EPR AND FLUORESCENCE STUDY OF INTERACTION OF *NAJA NAJA OXIANA* NEUROTOXIN II AND ITS DERIVATIVES WITH ACETYLCHOLINE RECEPTOR PROTEIN FROM *TORPEDO MARMORATA*

V. I. TSETLIN, E. KARLSSON*, A. S. ARSENIEV, Yu. N. UTKIN, A. M. SURIN, V. S. PASHKOV, K. A. PLUZHNIKOV, V. T. IVANOV, V. F. BYSTROV and Yu. A. OVCHINNIKOV

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, 117988, GSP-1, Moscow V-334, USSR and **Institute of Biochemistry, Box 576, S-75123 Uppsala, Sweden*

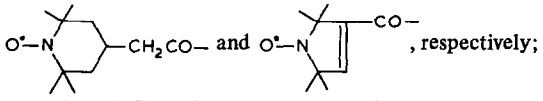
Received 28 June 1979

1. Introduction

Snake venom neurotoxins that specifically interact with the nicotinic acetylcholine receptor (AchR) of the postsynaptic membrane and thereby prevent the binding of acetylcholine and block neural transmission, have been successfully used to obtain highly purified preparations of the receptor, and to study its properties. Though much information on AchR and neurotoxins is available [1–3], the structural aspects of their interaction remain unclear. In particular, there are no direct data as to the sites of the neurotoxin molecule which bind to the AchR. We attacked this problem by using EPR and fluorescence spectroscopy for monitoring the binding of selectively spin- and fluorescence-labeled derivatives of the short (61 residues) neurotoxin II of *Naja naja oxiana* with AchR from the electric organ of *Torpedo marmorata*. The respective reporter groups were attached to lysine ϵ-amino groups, since upon acylation of the latter the spatial structure and biological activity are essentially preserved [4].

Earlier spectroscopic studies of AchR binding with fluorescence- [5–8] or spin-labeled [9–12] low molecular weight ligands were published; however, no such experiments were conducted with protein neurotoxins from snake venoms.

Abbreviations: AchR, acetylcholine receptor protein; NT-II, neurotoxin II from *Naja naja oxiana;* SL and SL', spin labels



, respectively;

Dns, dansyl; EPR, electron paramagnetic resonance

Address correspondence to: V. I. Tsetlin, Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, 117988, GSP-1, Moscow V-334, USSR

2. Materials and methods

NT-II was isolated by the procedure in [13]. Compounds I–III, VI and VIII–XII (see table 1) were obtained according to [4,14,15], the preparation of compounds IV, V and VII (synthesized via spin-labeled *N*-hydroxysuccinimide ester) will be published elsewhere. AchR from the electric organ of *Torpedo marmorata* was isolated by the procedure in [16]. The preparations of AchR solubilized in Triton X-100 demonstrated one band on polyacrylamide gel electrophoresis and possessed an activity of 10 000 nmol α-toxin binding sites/g receptor protein. In fluorescence and EPR studies, solutions of the receptor (0.3 M sodium phosphate buffer (pH 7.5), 0.025% Triton X-100) were used at $2.5–8.0 \times 10^{-7}$ M and 5×10^{-6} M, respectively. The stock solutions of neurotoxins were $\sim 10^{-4}$ M. Fluorescence measurements were made using the Aminco SPF-1000 instrument. For taking the difference fluorescence spectra (fig.1) AchR solution was added into the sample

cuvette, buffer into the reference cuvette, and equal quantities of NT-II or its derivative solutions were added into both cuvettes. Analysis of titration curves was carried out assuming mol. wt 250 000 for AchR [17]. Dissociation constants (K_d) of the AchR complexes with I–III and the number of binding sites n (fig.2) were calculated by using essentially the procedure in [18]. Displacement of the dansylated toxins from the AchR complex was analyzed using the formalism of [19]. EPR spectra were taken at 21 ± 1°C using the Varian E-109 (9 GHz) spectrometer equipped with the Varian E-900 EPR Data Acquisition System.

