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Interactions between α-conotoxin MI and the *Torpedo marmorata* receptor α–δ interface

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Abstract

The muscle-type nicotinic receptor has two distinguishable acetylcholine binding sites at the α–γ and α–δ subunit interfaces; α-conotoxins can bind them selectively. Moreover, we previously reported that α-conotoxin MI can interact with *Torpedo californica* and *Torpedo marmorata* receptors showing that conotoxins can also detect receptors from different species of the same genus [L. Cortez, S.G. del Canto, F. Testai, M.B. de Jiménez Bonino, Conotoxin MI inhibits the acetylcholine binding site of the *Torpedo marmorata* receptor, Biochem. Biophys. Res. Commun. 295 (2002) 791–795]. Herein, to identify *T. marmorata* receptor regions involved in α-conotoxin MI binding, a photoactivatable reagent was used and labeled sites were mapped by enzymatic proteolysis, MALDI-TOF-MS and Edman degradation. α-Conotoxin MI binding determinants were found and studies revealed a second binding motif at the α/δ interface. A proposal for receptor–toxin interaction is discussed based on experimental results and docking studies.

Keywords: Nicotinic receptors; *Torpedo marmorata*; α-Conotoxin MI; Ligand-binding sites; Conus

The nAChR belongs to the "Cys-loop" superfamily of ligand-gated ion channels. The muscle type of the pentameric α2βγδ phosphoglycoprotein is found at the neuromuscular junction and in the Torpedo electric organs. The receptor contains a large extracellular LBD containing ligand-binding sites for agonists and competitive antagonists, a membrane-spanning pore and a short intracellular domain.

Among competitive antagonists, conotoxins are small, tight peptide toxins, a major part of them being selectively targeted to specific receptors; some α-conotoxins can distinguish between the two acetylcholine-binding sites of a single receptor; moreover, we have reported that they can establish different interactions with receptors from various species of the same genus [1]. This work intends to enhance the knowledge of conotoxin-binding site environments; α-CnTx MI carrying a photoactivatable group at its N-terminal residue and proteolytic mapping followed by MS and sequence studies were used as tools to find receptor regions involved in the interaction. Moreover, a complex model was built and docking analysis allowed us to determine the interface involved in the interaction and estimate distances on the toxin-binding surface based on the conotoxin relatively rigid structure.

Materials and methods

Soybean L-α-phosphatidylcholine, carbamylcholine, Chloramine-T, *N*-hydroxysuccinimidyl-4-azido salicylic acid, and V8 proteinase (EC 3.4.21.19) were from Sigma Chemical Co., Saint Louis, MO, USA;

Abbreviations: α-CnTx, α-conotoxin; DMSO, dimethylsulfoxide; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MS, mass spectrometry; NHS-ASA, *N*-hydroxysuccinimidyl-4-azido salicilyc acid; LBD, ligand-binding domain; nAChR, nicotinic acetylcholine receptor.

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modified trypsin (EC 3.4.21.4), was from Promega, Madison, WI, USA; Affi-Gel 10 from Bio-Rad, Richmond, CA, USA, and the carrier-free ^{125}INa from NEN Research Products, New England Nuclear-DuPont Co., Boston, MA, USA. Bromoacetylcholine was synthesized from choline bromide and bromoacetyl bromide from Aldrich Chemical Co., Saint Louis, MO, USA. α-CnTx MI was from Peptides International, Louisville, KY, USA.

Receptor purification. It was affinity-purified according to Venera et al. [2] and stored at −70 °C in 10 mM phosphate buffer, 100 mM NaCl, 0.1 mM EDTA, and 0.02% NaN_3, pH 7.4.

α-CnTx MI radiolabeling. It was performed by the Chloramine-T method as per Lacorazza et al. [3] and stored at −20 °C. Specific activity was 36 Ci/mmol thus minimizing the formation of diiodo-α-CnTx MI.

Coupling of [^{125}I]CnTx MI and NHS-ASA. Selective α-amino derivatization was carried out by Myers et al. [4] as modified by Cortez et al. [1]. Briefly, 10 nmol [^{125}I]CnTx MI in 20 μl 100 mM ammonium bicarbonate, pH 7.4, was incubated with 200 pmol of NHS-ASA in 2 μl dried DMSO on ice for 40 min in the dark and then DMSO eliminated under nitrogen. To control the azide group incorporation to the conotoxin, a parallel experiment with unlabeled toxin was performed, followed by RP-HPLC. Due to the instability of radiolabeled photoactivatable derivatives, the nAChR-ASA-[^{125}I]CnTx MI was immediately used. Under these experimental conditions, the conotoxin can only bind at the α/δ interface.

