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A toxin from the green mamba *Dendroaspis angusticeps*: amino acid sequence and selectivity for muscarinic m4 receptors

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Abstract Muscarinic toxin 3 (MT3) (65 amino acids, four disulphides, M_r 7379) was isolated from the venom of the African snake *Dendroaspis angusticeps* (green mamba) and its amino acid sequence determined. Its ability to inhibit the binding of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) to Chinese hamster ovary cells stably expressing subtypes of muscarinic receptors was studied. MT3 displayed high affinity for the m4 receptor ($pK_i = 8.7 \pm 0.06$), 40-fold lower affinity at m1 receptors ($pK_i = 7.11 \pm 0.17$) whereas no inhibition of [^{3}H]NMS binding to m2, m3 and m5 receptors was observed at concentrations up to 1 μM. This makes MT3 the most selective m4 receptor ligand known to date.

Key words: Dendroaspis angusticeps toxin; Amino acid sequence; Muscarinic m4 receptor

1. Introduction

Muscarinic acetylcholine receptors are coupled to G-proteins, which in turn activate different second messengers and ion channels [1,2]. There are currently five subtypes of muscarinic receptors which are differentially distributed in tissues. For instance, the heart contains primarily m2 receptors whereas all five subtypes are found in the brain. The function of these different muscarinic receptors in the central nervous system remains unclear due, in part, to the lack of highly selective agonists and antagonists.

Natural toxins that are selective for subtypes of nicotinic acetylcholine receptors are known; α-neurotoxins from snakes [3,4] and α-conotoxins from predatory snails, genus *Conus* [4–6], are specific probes for receptors in skeletal muscles, and κ-bungarotoxin [3,4] and the newly discovered α-conotoxin ImI [7] are probes for neuronal nicotinic receptors.

Nature also provides toxins that discriminate among subtypes of muscarinic receptors. Mambas, African snakes of genus *Dendroaspis*, have such toxins. Already the first report on these so-called muscarinic toxins [8] indicated that they might have some selectivity as evidenced by the fact that these toxins inhibited only 50% of the binding of the nonselective radioligand [^{3}H]quinuclidinyl benzilate to synaptosomal membranes prepared from rat cerebral cortex. Later, a toxin called m1-toxin was isolated from the venom of the green mamba, *Dendroaspis angusticeps*, and shown to be highly selective for m1 receptors [9].

Here we report the isolation, characterization and amino acid sequence of a new toxin from green mamba venom, muscarinic toxin 3 (MT3), that is highly selective for m4 receptors, a subtype for which no specific ligand is currently available.

2. Materials and methods

2.1. Materials

Lyophilized *Dendroaspis angusticeps* venom was purchased from Jonathan Leaky Ltd, P.O. Box 1141, Nakuru, Kenya. Atropine and pirenzepine were from Sigma, Saint Quentin Fallavier, France, himbacine was a generous gift of Dr. W.C. Taylor, University of Sydney, Sydney, Australia and [^{3}H]NMS from Amersham, Les Ulis, France.

2.2. Isolation and characterization of the toxin

Isolation was carried out essentially as described earlier [5] by gel filtration on Sephadex G-50, chromatography on the cation-exchangers Bio-Rex 70 (polymer of methacrylic acid cross-linked with divinylbenzene) and SP-Sephadex C-25 (SP = sulphopropyl). Finally, the toxin was submitted to ion-exchange HPLC on a BioGel TSK SP-5-PW column (21.5 × 150 mm). The purity was checked by reversed-phase HPLC on a C4 and C18 column using a gradient of acetonitrile in 0.1% trifluoroacetic acid.

Amino acid analysis, plasma desorption mass spectrometry, hydrazinolysis, preparation of a pyridyl ethyl cysteine (PEC) derivative and Edman degradation were carried out as reported earlier [11,12]. For cleavage at tryptophan, the PEC derivative was dissolved in 75% acetic acid containing BNPS-skatole (50 times molar excess to tryptophan) 0.1% phenol as scavenger to protect tyrosine residues against modification [12]. The mixture was incubated in darkness at 20°C for 32 hours. The fragments were separated by gel filtration on a TSK HW-40 column (1.4 × 102 cm) in 50% acetic acid. The peak containing the terminal fragment 29–65 was identified by its spectrum (PEC and Tyr absorbance, maximun at 255 and 276 nm, respectively). It was purified further by cation exchange HPLC on BioGel TSK SP-5-PW in ammonium acetate, pH 6.7, and submitted to amino acid analysis and Edman degradation.

