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Interaction of protein ligands with receptor fragments

On the residues of curaremimetic toxins that recognize fragments 128–142 and 185–199 of the α-subunit of the nicotinic acetylcholine receptor

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Abstract

Using a solid-phase assay, we found that ^{3}H-labelled αCobtx from *Naja naja siamensis*, a long-chain curaremimetic toxin, and ^{3}H-labelled toxin α from *Naja nigricollis*, a short-chain toxin both bind specifically but with substantially different affinities (K_d = 4 10^{-7} M and 50 10^{-6} M) to fragment 185–199 (Tα185–199) of the α-subunit of the acetylcholine receptor (AcChoR) from *Torpedo marmorata* Then we show that monoderivatizations of residues common to both long-chain and short-chain toxins (Tyr-25, Lys-27, Trp-29, and Lys-53) or to long-chain toxins only (Cys-30 and Cys-34) do not affect the binding of the toxins to Tα185–199, suggesting that none of these invariant residues is implicated in the recognition of this AcChoR region αCobtx and toxin α bind to the fragment 128–142 (Tα128–142) with more similar affinities (K_d = 3 10^{-7} M and 1 4 10^{-6} M) and their binding is dramatically affected by the single abolition of the positive charge of Lys-53, an invariant residue that contributes to AcChoR recognition Therefore, the data indicate that Lys-53 more specifically recognizes the 128–142 region of AcChoR Other monoderivatizations have no effect on toxin binding The approach described in this paper may be of great help to identify toxin residues that establish direct contact with receptor fragments

Key words Curaremimetic toxins, Nicotinic acetylcholine receptor

1. Introduction

Identification of interaction surfaces between ligands and their receptors constitutes a fundamental step to understand the molecular mechanisms that are associated with the regulation of a number of essential physiological processes To examine ultimately the molecular events that allow snake curaremimetic toxins to block the cation-selective opening of nicotinic acetylcholine receptor (AcChoR) [1,2], extensive studies are currently undertaken in various laboratories to identify the complementary sites by which the toxins and the receptor interact

The nicotinic acetylcholine receptor (AcChoR) is a member of the ligand-gated ion channel family with five transmembrane subunits in a stoichiometry of $\alpha_2\beta\gamma\delta$. It regulates cation exchange through muscle membranes and hence muscular contraction [3–6] It has two binding sites for its competitive ligands, including curaremimetic toxins, which are mostly located on the α subunits [4,8–14]. More precise definition of the curaremimetic toxin binding site has been attempted with fragments of α subunits generated as proteolytic [15–18], synthetic [19–27] or fusion peptides obtained by genetic means [28–30] It is accepted that the region around the cysteine 192–193 is recognized by most AcChoR ligands. Systematic replacement of the residues by alanine suggest a special contribution of residues Tyr-190, Cys-192, Cys-193, Val-188, Thr-189, Ser-191, Pro-194, Asp-195 and Tyr-198 to formation of toxin–peptide complexes [31]. Other α subunit fragments may also be recognized by the toxins [19,20,32–34]; however, these data require further confirmation since they have sometimes been questionned Among the other possible toxin binding fragments is the loop between the disulfide 128–142 which is conserved in the family of ligand-gated channels, and has been predicted as interacting with cholinergic ligands [35] and controversially proposed as a target for snake toxins [19,20,33,34,36,37]

Curaremimetic toxins are small proteins from Elapidae and Hydrophiidae venoms that bind to AcChoR with high affinity ($K_d = 10^{-11}$ M) They are classified as long-chain or short-chain toxins when they respectively possess between 66 and 74 residues and five disulfides or

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Abbreviations αCobtx, α-cobratoxin, αBgtx, α-bungarotoxin, Ac-ChoR, *Torpedo* nicotinic acetylcholine receptor, ABTS, 2,2′-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid)

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between 60 and 62 residues and four disulfides [38]. All toxins are flat molecules similarly folded into three adjacent loops with a large β-pleated sheet that gives a two-faces containing aspect to the toxins [39–42] Based on chemical modifications of individual residues [43–47] and site-directed mutagenesis experiments [47,48], the receptor binding site of short-chain toxins appears to be located on one toxin face, including most residues of the central loop (loop II) and those at the tip of the two adjacent loops (loops I and III) In particular, Lys-27, Asp-31, Arg-33, Lys-53 [47,48] and Gln-10 (Ménez et al , unpublished results) seem to contribute predominantly to toxin function At present, however, the receptor residues with which these critical residues interact remain unknown