Rotation correlation times for spin-labeled derivatives (τ_{ob_s}) within the 5×10^{-11} –1×10^{-9} s interval were determined from the EPR spectra by the equation [20]:

$$\tau_{obs} = 6.65\ \Delta H_+ \left(\sqrt{[I_+/I_-]} - 1\right) \times 10^{-10}\ \text{s}$$

where ΔH_+ is the width of the low field component, I_+ and I_- are intensities of the extreme components located in the low and high field, respectively. For slower rotation rates the values of τ_{obs} were evaluated by comparison with calculated EPR spectra collection [21], assuming the isotropic rotation of labels. The correlation time of the spin-label rotation relative to the protein globule (τ_{rel}) was calculated in terms of isotropic rotation model by the equation:

$$\tau_{obs}^{-1} = \tau_p^{-1} + \tau_{rel}^{-1}$$

where τ_{obs} was determined from the spectra, and τ_p is the rotation correlation time of the protein macromolecule. For AchR $\tau_p = 0.4 \times 10^{-6}$ s as calculated from [22] and for the NT-II derivatives $\tau_p = 4.3 \times 10^{-9}$ s, as found from polarization and excited state life-time measurements with dansylated analog I (to be published elsewhere); this value is in accord with that ($\tau_p = 3.5 \times 10^{-9}$ s) calculated from the dimensions [23] of a homologous protein, erabutoxin b.

The accessibility of spin labels in free SL-derivatives of NT-II or in AchR complexes towards a paramagnetic probe $K_3Fe(CN)_6$ was estimated from the equation [24]:

$$\Delta H_o = 6.5 \times 10^{-8}\ k_p C$$

where ΔH_o is broadening of the central component in the EPR spectrum, C is concentration of the spin probe and k_p is exchange relaxation rate constant between the spin label and the spin probe. Table 2 gives the values of $k_w/2k_p$, where $k_w = 12 \times 10^{-8}$ M^{-1} s^{-1} [24] is the exchange relaxation rate constant between a free spin label in solution and the spin probe.

3. Results and discussion

Tables 1 and 2 show that all the spin- and fluorescence-labeled neurotoxin derivatives are biologically active retaining from 12–70% of the toxicity of the native molecule.

Figure 1 shows that binding of I is accompanied by a hypsochrome shift from 562–544 nm of the emission maximum and enhancement of the fluorescence intensity which are indicative of a more hydrophobic environment of the dansyl chromophore in the complex than in the unbound toxin. If the dansyl group is attached by means of the glycyl chain in the Lys 46 or Lys 26 positions (III and II), the spectral changes for III are similar (568–548 nm), while with

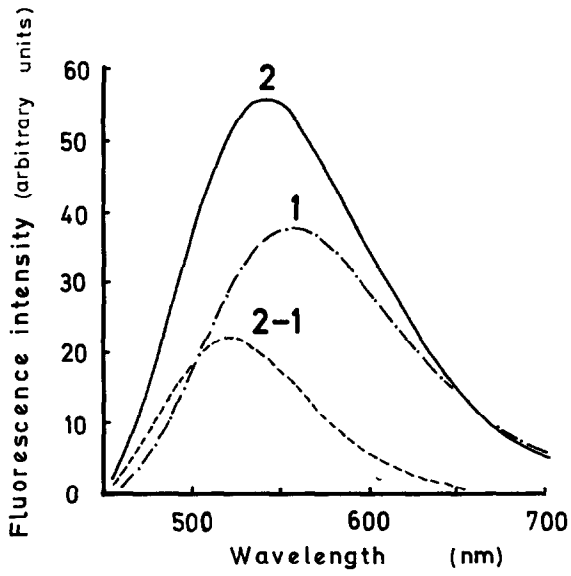


Fig.1. Fluorescence emission spectra of dansylated NT-II derivative I in buffer (.–.), AchR-bound (——) and the difference spectrum (– – –).

II there is virtually no shift of the emission maximum and the increase in intensity is considerably smaller, thereby indicating that the polarity of the environment of the dansyl group changes insignificantly upon binding of II to the AchR.