Autoradiography. A Kodak SAR-5 film was exposed to the dried gels for 8 days at −20 °C and densitometric patterns were taken (SigmaScan).

Proteolytic mapping. The method of Pedersen and Cohen [5] and V8 proteinase were used. After electrophoresis and staining, fragments from the modified α and δ subunits were electroblotted onto a PVDF membrane and sequenced.

Peptide sequencing. It was performed at the Facility for Peptide and Protein Sequencing (University of Buenos Aires). An Applied Biosystems sequencer model 477 A was used. Searches for similarities were performed through the Swiss Prot data bank. Peptides were aligned by using the "Multiple sequence alignment with hierarchical clustering".

In-gel digestion and MS. V8 fragments of the α and δ subunits native or modified with the adduct were digested "in-gel" with trypsin [6]. The peptide mixture was analyzed by MALDI-TOF-MS with a Bruker Biflex III instrument equipped with delayed extraction and reflector. The sample was prepared by the dried droplet technique using α-cyano-4-hydroxycinnamic acid as the matrix. The spectrum was obtained from 0.5 μl of a 1:1 (v/v) mixture of aqueous matrix solution and peptide mixture. Peptide mass fingerprinting analysis was carried out using ProFound Version 4.7.5.

Automatic docking of α-CnTx MI. The Z-dock program through the ClusPro server [7] were used to analyze toxin docking to the receptor structure refined by Unwin [8] at a 4 Å resolution. α-CnTx M1 was built by *in silico* mutating N11 for Y11 in the α-CnTx IA (1b45.pdb) [9] followed by energy minimization with MacroModel 8.6 software. Complexes in each run were visually screened and analyzed with Pymol [10] and Spdbv [11], taking into account our experimental results.

Results and discussion

V8-Proteolytic digestion of the photoaffinity-labeled α and δ subunits

The complex nAChR-ASA-[^{125}I]CnTx MI was submitted to the method of Pedersen and Cohen [5]. Gel bands were electroblotted onto a PVDF membrane, subjected to autoradiography and then sequenced. The autoradiography detected 4 and 3 labeled fragments from modified α and δ subunits, respectively (Fig. 1). Table 1 summarizes N-terminal sequences. The position of the low Mr radiolabeled band in Fig. 1B is coincident with that of the tracking dye. This band also contains a peptide mixture.

Localization of V8 labeled fragments

It was performed by MS of tryptic peptides to provide a better definition of toxin-binding determinants. All radioactive fragments from V8 digestion were cut, digested in gel and the peptide mixture submitted to MALDI-TOF-MS. Fragments from the native α and δ subunits were processed in parallel. Mass values obtained from labeled αV8 and δV8 fragments as well as those from the control were correlated with tryptic peptide sequences by the GPMAW 5.10 program. Despite using a sequencing grade trypsin, several values corresponded to masses of chymotryptic peptides due to the trypsin residual chymotryptic activity; besides, several masses from V8 or keratin tryptic digestion were found.

Data from the N-terminal sequence and molecular mass of V8 fragments, enzyme specificity and peptide mass of tryptic peptides, allowed completion of Table 2.

As to αV8–13, its N-terminal sequence showed peptides starting at positions V46 and T52. However, mass values denoted the presence of peptides 116–125, 146–155 and 152–164, among others (online Supplementary material A). Based on enzyme specificity, the αV8–13 fragment should end up at E172; nevertheless, αV8–13—with glycosylation site αN141—could only justify its 13 kDa mass if partially deglycosylated. Something similar occurs with δV8–13 including glycosylation site δN143; as the mass of peptide 137–147 appears in δV8–13 mass list (online Supplementary material B) we infer that at least part of the δ subunit is also deglycosylated.

Fragments L263-E377 and N339-E432 from the α subunit and T281-E486, I192-E345, I346-A501, K333-E442, and R389-A501 from the δ subunit are not considered as they do not belong to the LBD.

