INTERACTION OF SPIN-LABELED *NAJA NAJA SIAMENSIS* α-NEUROTOXIN WITH ACETYLCHOLINE RECEPTOR FROM *TORPEDO CALIFORNICA*

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1. Introduction

The binding of acetylcholine to the nicotinic acetylcholine receptor (AChR) results in an increase in cation permeability of postsynaptic membranes. The permeability responses are blocked by specific antagonists, including snake α-neurotoxins. The binding sites for acetylcholine and other cholinergic ligands are probably the same sites to which the snake α-neurotoxins specifically bind [1] and there are 2 α-toxin binding sites/acetylcholine receptor monomer [2]. Of these 2 sites, only one is specifically labeled by the affinity alkylating agents, 4-(*N*-maleimido) benzyltrimethylammonium iodide (MBTA) and bromoacetylcholine bromide [3,4]. Disagreement exists as to the no. reversible cholinergic ligand sites/receptor monomer with values of 1 or 2 reported [1]. Here we report the preparation of a spin labeled *Naja Naja siamensis* α-neurotoxin and the use of this toxin to examine the relationship between the 2 α-toxin binding sites. The results indicate that the 2 α-toxin binding sites are structurally very similar.

2. Materials and methods

2.1. *Torpedo membrane vesicles*

Acetylcholine receptor-rich membranes (AChRM) were prepared from *Torpedo californica* electroplax as in [5]. The final membrane pellet was suspended in vesicle dilution buffer (VDB-255 mM KCl, 1.5 mM $NaPO_4$, 4 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.0) to ~3 mg protein/ml; 200 g tissue yielded 50 mg membrane protein having an ^{125}I-labeled α-bungarotoxin (^{125}I-αBgTx) binding activity of 1 nmol/mg protein.

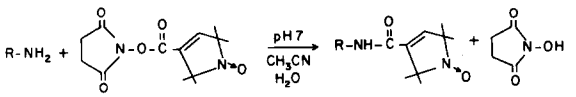

Fig.1. Reaction of 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid *N*-hydroxysuccinimide ester with the principal α-neurotoxin from *Naja naja siamensis* venom (R).

2.2. *Preparation of spin-labeled toxin*

2,2,5,5-Tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid *N*-hydroxysuccinimide ester, 13 mg (46 μmol) in 0.60 ml acetonitrile, was added to 24 mg (3.1. μmol) purified *Naja naja siamensis* α-neurotoxin [6] in 7.0 ml 50 mM $NaPO_4$ (pH 7.0) (fig.1). The mixture was incubated for 5 min at room temperature and then applied to a 1.5 × 50 cm column of Sephadex G-10 equilibrated with 0.1 M NH_4Ac (pH 6.7). The protein peak was lyophilized and suspended in 2.5 ml 0.01 M NH_4Ac (pH 6.7). This material was applied to a 0.9 × 18 cm column of CM-25 Sephadex equilibrated with 0.01 M NH_4Ac (pH 6.7). The column was first eluted with 3 column vol. 0.01 M NH_4Ac (pH 6.7) and then with an 80 ml 0.10–1.0 M NH_4Ac (pH 6.7) gradient. The presence of spin label was monitered by EPR spectroscopy. In one preparation, 5.3 mg pure spin labeled α-toxin (SL toxin) from the CM-25 Sephadex column was applied to a 0.7 × 15 cm column of Bio-Rex 70 (–400 mesh) equilibrated with 0.005 M NH_4Ac (pH 6.5). 3 column vol. NH_4Ac (0.005 M) were run through the column. This was followed by a 60 ml 0.01–0.10 M NH_4Ac gradient.

2.3. *Binding of spin-labeled toxin to AChRM or pure AChR and separation of bound and free toxin*

AChRM or pure AChR containing 3–15 nmol

α-toxin binding sites was incubated with various amounts of SL toxin or a mixture of SL toxin and native *N.n.siamensis* α-toxin for 30 min at room temperature. Free toxin was separated from toxin bound to AChRM by layering the incubation mixture, typically 2 ml, on top of 2.4 ml 10% sucrose in VDB and centrifuging at 35 000 rev./min for 30 min in an SW-60 rotor. The membrane pellet was suspended in a small volume of VDB for EPR spectroscopy. Protein was typically 30 mg/ml. SL toxin bound to pure AChR was separated from free SL toxin by chromatographing the mixture on Bio-Rad P-30. Fractions containing SL toxin bound to AChR were concentrated with an Amicon Minicon A-25 concentrator.

2.4. *Other procedures*

αBgTx was a gift from Michael Hanley (Chemical Biodynamics Lab, University of California, Berkeley). ^{125}I-αBgTx was prepared as in [7]. MBTA labeling of AChRM was done as in [8]. AChR from *Torpedo californica* was purified as in [9] except that bromoacetylcholine rather than (*p*-carboxyphenyl) trimethylammonium was coupled to the affinity matrix. ^{125}I-αBgTx binding activity was determined by the method developed for tritated *N.n.siamensis* α-toxin binding in [5]. EPR spectra were obtained using a Varian E-4 spectrometer equipped with a temperature control device. All spectra were recorded at 25°C. The no. spin label groups/SL toxin molecule was determined by double integration of the EPR spectra of the SL toxin and a set of spin label standards. Amino acid sequence analysis was done by using a Beckman 890C sequencer with a DMAA program (Beckman no. 102974). Analysis was done both with and without an acid wash before the initial coupling.