The aim of this paper is threefold. First, we compare the ability of a short-chain toxin and a long-chain toxin, both labelled with tritium to recognize the *Torpedo marmorata* receptor fragments Tα185–199 (that involves the cystine 192–193) and Tα128–142 (which comprises the cystine 128–142) Our data reveal that the two fragments are specifically recognized by both short-chain and long-chain toxins but with different affinities Second, we examined the binding of a set of long-chain toxins toward both fragments We show that binding affinities substantially vary from one long-chain toxin to another. Third, we determined the affinities of several monoderivatized toxins for the receptor fragments Our data indicate that the conserved lysine 53 interacts with the fragment 128–142 whereas other conserved and functional toxin residues do not recognize Tα185–199 nor Tα128–142

2. Materials and methods

2 1 Toxins

αCobtx from *Naja naja siamensis* venom (Latoxan, France) was purified as described by Karlsson et al [49] Toxin α was prepared from the venom of *Naja nigricollis* (Institut Pasteur, France) by the procedure of Fryklund [50] Toxin α and αCobtx were respectively labelled with tritium according to Ménez et al [51] and Charpentier et al [52] with respective specific radioactivity of 14 1 and 11 2 Ci/mmol αBgtx from *Bungarus multicinctus* and Toxin I from *Naja naja oxiana* were obtained from Sigma Toxin III from *Naja haje annulifera* and LS III from *Laticauda semifasciata* were kindly provided to us by Dr Joubert and Dr Tamiya, respectively Toxin α was covalently linked to peroxydase and used as a tracer in enzyme-linked receptor solid-phase assays according to Léonetti et al [53] The yield of scintillation counting was 36%

Toxin α and αCobtx were both submitted to monoderivatizations at Tyr-25, Lys-27, Trp-29 and Lys-53 according to procedures previously described in details [46,47,52] Furthermore, the additional disulfide Cys-30–Cys-34 of αCobtx was submitted to selective reduction and carboxymethylation with iodoacetamide according to a procedure described in Charpentier et al [52]

2 2 AcChoR and receptor fragments

The acetylcholine receptor (AcChoR) from the electric organ tissue of *Torpedo marmorata* was prepared as described by Saitoh et al [54] and solubilized according to Sobel et al [55] The two receptor fragments Tα185–199 (KHWVYYTCCPDTPYL) and Tα128–142 (CEIIVTHFPFDQQNC) and the non-relevant peptide control (Peptide 32–49 of toxin α HRGTIIERGGGCPTVKPG) were prepared by the classical solid-phase method described by Merrifield [56] The peptides were synthesized on an Applied Biosystems 430A peptide synthesizer using the Boc/Benzyl strategy and symmetrical anhydrides or hydroxybenzotriazole esters Each cysteine was protected with the *S-p*-methoxybenzyl group Free thiols could thus be readily generated by deprotection with hydrogen fluoride (HF) Oxidation of cysteines in disulphides 128–142 and 192–193 was performed by air oxydation Crude materials were purified to homogeneity by reverse phase HPLC on C18 column using a CH_3CN gradient in 0 1% TFA Quality of the peptides was assessed by both amino acid analysis and mass spectrometry

2 3 Binding experiments

In the assay with the complete receptor (AcChoR), the solubilized receptor was diluted in carbonate buffer 0 1 M, pH 9 6 to a concentration of 50 μg/ml and 100 μl of this solution was incubated in 96-well polystyrene microtitrated plates (Immunosorb, Nunc) Adsorption to the walls was conducted overnight at 4°C The plate was then washed 5 times with phosphate buffer (0 01 M, pH 7 4 + 0 005% Tween 20) and subsequently saturated with 300 μl phosphate buffer containing 0 3% BSA Eight hours later the plates were washed 5 times with phosphate buffer and the conjugate toxin α-peroxydase (50 μl) was incubated overnight at 4°C, in the presence of different amounts of toxins dissolved in 50 μl phosphate buffer The plates were washed and 200 μl of ABTS (10^{-3} M) was added 30 min later, the absorbance at 414 nm was determined