Identification of the tryptic peptides bound to the adduct

α-CnTx MI has R at position 2 and K at position 10 and, therefore, the mass of the fragment able to bind to ASA-[^{125}I]CnTx MI may increase by 364.36; 1163.44 or 1629.61 Da (Fig. 2). These values were added to each experimental mass of peptides from tryptic digestion, the resulting values being checked against those in mass lists. Only αV8–13, δV8–13, and αV8–18 yielded peptides bound to the adduct; the corresponding data appear in Table 3. Those from unlabeled tryptic peptides appear as online Supplementary materials A, B and C.

A value of 2367.32 Da was found (Table 3); this corresponds—with an error of 0.22 Da—to the mass of peptide α ^{116}IMWTPPAIFK^{125} (1203.66 Da) plus that of the adduct cleaved at K10 (1163.44 Da, Fig. 2). Besides, the molecular mass 2367.31 Da corresponds to the same peptide bound to the adduct cleaved in the same way. We could also detect the peptide molecular mass without the adduct addition and the mass of the modified fragment does not appear in the control.

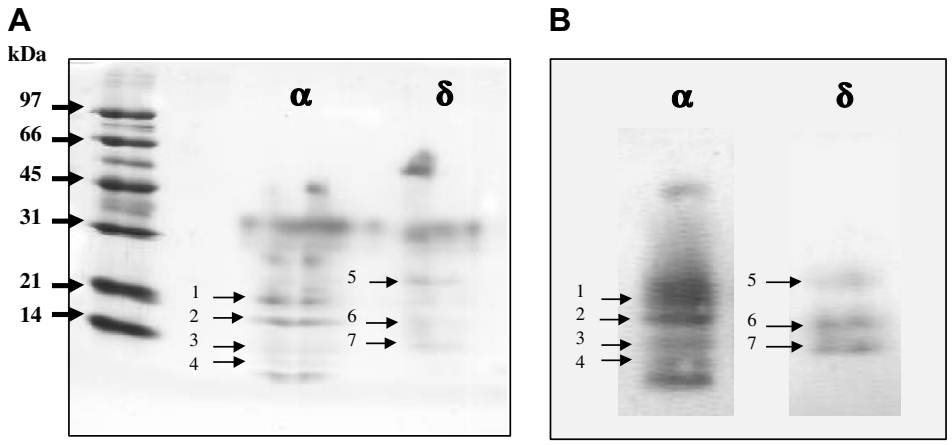

Fig. 1. (A) SDS–PAGE of fragments from α and δ subunits bound to the adduct and V8 digested. Coomassie staining. (B) Autoradiography. 1, αV8–20; 2, αV8–18; 3, αV8–13; 4, αV8–10; 5, δV8–23; 6, δV8–18; 7, δV8–13.

Table 1
N-terminal sequence of V8 fragments (Fig. 1)

Gel band (Fig. 1)	N-terminal sequence
1: αV8–20[a]	^{173}SGEWVMKDYT
2: αV8–18	^{46}VNQIV
3: αV8–13	^{46}VNQIV
	^{52}TNVRL
	Other minor signals
4: αV8–10	^{1}SEHETR
	Other minor signals
5: δV8–23	Ambiguous results
6: δV8–18	^{192}II X X P A X K N I
7: δV8–13	Insufficient amount

[a] Numbers indicate apparent molecular mass in kDa.

Table 2
Localization of V8 fragments on the basis of their N-terminal sequence, enzyme specificity and mass of their tryptic peptides

Gel band (Fig. 1)	Fragment/s
1: αV8–20	S173-E338
2: αV8–18	V46-E172
3: αV8–13	V46-E172
	T52-E172
	L263-E377
4: αV8–10	S1-D84
	N339-E432
5: δV8–23	W176-E380
	T281-E486
6: δV8–18	I192-E345
	I346-A501
7: δV8–13	T48-E157
	K333-E442
	R389-A501

As regards the δ subunit a value of 2206.95 Da was found corresponding (error 0.08 Da) to the addition of the mass of the chymotryptic peptide δ ^{118}VTWLPPAIF^{126} (1043.59 Da) plus that of the adduct cleaved at K10 (1163.44 Da). The 2206.95 Da value does not appear in the control.