3. Results

3.1. *Characterization of the spin-labeled toxin*

Three protein peaks were obtained during gradient elution of the CM-25 Sephadex column (fig.2). The first 2 were spin labeled α-toxin and the final 1 was native α-toxin. The middle peak was used for all experiments to be described and will be refered to as SL toxin. The EPR spectrum of SL toxin is shown in fig.3a. The spectrum is characteristic of a homogeneous, highly mobile species, and from the spectrum a rotational correlation time of 1.0 ns was calculated for the SL toxin [10]. In order to test the homogeneity of the SL toxin it was rechromatographed on Bio-Rex 70. Bound protein was eluted from the column in 2 minor peaks and 1 major peak. The major peak contained 94% of the protein. EPR spectra of the SL toxin before and after chromatography were identical. It has been shown that 1 can acetylate the 6 free amino groups of *N.n.siamensis* α-toxin and separate the 6 monoacetyl toxins by chromatography on Bio-Rex 70 [11].

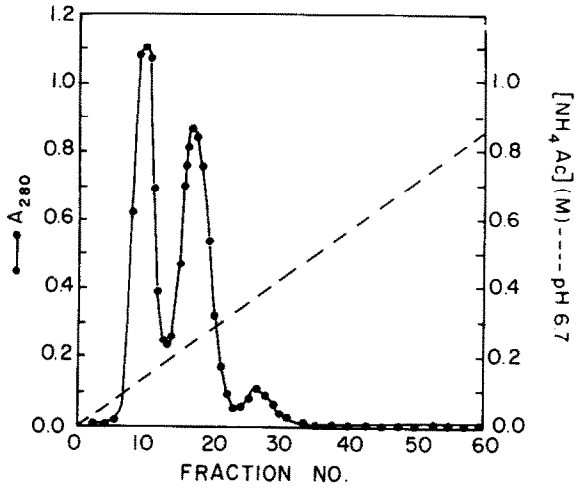

Fig.2. Fractionation of spin labeled and native *N.n.s.* α-toxin on CM-25 Sephadex. Spin labeled and native *N.n.s.* α-toxin in 0.01 M NH_4Ac pH 6.7 were applied to a 0.9 × 18 cm column of CM-25 Sephadex equilibrated with 0.01 M NH_4Ac pH 6.7. The column was eluted with 3 column volumes of 0.01 M NH_4Ac pH 6.7 (not shown) and an 80 ml. 0.01 M–1.0 M NH_4Ac pH 6.7 gradient. The middle peak was used for all experiments.

The no. spin label groups/toxin molecule was found to be 0.91 by double integration of the EPR spectrum. Amino acid sequence analysis was carried out on the first 5 residues of the N-terminus. The results agreed with those for *N.n.siamensis* α-toxin in [11] and showed that the spin label was not attached to the N-terminus. The absence of any change in the EPR spectrum after base extraction (pH 12) indicated that the spin label was not attached to a tyrosine [13]. The spin label was probably attached to the ε-amino group of a lysine [13]. Preincubation of AChRM with native *N.n.siamensis* α-toxin completely blocked the binding of SL toxin. AChR or AChRM was saturated with SL toxin and a 50-fold excess of *N.n.siamensis* α-toxin was added to the saturated receptors. After 7 h, >90% of the SL toxin was displaced from the

