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α-Toxin binding to acetylcholine receptor α179–191 peptides: intrinsic fluorescence studies

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Interactions between two α-toxins and the synthetic peptides α179–191 from both calf and human acetylcholine receptor α-subunit sequences have been studied by measurements of quenching of intrinsic fluorescence after toxin addition. Dissociation constants of approx. 5×10^{-8} M for binding of calf peptide by both α-cobratoxin and erabutoxin a have been estimated. The binding of α-cobratoxin to calf peptide, which leads to marked quenching of fluorescence intensity, is inhibited by a 10^4 molar excess of acetylcholine. The human α179–191 peptide binds to α-cobratoxin, but not, under comparable conditions, to erabutoxin a.

Acetylcholine receptor α-subunit; α-Toxin binding region; α-Cobratoxin; Erabutoxin a; Synthetic peptide; Intrinsic fluorescence

1. INTRODUCTION

The nicotinic AChR, a multi-subunit membrane protein, $\alpha_2\beta\gamma\delta$, is a pentameric array of four highly homologous subunits. The α-subunit contains the competitive binding site for both normal neurotransmitter, ACh, and for the protein snake venom α-toxins, which block neuromuscular transmission [1]. In a series of studies, the structure of the reactive site of one toxin has been employed as prototype stereochemical probe of receptor [2–4]. These studies have led directly to identification of one AChR α-subunit sequence as the probable complementary site of toxin binding [4]. The initial requirement imposed by the toxin reactive site stereochemistry was that the extracellular α-subunit toxin-binding domain should include a unique or uniquely located Trp residue absent from the corresponding β-, γ- or δ-subunit sequences. Two such residues, Trp-184 and Trp-187, occur in the unique sequence α Val-177–Cys-193 of *Torpedo californica* [5–7], an α-segment, highly conserved in five other species, which satisfies all conformational and interactive requirements imposed by the toxin stereochemistry [4]. In the anomalous human α-subunit sequence, residue 187 is Ser.

This communication describes evidence which confirms our designation of one continuous peptide chain segment, α Val-177–Cys-193, as prime toxin-binding domain. Two mammalian sequence peptides have been synthesized and their binding interactions with both long chain α-CbTX and short chain Ea, studied using intrinsic fluorescence techniques. These techniques have been used to study AChR binding of both ACh and toxins [8,9].

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Abbreviations: AChR, acetylcholine receptor; ACh, acetylcholine; α-CbTX, α-cobratoxin; Ea, erabutoxin a; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; FPLC, fast performance liquid chromatography

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2. MATERIALS AND METHODS

α-CbTX (*Naja naja siamensis*) was obtained from the Miami Serpentarium, Salt Lake City, UT; Ea (*Laticauda semifasciata*) was a gift from Professor Nobuo Tamiya. The purity of both toxins was confirmed by reverse-phase HPLC. Peptides were synthesized with both calf and human AChR α-subunit 179–191 sequences [10]. The human sequence synthesized differs from the calf sequence shown below only at α Ser-187 and α Thr-189.

Calf: Lys(179)-Glu-Ser-Arg-Gly-Trp(184)-Lys-His-Trp(187)-
Val-Phe-Tyr-Ala(191)

To enhance peptide solubility and to avoid possible disruptive conformational effects, neither the N-terminal Val-177 Ile-178 nor the C-terminal Cys-192 Cys-193 dipeptide regions of the target segment 177–193 were included in the syntheses. To avoid problems in synthesis of human peptide, Ser-191 was replaced by Ala as in calf. The calf α 179–191 AChR peptide was synthesized with an Applied Biosystems 430A automated synthesizer, and purified by ion exchange (Pharmacia FPLC mono-Q column) followed by reverse-phase HPLC. The human α 179–191 AChR peptide was synthesized by an updated procedure [11], and purified by gel filtration followed by two reverse-phase HPLC separations. Amino acid and sequence analyses were used to establish purity and identity of both peptides.

Fluorescence emission spectra were obtained at 23°C with the Perkin Elmer MFP66 spectrofluorometer using a 1 cm cell. Spectra were recorded over the range 320–495 nm and averaged over 3–10 scans. The excitation wavelength associated with tryptophan fluorescence, 280 nm, was used except where noted. For studies with calf peptide, 2 × 5 nm or 3 × 5 nm slits were used to minimize photochemical decay; with the more weakly fluorescing human peptide, 5 × 5 nm slits were used.

In each experiment, fluorescence spectra were obtained for a sample containing a fixed concentration (10^{-8}–10^{-7} M) of calf or human peptide and sequentially increased toxin concentrations. Stock solutions ($\sim 10^{-5}$ M) of peptides and toxins were prepared in deionized and millipore filtered water (concentrations were established by amino acid analysis). In a typical experiment, after a baseline was established for 3.00 ml PBS at pH 7.4 in the sample cell, 5 μl of peptide stock solution were added and the peptide spectrum recorded; samples of toxin stock solution were then added sequentially, and the spectra recorded 20–40 min after each addition. Corresponding spectra for toxin alone were obtained either in separate experiments, or by interpolation between standards. Total toxin to total peptide molar ratios varied from 0.1 to 6.