Both hydrophobic peptides—α ^{116}IMWTPPAIFK^{125} and δ ^{118}VTWLPPAIF^{126}—belong to the LBD and might

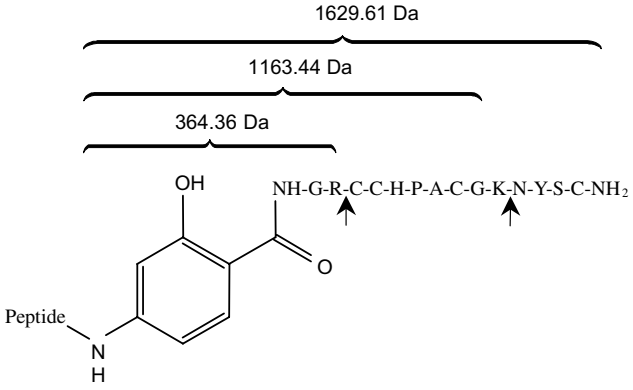

Fig. 2. Adduct covalently bound to a receptor tryptic peptide. Arrows indicate possible tryptic cleavage sites; mass values which can be added to tryptic peptides are indicated.

be considered two of the determinants for α-CnTx MI binding. Results agree with those of Bren and Sine [12] who stated that hydrophobic or aromatic residues are essential to form the high-affinity complex. Close to this region, αY113 of muscle mouse receptor was considered by the authors as an important selectivity determinant; however, R substitutes αY113 in the *Torpedo marmorata* receptor without having any influence on selectivity since both receptors interact with a higher affinity with the α/δ interface.

On the other hand, despite finding the radioactive fragment αS173-E338 as well as δW176-E380 and I192-E345 (Table 2), we were unable to find any labeled peptide from their digestion. The extracellular LBD extends up to positions 212 (α) or 227 (δ) and then the chain enters the membrane so that the adduct binding indicated by the label of αV8–20, δV8–23, and δV8–18 is just possible in the S173-Y212 (α subunit) and W176-F227 (δ subunit) regions. Additionally, several amino acids of these fragments are known to be key residues in delineating the ligand-binding sites. Bren and Sine [12] detected the interaction of α-CnTx

Table 3
Mass of labeled peptides and corresponding sequences

Gel band (Fig. 1)	*m/z* (Da) of labeled peptide	Sequence of labeled peptide	Localization	Mass error	
2: αV8–18	2367.32	IMWTPPAIFK	116–125	0.22	T–T[a]
3: αV8–13	2367.31	IMWTPPAIFK	116–125	0.21	T–T
7: δV8–13	2206.95	VTWLPPAIF	118–126	−0.08	Q–Q

[a] T, Q: tryptic and chymotryptic cleavage.

MI with αY190, αY198, and δI178 of the muscle receptor; the first two amino acids being proposed as critical in binding-site stabilization. It should be noted that photoactivatable reagents have a low cross-linking yield, mainly those with arylazide groups, making the identification of labeled fragments difficult. This low yield is likely to hamper the isolation of other labeled peptides.

Automatic docking of α-CnTx MI

In most of the 20 toxin–receptor complexes obtained the conotoxin is located at the α/δ receptor interface, which agrees with our experimental results. However, and surprisingly, several of them are positioned at the inner face of the LBD and the others at the outer face (Fig. 3).

The LBD is built around a β sandwich core (10 β strands forming the inner and outer sheets) and also contains several loops relevant for receptor function [8].

Fig. 4A shows one of the complexes where the toxin binds to the outer side of the receptor surrounded by loops A, B, and C of the α subunit (connecting β strands) known to form the Ach-binding pocket of the receptor in its closed state. The region α173–212—labeled by the adduct—contains the β9–β10 hearpin connected by loop C in the outer sheet of the β sandwich. Results indicate that fragment δ118–126 including β5, part of β6 of the inner sheet, and the connecting loop was also labeled by the probe bound to the N-terminal G of α-CnTx MI. Analysis of the model complex shows that such G is located at 10.5 Å from δT119 and δL121, both exposed to the solvent. As the probe is 7.5 Å long, the photoactivatable group is very likely to be at 3 Å, making their interaction feasible. Moreover,