2.3. Receptors

Chinese hamster ovary (CHO) cells stably expressing one of the five muscarinic receptor subtypes were cultured using standard techniques. Upon confluency, cells were scraped and homogenized with a polytron, setting 6 for 15 sec in 10 ml 50 mM sodium potassium phosphate buffer, pH 7.4 (used throughout in the following steps). The homogenate was centrifuged at 40,000 × *g* for 10 min at 4°C. The pellet was resuspended to obtain 50–400 mg/ml depending on the receptor subtype. The concentration of MT3 was determined spectrophotometrically and 11 dilutions were made for each experiment. Incubation tubes contained 200 μl buffer, 50 μl [^{3}H]NMS (final concentration 0.3 nM), 50 μl toxin, pirenzepine or himbacine and 200 μl homogenate. Nonspecific binding was determined as the binding in the presence of 2 μM atropine. After a 1 h (CHO-m1-m4) or 1.5 h (CHO-m5) incubation at room temperature (19–21°C) bound and free radioligand were separated by rapid filtration through glass fibre filters using a 96-well cell harvester. Filter-bound radioactivity was quantified by solid scintillation spectrometry. Competition curves were analyzed by fitting the data to a four-

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parameter logistic equatior ıg the curve-fitting program InPlot (version 4, GraphPad Software, San Diego, CA). K_i values were calculated using the Cheng–Prusoff equation $K_i = IC_{50}/(1+[L]/K_d)$ [13], where [L](K_d); concentration (dissociation constant) of [^{3}H]NMS. K_d values were determined from saturation experiments performed under the same conditions as described above.

3. Results

3.1. Isolation of toxin MT3

The two first isolation steps using gel filtration on Sephadex G-50 and chromatography on the cation-exchanger Bio-Rex 70 are described in detail elsewhere [6]. In chromatography on SP-Sephadex C-25, more shallow gradients were used than before which considerably improved the resolution. With the ion-exhanger, equilibrated at pH 5.2, two peaks (Fig. 1A) were obtained that displaced [^{3}H]NMS from its binding sites on synaptosomal membranes from porcine brain. The peaks were pooled, pH adjusted to 7 with ammonia, freeze-dried and submitted to chromatography on SP-Sephadex C-25 at pH 6.7. Peak I (Fig. 1A) contained four toxins (Fig. 1B) and peak II three (Fig. 1C). Only two of the toxins, MT1 and -2, were known earlier [8]. The toxins were called in the order in which they were detected. Because of its subtype specificity muscarinic toxin 3 (MT3) was characterized further. Data on the other new toxins will be reported later.

After HPLC ion-exchange on BioGel SP-5-PW (buffer A: H_2O, buffer B: 1.00 M ammonium acetate, pH 6.7) the toxin was homogeneous in analytical reversed phase HPLC on both a C4 and C18 column.

3.2. Amino acid composition and sequence

A molar ratio of 8.5 for Thr was repeatedly obtained in the analysis of MT3 (Table 1). Mass spectrometry gave a molecular

Table 1
Amino acid composition of MT3

Amino acid	Acid hydrolysis Molar ratio (Integer)*		Sequence
Asp	7.12 ± 0.12	(7)	2
Asn			5
Thr	8.47 ± 0.07	(9)	9
Ser	1.26 ± 0.01	(1)	1
Glu	5.18 ± 0.05	(5)	4
Gln			1
Pro	3.98 ± 0.11	(4)	4
Gly	3.08 ± 0.02	(3)	3
Ala	2.93 ± 0.08	(3)	3
Half-cys	7.98 ± 0.21	(8)	8
Val	2.16 ± 0.05	(2)	2
Met	0.00	(0)	0
Ile	5.65 ± 0.00	(6)	6
Leu	2.14 ± 0.00	(2)	2
Tyr	3.00 ± 0.02	(3)	3
Phe	2.00 ± 0.01	(2)	2
His	1.94 ± 0.04	(2)	2
Lys	4.10 ± 0.01	(4)	4
Arg	3.00 ± 0.03	(3)	3
Trp		(1)	1
No. of residues		65	65
Molecular weight			7379
Molar absorptivity at 276 nm			11,100
$A^{0.1\%}$			1.50

*Average of two analyses. Hydrolysis; 6 M HCl, 24 h, 110°C. 9 Thr concluded from mass spectrometry. Absorption maximum at 276 nm.