Titration of AchR by the compounds I–III shows saturable binding. Analysis of the titration curves in fig.2 shows that ~2 mol compound I are bound/mol AchR, while K_d determined in several experiments (with various AchR preparations and concentrations), equals $(7 \pm 3) \times 10^{-8}$ M (table 1).

Comparison of emission spectra of I at excitation wavelengths 330 and 298 nm (absorption band of tryptophan residues) revealed the energy transfer from tryptophan(s) in the toxin binding site of the AchR to the Dns group of the neurotoxin.

The EPR spectra (fig.3) and the values of τ_{obs} and τ_{rel} (table 2) show that the spin labels on different sites of the neurotoxin molecule differ in mobility. Binding of compounds IV–VIII to AchR is accompanied by broadening of the EPR spectra. The calculations show that complexation increases the τ_{rel} values to various extents, the relative rotation of spin labels in the toxin–receptor complex being most noticeably hindered with the compounds V and VI (Lys 25 and Lys 26). However, even in these two cases τ_{rel} is ~20-fold lower than τ_p for AchR

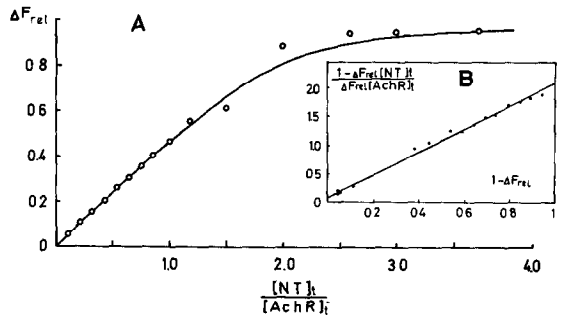


Fig.2. Titration of AchR (7.8×10^{-7} M) by NT-II derivative I; excitation and emission (in difference spectrum) wavelengths are 330 and 525 nm, respectively. (A) Circles, experimental values; solid line, a theoretical curve calculated for $K_d = 7.3 \times 10^{-8}$ M and $n = 2$ found from plot B in linear coordinates; ΔF_{rel}, the ratio of fluorescence intensity at given concentration of neurotoxin to maximal fluorescence at complete occupancy of AchR neurotoxin binding sites; $[NT]_t$ and $[AchR]_t$, the total concentration of a neurotoxin and AchR, respectively.

$(0.4 \times 10^{-6}$ s), which means that in all complexes the spin labels preserve considerable mobility.

According to the EPR titration data, similar to the above results for the Dns-derivatives, 1 molecule of AchR binds 2 molecules of spin-labeled toxin. High specificity of binding is indicated by the experiments

Table 1
Toxicity of the *Naja naja oxiana* neurotoxin II derivatives and dissociation constants for their complexes with acetylcholine receptor from *Torpedo marmorata*

Modification to neurotoxin II	Designation	LD_{50} (μg/kg)	K_d ($\times 10^8$ M)
None	NT-II	60	0.3[b]
N^{ϵ}–Dns on Lys 26	I	500	7 ± 3
N^{ϵ}–Dns–Gly on Lys 26	II	430	8[c]
N^{ϵ}–Dns–Gly on Lys 46 and N^{ϵ}–Ac on Lys 26	III	–[a]	8[c]
N^{ϵ}–Ac on Lys 25	IX	100	0.3[b]
N^{ϵ}–Ac on Lys 26	X	250	1.5[b]
N^{ϵ}–Ac on Lys 44	XI	60	0.3[b]
CF_3CO on N^{α} of Leu 1 and N^{ϵ} of Lys 15, Lys 25, Lys 26, Lys 44 and Lys 46	XII	6000	–[a]