The spectra of the mixtures were interpreted by the use of quenching curves, where the quenching, Q, at a given wavelength is given by the expression: $Q = F_p + F_t - F_m$. Here F_p and F_t represent fluorescence intensity for peptide and toxin solutions, respectively, and F_m the fluorescence intensity of the solutions when mixed. For simple 1:1 peptide/toxin binding, the fraction of peptide bound, x, is given by $x = Q/Q_L$, where Q_L is the limiting value of Q at high toxin concentrations [12]. If Q_L is estimated, the fraction x for each mixture can be evaluated. The dissociation constant, K_d, can then be calculated from the equation $K_d = [P]\cdot(b - x)\cdot(1 - x)/x$, where b = [T]/[P], and [P] and [T] are the overall peptide and toxin concentrations respectively. For interpretation of data from peptide-α-CbTx solutions, where there were 6–10 separate observations, values for K_d and for a scale factor $f = Q_L/[P]$ were obtained by non-linear least squares fit of observed and calculated quenchings at the chosen wavelength (locally written program).

In solutions containing a large excess of toxin, almost all peptide is bound, and fluorescence is mainly that from peptide–toxin complex plus unbound toxin. Thus, if toxin fluorescence remains relatively unchanged on binding, the difference spectrum between that of the mixture and that of toxin alone may be considered the fluorescence spectrum of bound peptide. Changes in peptide Trp fluorescence on binding may thus be monitored as in studies with whole receptor [9]. For solutions containing a large excess of ACh, quenching was defined as before by $Q = F_t + F_p - F_m$ where F_t, F_p and F_m now refer to fluorescence intensities in the presence of ACh.

3. RESULTS

3.1. *Calf peptide α-CbTX studies*

Spectra obtained after sequential addition of α-CbTX to calf peptide solution show an initial decrease in peak fluorescence intensity, followed by an increase (fig.1A). Associated quenching curves all have the same shape and differ only in scale as expected for 1:1 binding of toxin to peptide (see fig.2). Near saturation after final toxin addition is evident in fig.1B. Least squares analyses of the quenching data at 353 nm and at 343 nm gave estimated K_d values of 6×10^{-8} M and 11×10^{-8} M, respectively. Analyses of data at 353 and 340 nm from an independent experiment have K_d values of 2×10^{-8} M at each wavelength. The average dissociation constant for these runs was approx. 5×10^{-8} M.

In one experiment calf peptide was preincubated for 45 min with a 10^4 molar excess of ACh before toxin addition. This led to a sharp reduction in the toxin quenching effect (cf. 1a and 1b in fig.2). Fluorescence of peptide solution alone was reduced 20% in the presence of ACh. When ACh binds to the whole receptor there is a comparable 7–8% reduction in fluorescence intensity [8]. The observed inhibition of peptide binding by excess ACh and the ACh quenching of peptide fluorescence are both evidence of ACh binding by calf peptide.

3.2. *Calf peptide-Ea studies*

Data from two experiments with calf peptide and Ea, at 278 and 280 nm excitation wavelengths,

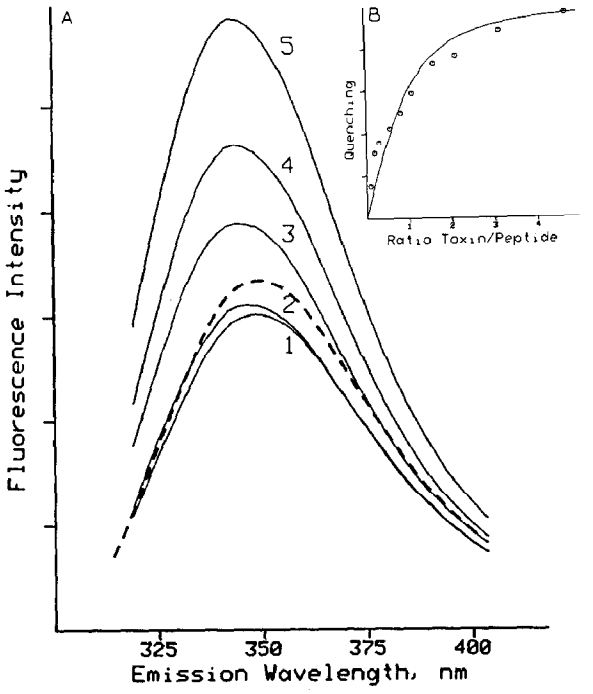

Fig.1. (A) Fluorescence spectra: (---) calf peptide (1.9×10^{-7} M), alone. Calf peptide after α-CbTX addition at selected concentrations of (1) 0.4, (2) 1.1, (3) 3.0, (4) 5.8 and (5) 8.7×10^{-7} M. Note blue shift in peak positions as toxin concentration is increased. (B) Saturation: quenching at 353 nm as function of toxin concentration. Smooth curves calculated using $K_d = 5 \times 10^{-8}$ M.