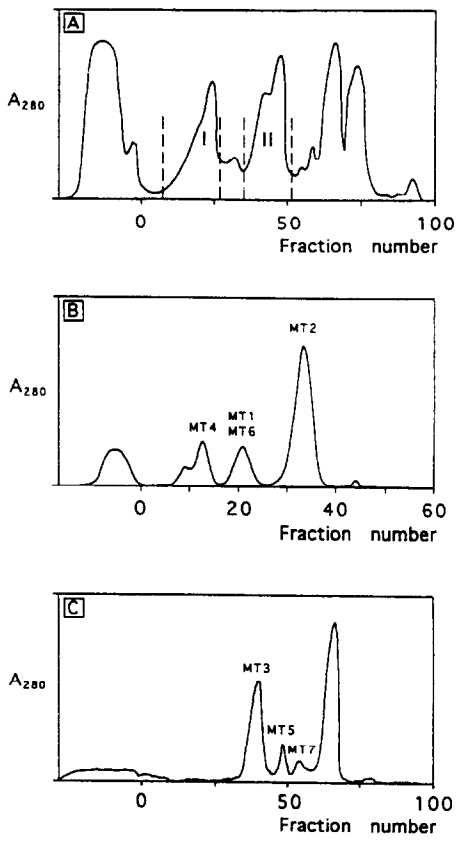

Fig. 1. Ion-exchange chromatography on SP-Sephadex C-25 (2.0 × 28 cm) equilibrated with buffer A. Freeze-dried sample dissolved in buffer A applied to column. Elution with buffer A until all non- adsorbed material had come out and then with a concave gradient of 900 ml buffer A vs. 400 ml buffer B. Gradient formed by two connected cylinders of diameters 9 cm (buffer A) and 6 cm (buffer B). Flow rate 25 ml/h. Fraction collection started at beginning of gradient elution. A_{280} (absorbance at 280 nm), arbitrary scale. (A) Sample from totally 9 g venom: non-retarded fraction from run on Bio-Rex 70 [8]. Buffer A: 0.05 M AmOAc (pH 5.2), buffer B: 1.00 M AmOAc (pH 6.5). 13 ml fractions. Activity, inhibition of [^{3}H]NMS binding to synaptosomal membranes, in peaks I and II. (B) Sample peak I (1A). Buffer A: 10 mM AmOAc (pH 6.7), B: 1,00 M AmOAc (pH 6.7). Buffers by dilution of 5 M stock solution without pH adjustment. 12 ml fractions. MT = muscarinic toxin. (C) Sample peak II (1A). Buffers as in B. 9 ml fractions.

weight of 7360, a value in better agreement with the molecular weight from amino acid analysis (7385) assuming 9 Thr rather than 8 Thr (7284). Sequence determination confirmed that the toxin has 9 Thr residues and 65 amino acids.

The molar absorptivity at pH 7 and 276 nm (absorbance maximum) was 11,100 $M^{-1} \cdot cm^{-1}$. The toxin has one tryptophan, since the sum of the molar absorptivities of 3 Tyr (4101), 4 cystine (440) and 1 Trp (5431) = 9972. The difference 128 (10%) is the contribution from secondary and tertiary structures.

Two nanomoles PEC derivative was sequenced to amino acid 43 without any impurities being detected. The yield of the first phenylthiohydantoin amino acid was 50% and the repetitive yield, i.e. the yield in one step compared to that of the preceding step, averaged 94%. The fragment from cleavage at Trp was sequenced from amino acid 29 to 65, glutamic acid. The

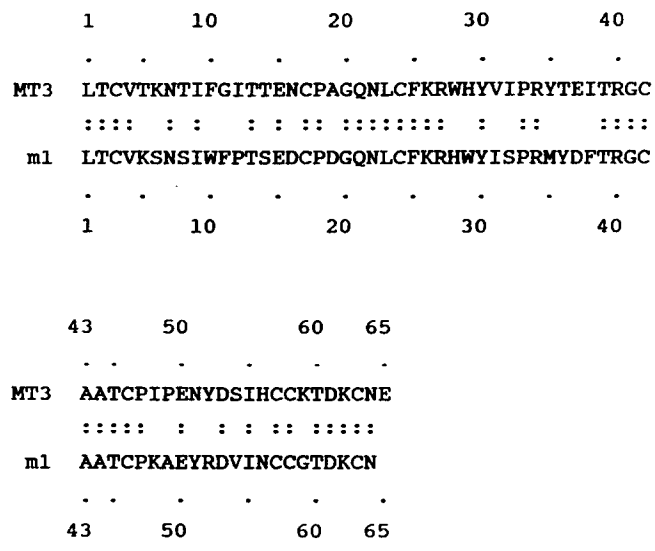

Fig. 2. Primary structures of MT3 (selective for muscarinic receptors of subtype m4) and m1-toxin (selective for m1) [9]. Invariant amino acids are indicated by two dots.

C-terminal amino acid was Glu, since hydrazinolysis of the native toxin gave Glu in a yield of 94% and no traces of other amino acids.