[a] Not determined
[b] Found by displacement experiments
[c] One measurement

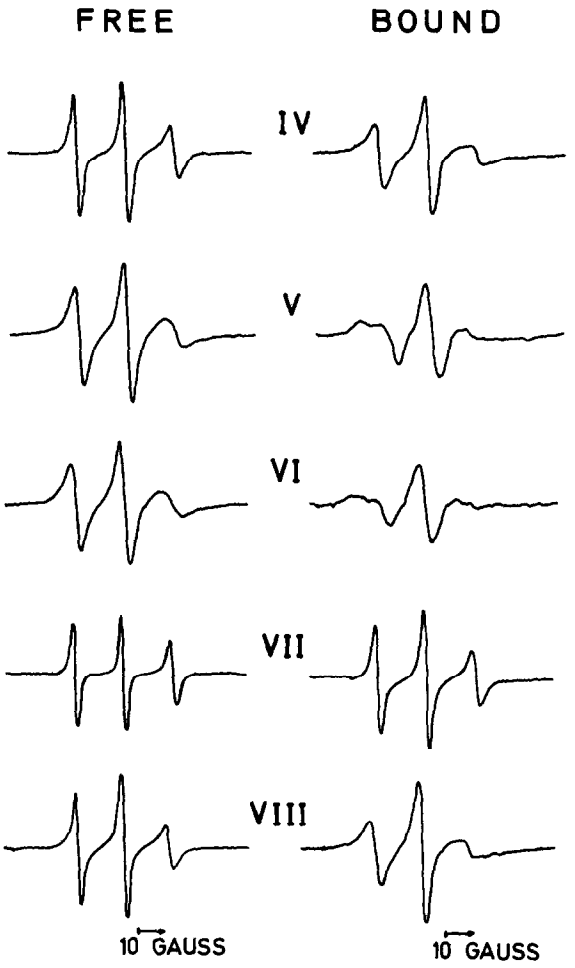


Fig.3. EPR spectra of NT-II spin-labeled derivatives in solution and bound to the AchR.

on displacement of the labeled compound from the AchR complex by excess of the native NT-II, which results in the fluorescence and EPR spectra corresponding to those of free labeled derivatives. K_d of NT-II determined from the displacement of I is $\sim 0.3 \times 10^{-8}$ M, which approaches to the K_d value obtained from the binding of radioactively-labeled α-toxin of *Naja naja siamensis* to similar preparations of solubilized AchR [25,26].

Displacement of Dns- or SL-derivatives by NT-II and its acetylated analogs IX–XI allows one to assess the effect of amino-group modification on the binding to AchR. Acetylation of Lys 25 and Lys 44 residues has no effect on the binding, while modification of Lys 26 and Lys 46 residues lowers the binding, the effect being more pronounced for Dns- than for Ac-derivatives (table 1). Introduction of SL- and Dns-groups also impairs the toxicity more severly than acetylation; however, in general the weaker binding to AchR (larger K_d) is accompanied by lower toxicity. The same was observed for modified related neurotoxins [19,27].

The nontoxic hexatrifluoroacetyl derivative XII has a low affinity for AchR and displaces only ~50% of the bound quantity of compound VIII. This can be rationalized in the light of the present-day concept that 2 α-toxin binding sites, equivalent in the free AchR can show nonequivalence due to cooperative interactions with neurotoxins or low molecular weight ligands [28].

The $k_w/2k_p$ values (table 2) obtained with the paramagnetic probe $K_3Fe(CN)_6$ indicate that for spin-labeled derivatives IV, VII and VIII (labels on

Table 2
Spin-labeled neurotoxin II derivatives

Modification to neurotoxin II	Desig-nation	LD_{50} (μg/kg)	Correlation time				Broadening by paramagnetic probe	
			Free derivative		AchR-bound			
			τ_{obs} (ns)	τ_{rel}	τ_{obs} (ns)	τ_{rel}	Free toxin	AchR-bound ($k_w/2k_p$)
N^{ϵ}–SL on Lys 15	IV	110	0.8	1.0	2	2	1.29	1.37
N^{ϵ}–SL on Lys 25	V	85	2.0	3.7	~20	~20	2.29	4.23
N^{ϵ}–SL on Lys 26	VI	300	2.0	3.7	~20	~20	2.93	>>10
N^{ϵ}–SL on Lys 44	VII	152	0.5	0.56	0.9	0.9	1.02	1.25
N^{ϵ}–SL′ on Lys 46	VIII	240	0.9	1.1	2	2	1.13	9.26

Lys 15, 44 and 46) the microviscosity in the environment of these labels is close to that of pure water ($k_w/2k_p = 1$) [24]. This should indicate that the labels point out from the surface of the molecule. The $k_w/2k_p$ values for the compounds V and VI (Lys 25 and 26) are typical of labels located on the surface of the protein molecule surrounded by structurated water [24]. This differentiation of spin labels according to the microviscosity of the environment is in accord with the correlation times, lower viscosity corresponding to shorter correlation time (table 2).