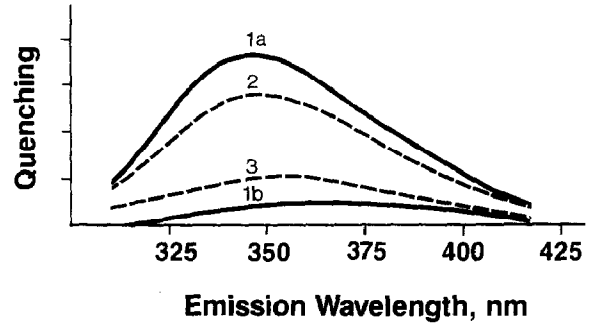

Fig.2. Fluorescent quenching: ACh inhibition. Quenching curves for calf peptide (9.0×10^{-8} M) with α-CbTX (4.8×10^{-7} M) in the absence (1a) and in the presence (1b) of 1.5×10^{-3} M ACh, and, in the absence of ACh, with α-CbTX (1.7×10^{-8} and 0.3×10^{-8} M), curves 2 and 3 respectively. Curves comparable to 2 and 3 (not shown) obtained in the presence of ACh lie very close to the baseline.

established binding, with a dissociation constant between 2 and 5×10^{-8} M. This range of values is similar to that estimated for calf peptide-α-CbTX binding. As fig.3 shows, calf peptide fluorescence is more strongly quenched over the whole spectral range when long chain α-CbTX binds than when short chain Ea binds.

3.3. *Human peptide-toxin studies*

It was more difficult to obtain quantitative data from studies with this peptide, since the intrinsic fluorescence of human peptide, measured under comparable conditions, is lower than that of calf peptide (fig.3). There is only one weakly fluorescing Trp residue α 184 in human peptide. A tentative analysis of the results of preliminary investigations of human peptide-α-CbTX interactions (278 nm excitation wavelength) suggested a K_d of approx. 10^{-7} M, which is somewhat larger than that for calf peptide-α-CbTX interactions. Under comparable conditions no evidence of Ea binding by human peptide was found. Exhaustive studies were not made.

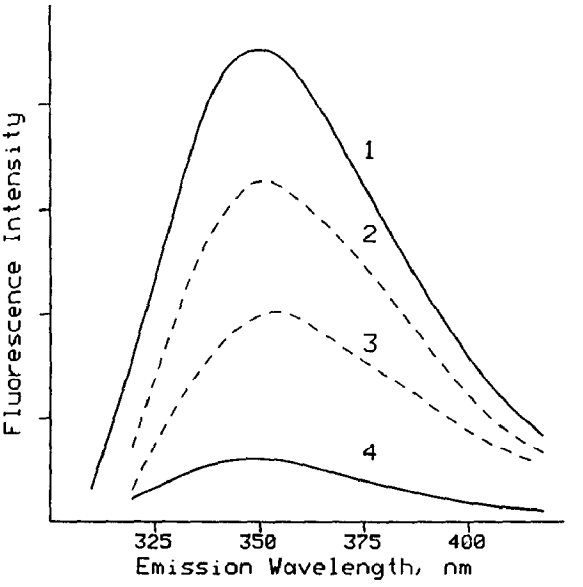

Fig.3. Peptide fluorescence before and after binding. (1) Calf peptide fluorescence (0.9×10^{-7} M); (2) calf peptide fluorescence in association with Ea; (3) calf peptide fluorescence in association with α-CbTX (2 and 3 prepared as described in text); (4) human peptide fluorescence (0.9×10^{-7} M).

4. DISCUSSION

These experiments clearly establish that the AChR calf α-subunit synthetic peptide, α 179–191, binds both long chain α-CbTX and short chain Ea, with dissociation constants of $\sim 5 \times 10^{-8}$ M in each case. Thus a short isolated segment of the α-subunit, first identified in terms of stereochemical requirements imposed by the toxin, binds these toxins as tightly as AChR binds both ACh and the curare alkaloids [13,14]. Further, with one toxin, α-CbTX, competitive binding by ACh has been demonstrated. The studies strongly support identification of the α 179–191 segment as prime competitive AChR toxin-binding region [4].

