroism. This problem might be ameliorated in future studies by using synthetic yeast codons instead of native cDNA codons for expression. The second problem was that it was necessary to use much larger quantities of recombinant m1-toxin1 and K65E for irreversible blockade (by a factor of 100 or more) than should be required for stoichiometric blockade. By comparison, M_1 receptors can be irreversibly blocked with synthetic m1-toxin1 or radioiodinated synthetic m1-toxin1 using only a slight molar excess of toxin over receptor (Potter, 2001). This second problem could have appeared in the present studies because of incorrect folding of m1-toxin1 and K65E. However, the fact that we obtained a single toxin protein by HPLC and by mass spectrography makes it unlikely that there was variable folding or impure protein. Moreover, the problem of nonstoichiometric binding has been present in every study in this laboratory with *native* m1-toxin1 (Max et al., 1993a; Liang et al., 1996; Carsi and Potter, 2000; Potter, 2001), and we think it is due to extensive surface denaturation of toxins when they are lyophilized. We attempted to avoid the inactivation of recombinant toxins by adding BSA before lyophilizations, but were unable to prevent major losses of the active toxins. We now know that losses can be avoided by concentrating each toxin by centrifugation over molecular cutoff filters, but this was not done in the present studies. It may be noted that the partial denaturation of K65E does not explain the inability of the active toxin to bind to [^{3}H]NMS-receptor complexes and to slow the dissociation of [^{3}H]NMS, because we used concentrations of this toxin for dissociation rate experiments (Fig. 10) that were capable of causing full blockade of unoccupied M_1 receptors.

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