The $k_w/2k_p$ values indicate that the spin label on Lys 44 (compound VII) is almost equally accessible for the broadening paramagnetic probe $K_3Fe(CN)_6$ in the free toxin as in its AchR complex. For the spin labels on the other lysines the complexation decreases the possibility of contacts with the paramagnetic probe, the screening effect being most pronounced for Lys 26 and Lys 46 derivatives of NT-II (VI and VIII).

Our findings show the orientation of the neurotoxin molecule in its complex with the receptor. To summarize, the EPR data provide the direct evidence for the fact that the labels covalently bound to ϵ-amino groups of Lys 26, Lys 46, Lys 15 and Lys 25 residues, on formation of toxin–receptor complexes, are in contact with the surface of the AchR molecule. Fluorescence measurements support this conclusion for Lys 26 and 46. The τ_{rel} values are indicative of restricted mobility in the complex of the spin labels on Lys 25 and 26, whereas the labels on Lys 15 and 46 are less restricted although inside the complex, perhaps situated in a large 'pocket' or cavity. The cavity for Lys 26 can accommodate a dansyl group but not if it is attached by a glycyl arm, while the 'pocket' for Lys 46 can hold even the dansyl–glycyl group. The neurotoxin binding site is apparently considerably extended and the neurotoxin–receptor interaction, as was supposed [2] is of a multipoint character involving at least the 3 loops A, B and C confined with disulfide bonds (fig.4). The present data support in particular our earlier supposition that the loops B and C are involved in binding [4].

Since all monolabeled toxin derivatives are more or less effective in their interaction with the receptor, no single charged ϵ-amino group in the native toxin is absolutely essential for the binding and biological activity. This conclusion agrees with earlier results

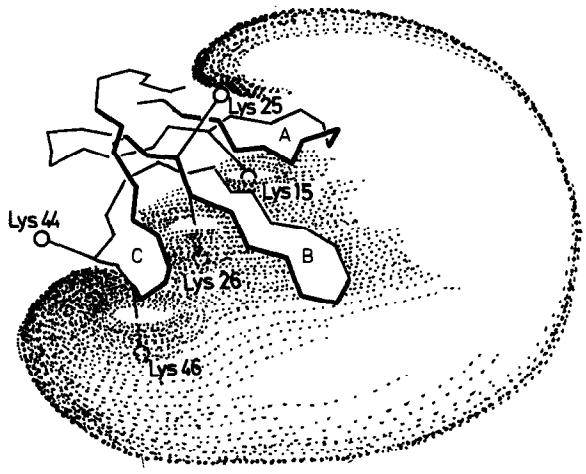


Fig.4. A scheme for neurotoxin binding to AchR as inferred from the EPR and fluorescence studies.

obtained with other neurotoxins [2,29,30]. Apparently, the interaction with the receptor also involves residues in the neurotoxin molecule other than lysines, which enable the ϵ-amino groups with their attached labels to approach the binding area of the receptor. Modification of such a group taking part in the interaction should only weaken the binding, not abolish it, since the interaction from the remaining intact sites still is sufficient to bind the toxin to the receptor.

As the solution structure of neurotoxin II might be essentially similar [4,15] to the crystal structure of erabutoxin b [23,31,32] we have used the crystal structure to illustrate the binding of neurotoxins to AchR (fig.4). The figure shows the association of one neurotoxin molecule. Binding of the second toxin molecule, at least with respect to the lysine residues, could be represented in a similar way.

Acknowledgements

This research was one within the program 'Physico–chemical methods in biology', sponsored by the USSR Academy of Sciences, Royal Swedish Academy of Sciences and the Royal Swedish Academy of Engineering Sciences.

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