The following sequence was obtained:

$L^{1}TCVTKNTIF^{10}GITTENCPAG^{20}$
$QNLCFKRWHY^{30}VIPRYTEITR^{40}GCAATCPIPE^{50}$
$NYDSIHCCKT^{60}DKCNE^{65}$

It agrees with data from amino acid analysis, mass spectrometry and hydrazinolysis.

3.3. Binding to subtypes of muscarinic receptors

In saturation experiments, it was shown that the radioligand, [^{3}H]NMS, displayed dissociation constants, K_d, of 96, 140, 74, 72 and 240 pM for m1, m2, m3, m4 and m5 receptors, respectively.

MT3 displayed high affinity (pK_i = 8.7) for the m4 receptor which was 40 times higher than for the m1 receptor (pK_i = 7.11) and no binding of the toxin to m2, m3 and m5 receptors (pK_i < 6) could be detected (Table 2). The Hill coefficients are close to 1, indicating binding to one site. For comparison, the affinity values are reported for two compounds,pirenzepine and himbacine, that have been used as pharmacological tools to distinguish m1 and m4 receptors (Table 2). It is evident that MT3 is considerably more selective for one receptor than pirenzepine and himbacine.

4. Discussion

Ammonium acetate buffers were used in the isolation because the samples can be freeze-dried directly without prior desalting. Buffers with high capacity are usually recommended for ion-exchange [14], but buffers with very low capacity can, evidently, also give good resolution (Fig. 1B and C). Buffering capacity of ammonium acetate is minimal at neutral pH. The nature of the buffer ions can also be important, for instance, acetate often gives a better resolution than chloride [15].

Several remarkable neurotoxins have been isolated from the venom of the green mamba: dendrotoxins, blockers of voltage-dependent potassium channels [16]; fasciculins, non-competitive inhibitors of acetylcholinesterase [17] and muscarinic toxins. An important aspect of these toxins is their selectivity for various subtypes of the target molecules. Dendrotoxins can distinguish between different types of voltage-dependent potassium channels [18], fasciculins inhibit mammalian but not insect acetylcholinesterases [17]. Some of the muscarinic toxins isolated from green mamba venom are also selective; MT3 for m4 receptors (this report) and m1-toxin for m1 receptors [9].

A comparison of the sequences of the two toxins (Fig. 2) shows a high homology; they have the same amino acidin 40 of the 65 positions. The amino acids that determine the subtype specificity should be in the parts of the molecules that are different. The central part, residues 21–38, contains 6 or 7 hydrophobic amino acids (F, I. L, V, W, Y) which is characteristic of muscarinic toxins [11].

Currently, no ligands selective for m4 receptors are available. In binding studies using human recombinant muscarinic receptors, the affinity of pirenzepine, a compound used as an m1 receptor selective ligand, for m4 receptors was only 5-fold lower than its affinity for m1 receptors (Table 2) [19]. The alkaloid himbacine isolated from the bark of Australian trees of *Galbulimima* species (Ritchie and Taylor, 1967, cited in [20]) displayed the same (Table 2) [19] or slightly lower (about half [21]) affinity for m4 than for m2 receptors. The new toxin MT3 is highly selective (> 500-fold) for m4 receptors compared to m2 and has a sufficiently high selectivity for m4 compared to m1 (40-fold) and m3 and m5 (> 500-fold) that it should be a useful tool for investigating the distribution and function of m4 receptors in various organs and tissues. Specifically, it should be useful to characterize autoreceptors in brain which have been proposed to be of the subtype m2 [22], m3 [24] or m4 [23].

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Table 2
Affinity constants (pK_i) and Hill constants (nH) of MT3, pirenzepine and himbacine for subtypes of muscarinic receptors. Values are expressed as mean ± S.D., n = 4 (MT3) or 3–4 (pirenzepine, himbacine)

Compound	MT3		Pirenzepine		Himbacine	
	pK_i	nH	pK_i	nH	pK_i	nH
m1	7.11 ± 0.11	0.94 ± 0.01	7.97 ± 0.09	0.88 ± 0.03	6.68 ± 0.05	0.95 ± 0.04
m2	<6		6.33 ± 0.07	0.89 ± 0.02	7.92 ± 0.15	0.94 ± 0.05
m3	<6		6.65 ± 0.09	1.07 ± 0.16	6.85 ± 0.05	0.99 ± 0.11
m4	8.70 ± 0.06	1.00 ± 0.11	7.30 ± 0.17	0.91 ± 0.04	7.88 ± 0.07	1.38 ± 0.26
m5	<6		6.88 ± 0.04	0.95 ± 0.08	5.35 ± 0.38	0.88 ± 0.03

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