The dynamic mode of toxin-receptor binding proposed earlier [4], which required that a receptor α Trp residue enter and be bound in the 'Trp' cleft of the toxin reactive site, favored α Trp-187 rather than α Trp-184 as cleft occupant. The experiments reported here suggest that α Trp-187 has indeed a major role in peptide/toxin binding interactions. The fluorescence intensity of calf peptide, with two α Trp fluorophores, α Trp-184 and Trp-187, is, on a molar basis, approx. 8-times greater than of human peptide, which lacks α Trp-187. The enhanced calf peptide fluorescence may reasonably be associated with the Trp-187 contribution as dominant fluorophore. The sharp reduction of calf peptide fluorescence on binding, almost 50% for α-CbTX interactions, therefore reflects, in large part, a marked change in α Trp-187 environment.

Although the human α 179–191 peptide lacks Trp-187 and does not bind the short chain Ea, it does bind, almost as tightly as calf peptide, to long chain CbTX. These findings follow the unusual pattern which characterises binding by intact human receptor. Thus, clinical observations [15] and biochemical experiments [16] have both implied or shown that long chain toxins do, and short chain toxins do not, bind to human receptor. The dilemma imposed by the assumption of one common α-toxin binding mode [3] remains unsolved. It is possible that the long chain toxins can adopt a conformation adjusted to an atypical adaptive mode of binding to human AChR [4].

Designation of the α 179–191 segment as prime competitive toxin-binding site is generally consistent with results of other studies. The whole extracellular region of the α-subunit has been explored sequentially [17–20]. The principal toxin-binding regions so identified overlap, at least partially, the segment α 179–191. In one study a K_d of 4×10^{-5} M was reported for α-CbTX binding to *T. marmorata* synthetic peptide, α 185–196 [20]. The binding reported here for segment α 179–191, which is three orders of magnitude tighter, emphasizes the importance in multipoint toxin binding interactions of other residues besides α Trp-187 along the whole 179–191 segment. Work in progress seeks to establish structural details of these interactions.

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REFERENCES

[1] Changeux, J.-P., Devilliers-Thiery, A. and Chemoulli, P. (1984) Science 225, 1335–1345.
[2] Low, B.W. (1979) in: Snake Venoms; Handbook of Experimental Pharmacology (Lee, C.Y. ed.) vol.52, pp.213–257, Springer, Berlin.
[3] Low, B.W. and Corfield, P.W.R. (1986) Eur. J. Biochem. 161, 579–587.
[4] Low, B.W. and Corfield, P.W.R. (1987) Asia Pac. J. Pharm. 2, 115–127.
[5] Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T. and Numa, S. (1982) Nature 299, 793–799.
[6] Claudio, T., Ballivet, M., Patrick, J. and Heinemann, S. (1983) Proc. Natl. Acad. Sci. USA 80, 1111–1115.
[7] Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Hirose, T., Asai, M., Takashima, H., Inayama, S., Miyata, T. and Numa, S. (1983) Nature 301, 251–255.
[8] Kaneda, N., Tanaka, F., Kohno, M., Hayashi, K. and Kagi, K. (1982) Arch. Biochem. Biophys. 218, 376–383.
[9] Endo, T., Nakanishi, M., Furukawa, S., Joubert, F.J., Tamiya, N. and Hayashi, K. (1986) Biochemistry 25, 395–404.
[10] Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Tanabe, T., Shimizu, S., Kikyotani, S., Kayano, T., Hirose, T., Inayama, S. and Numa, S. (1983) Nature 305, 817–823.
[11] Hashim, G.A., Day, E.D., Fredane, L., Intintola, P. and Carvalho, E. (1986) J. Neurosci. Res. 16, 467–478.
[12] Deranleau, D.A. (1969) J. Am. Chem. Soc. 9, 4044–4049.

[13] Neubig, R.R. and Cohen, J.G. (1979) Biochemistry 18, 5465–5475.
[14] Weber, M. and Changeux, J.-P. (1974) J. Mol. Pharmacol. 10, 15–34.
[15] Reid, A. (1979) in: Snake Venoms; Handbook of Experimental Pharmacology (Lee, C.Y. ed.) vol.52, pp.940–943, Springer, Berlin.
[16] Ishikawa, Y., Kano, M., Tamiya, N. and Shimada, Y. (1985) Brain Res. 346, 84–88.
[17] Wilson, P.T., Lentz, T.L. and Hawrot, E. (1985) Proc. Natl. Acad. Sci. USA 82, 8790–8794.
[18] Mulac-Jericevic, B. and Atassi, M.Z. (1986) FEBS Lett. 199, 68–74.
[19] Ralston, S., Sarin, V., Thanh, H.L., Rivier, J., Hox, J.L. and Lindstrom, J. (1987) Biochemistry 26, 3261–3266.
[20] Neumann, D., Gershoni, J.M., Fridkin, M. and Fuchs, S. (1986) Proc. Natl. Acad. Sci. USA 82, 3490–3493.