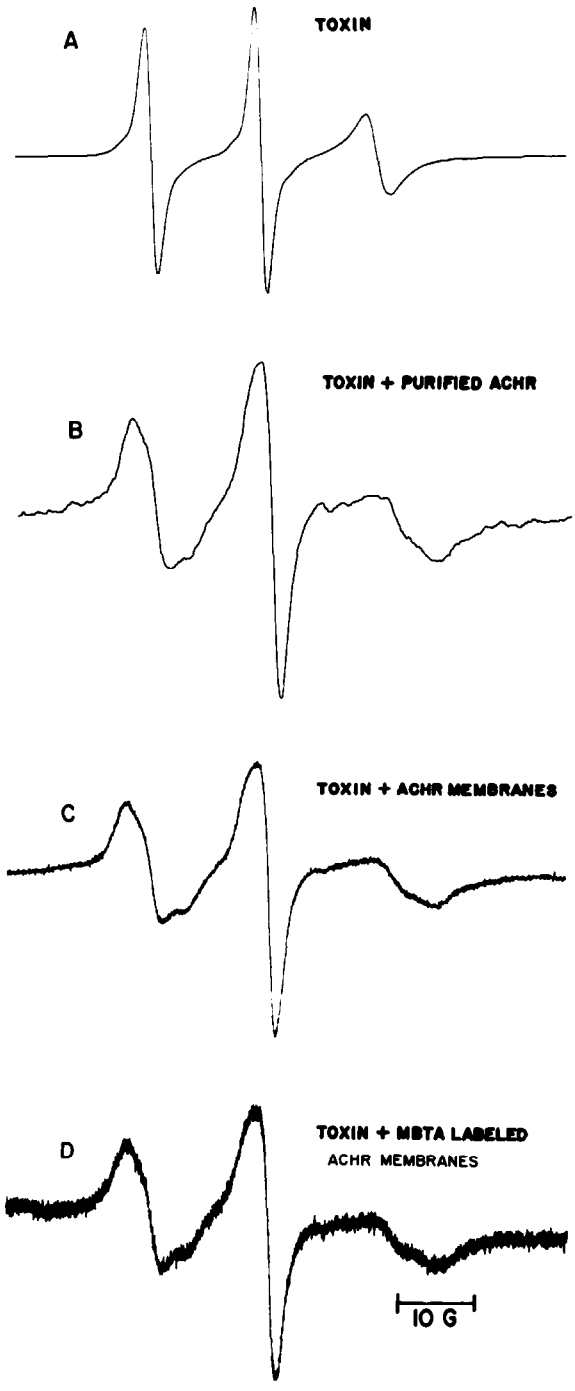

Fig.3. EPR spectra of a) spin labeled α-toxin, b) pure acetylcholine receptor saturated with spin labeled α-toxin, c) acetylcholine receptor-rich membranes saturated with spin labeled α-toxin, and d) MBTA-labeled AChRM saturated with spin labeled α-toxin. For all spectra the field set was 3260 G, microwave frequency was 9.15 GHZ, and modulation amplitude was 1.00 G.

receptors. Titration of AChRM with SL toxin showed that SL toxin binding was saturable and that AChRM contained the same number of sites for SL toxin as for ^{125}I-αBgTx. The abilities of SL toxin and native *N.n.siamensis* α-toxin to decrease the initial rate of ^{125}I-αBgTx binding to AChRM were the same. The above evidence showed that spin labeling of the α-toxin did not alter its receptor-binding properties.

3.2. *EPR spectroscopy of spin-labeled toxin bound to AChRM and AChR*

The EPR spectra of SL toxin bound to AChR and AChRM appeared to be the composite of a moderately immobilized and a weakly immobilized component (fig.3b,c). In order to insure that the SL toxin-AChRM contained no free SL toxin it was washed twice with VDB. No change in the lineshape or amplitude of the EPR spectrum was observed after washing. The more mobile component had an EPR spectrum different from the free toxin since its contribution could not be removed by computer subtraction of free toxin signal.

The EPR spectrum of SL toxin bound to AChRM or AChR were not similar to the spectra of SL toxin in various concentrations of glycerol. This suggested that the receptor environment was important in determining the spectrum of SL toxin bound to the receptor. The presence of >1 component could result if SL toxin was binding to 2 different sites, or if it was able to bind to the receptor in >1 conformational state, or if the spectra represented a complex motion of the bound spin label.

The EPR lineshape of the spectrum of SL toxin bound to MBTA labeled AChRM was the same as the lineshape of SL toxin bound to native AChRM (fig.3d) although the amplitude was reduced by 50% as expected. ^{125}I-αBgTx binding assays and comparison of spectral amplitudes indicated that MBTA labeling was 80–100% complete. Spectra of SL toxin occupying different amounts of the total number of AChRM α-toxin binding sites had lineshapes that were identical. The EPR spectrum of membranes incubated with a 1:1 mixture of native *N.n.siamensis* α-toxin and SL toxin also had the same lineshape as the spectrum of AChRM saturated with SL toxin alone. Addition of carbamylcholine to AChRM half-saturated with SL-toxin for several minutes or for 30 min before EPR spectroscopy did not alter lineshapes. The concentration of carbamylcholine used (100 μM) will shift the receptor to the desensitized state [13]. In addition,

progressive reduction of the spin label with ascorbate (3.2 mM at pH 7) reduced both components of the EPR spectrum at equal rates, indicating that there were no large differences in the accessibility of toxin to the aqueous environment.

4. Discussion

Our results provide the first evidence that the physical environments of the 2 α-toxin binding sites are very similar. MBTA labeling of AChRM specifically blocks only 1 of the 2 α-toxin binding sites on the receptor molecule [3]. If the environment of the 2 toxin sites was very different one would expect that the lineshape of the EPR spectrum of SL toxin bound to AChRM would be different than that of SL toxin bound to MBTA labeled AChRM. The results are consistent with studies showing that the affinities of solubilized receptor, membrane-bound receptor, and desensitized receptor for α-toxin are very similar [13, 14] and that membrane-bound receptor has a kinetically homogeneous population of α-toxin binding sites [15]. Each toxin binding site may represent an independent, functional site for activation of increased ion permeability (Delegeane, M. G. McN. submitted). The structural basis for the half-to-the-site reactivity of the affinity labels remains obscure, but could reflect different subunit contacts for each binding subunit of the receptor.

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