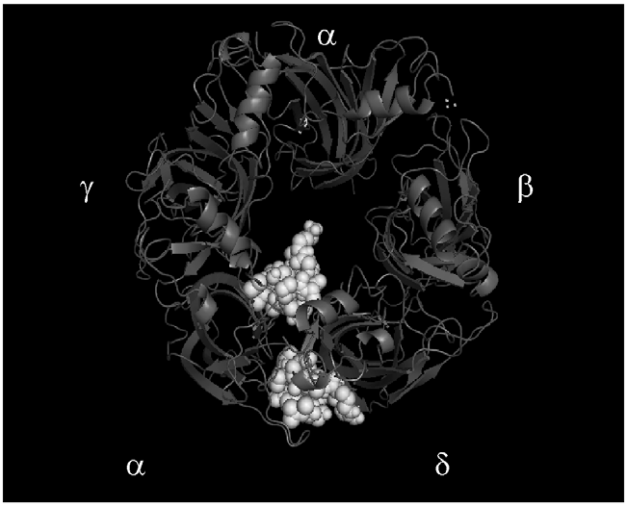

Fig. 3. Interaction model of α-CnTx MI (sphere representation) with the α/δ receptor interface. The *T. marmorata* receptor structure (ribbon representation) refined by Unwin [8] at a 4 Å resolution was used. View from the synaptic cleft.

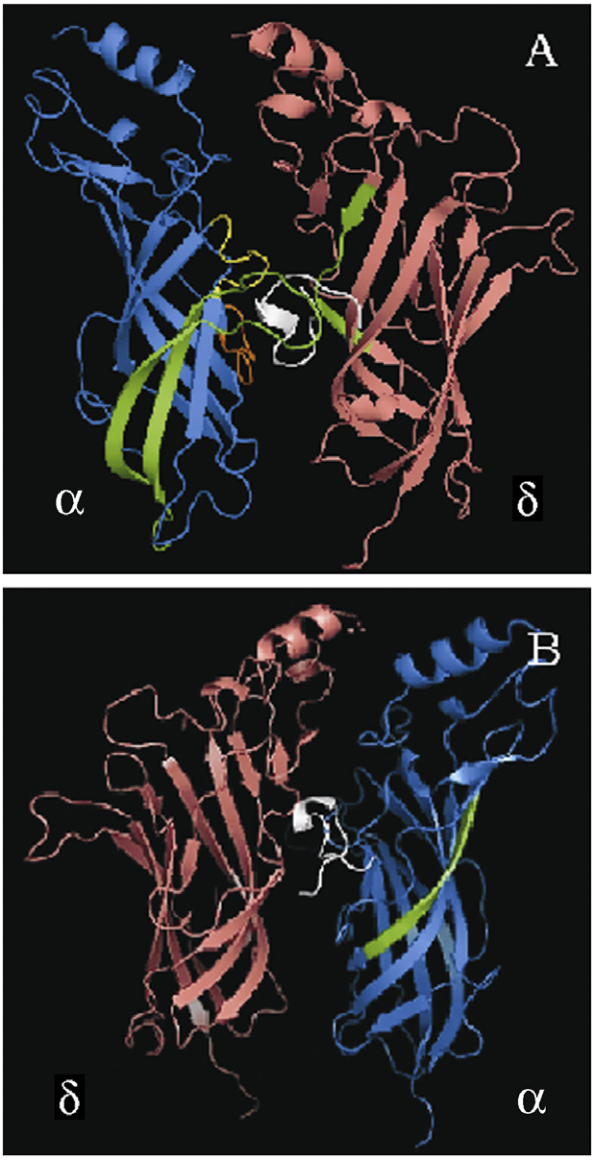

Fig. 4. Interaction example of α-CnTx MI (white) with the outer (A) and inner (B) face of α (blue) and δ (pink) subunits of the *T. marmorata* receptor. In green: fragments δ118–126, α173–212 (including loop C) and α116–125. Loops A (orange) and B (yellow) are also shown.

αP121, δP122, and δP123 were shown [8] as interacting with residues of neighboring subunits at the interfaces assuming a 3.9 Å cut off for interacting atoms.

Fig. 4B shows the complex where the toxin binds to the inner side of the receptor and fragment α116–125. αI123 is likely to be at about 3 Å from the photoactivatable group making their interaction also possible.

Summarizing, results herein lead to the identification of binding determinants for the interaction between α-CnTx MI and the *T. marmorata* nicotinic receptor. Moreover, experimental data in this work plus those from the literature lead us to suggest—despite the low resolution of complexes from automatic docking—the existence of two different α-CnTx MI-binding sites on the α/δ receptor interface.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.01.154.

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