In the assay with synthetic fragments, the peptides were first dissolved in a mixture of acetonitrile/H_2O (50/50) at a concentration of 0 5 mg/ml and then diluted at 2 μg/ml (1 μM) in acetonitrile/H_2O (80/20) The solution (50 μl) was placed in the wells and incubated overnight at 37°C 200 μl of phosphate buffer containing 2% BSA was then added for 1 h at room temperature and the wells were washed 5 times In saturation experiments the wells were incubated with 100 μl of various quantities of labelled toxins and the nonspecific binding was determined in the presence of an excess of unlabelled corresponding toxin In competition experiments, [^{3}H]toxin was dissolved in phosphate buffer 0 05 M, pH 7 4, supplemented with 0 1% BSA and 0 01% SDS and 50 μl of the solution was incubated in the presence of various amounts of competitive toxins dissolved in 50 μl of the same buffer, for 4 h The mixtures were washed 5 times with 300 μl of phosphate buffer Bound labelled-toxin was dissolved from the wells in 200 μl of NaOH 0 25 N (30 min) and the dissolved radioactivity was measured Each experiment was done in triplicate

3. Results and discussion

3 1 Choice of the toxins

The toxins that were selected for this study are well-characterized at a molecular level α-Cobratoxin (αCobtx) from venom of *Naja naja siamensis* is a typical curaremimetic long-chain toxin with 72 amino acids and 5 disulfides (see Table 1). It binds to AcChoR with a high affinity characterized by a K_d value approximately equal to 10^{-10} M and has the specific immunological properties of long-chain toxins [52] The high resolution three-dimensional structure of αCobtx is known both in crystals [40] and in solution [57] Toxin α from *Naja nigricollis* is a short-chain toxin with 61 residues and four disulfides It binds to AcChoR with a K_d value close to 10^{-10} M Immunological properties of toxin α have been subjected to extensive studies [58] and are typical of the family of short-chain toxins The 3D structure of toxin α has been solved at high resolution by NMR spectroscopy [42] Several monomodified derivatives of both tox-

Table 1
Amino acid sequences of α-cobratoxin from *Naja naja siamensis* and Toxin α from *Naja nigricollis*

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O  0   1   1   2   2   3   3   4   4   5   5   6   6   7   7
1  5   0   5   0   5   0   5   0   5   0   5   0   5   0   5

IRCFITPDITSKD    CPNGHVCYTKTWCDAFCSIRGKRVDLGCAATCPTVKTGVDIQCC SDNCNPFPTRKRP   αCobtx

LECHNQQSSQPPTTKT CPGETNCYKKVWRD H   RGTIIERGC  GCPTVKPGIKLNCCTTDKCNN          Toxin α

   I-----------I        I-------------------I    I--------I
       loop I                   loop II          loop III
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The two amino acid sequences have been aligned in such a manner that the resulting numbering corresponds to what is currently used in the literature for conserved residues The underlined Tyr-25, Lys-27, Trp-29, Cys-30, Cys-34 and Lys-53 (Lys-46 in Toxin α and Lys-47 in erabutoxins) are residues that were monomodified The three loops that are defined by structural analysis are indicated

ins are available, especially at residues that are implicated in the recognition of AcChoR [45,52,59]. Moreover, both toxins can be radiolabelled with tritium at high specific radioactivity [51,52].

3 2 *Specific binding of [^{3}H]αCobtx and [^{3}H]toxin α to AcChoR peptides*

Previous binding experiments indicated that labelled toxins bind only to the AcChoR α subunit and not to the β, γ and δ subunits [60]. The receptor fragments selected for the present study have the amino acid sequence of the *Torpedo marmorata* α subunit. Binding of increasing amounts of labelled toxins toward Tα185–199 and Tα128–142 coated on a solid phase was determined at equilibrium. Specific and saturable binding was observed with both fragments, as shown typically in the case of binding of [^{3}H]αCobtx and [^{3}H]toxin α to Tα128–142 (Fig. 1A and B) Binding specificity was confirmed by two control experiments. First, we found no specific binding of either toxin to a synthetic fragment with an irrelevant amino acid sequence (not shown) Second, when the peptides were solubilized and incubated with radioactive toxins prior to incubation with the coated AcChoR, binding of labelled toxins to AcChoR decreased in a manner depending on the concentration of the soluble peptides (data not shown) Therefore, these two fragments are able to bind the toxin either coated on a solid support or free in solution Whether or not the coating interferes with the conformation of the fragments remains unclear, however, it was previously shown that coating of the complete receptor does not significantly modify its affinity for receptor ligands [23] Derived Scatchard plots of binding curves (Fig. 1) indicated apparent binding dissociation constants in the micromolar range (Table 2). Toxin α, however, had a nearly 100 times lower affinity for Tα185–199 as compared to αCobtx, suggesting substantial differences between the surfaces by which the two toxins recognize this fragment In contrast, αCobtx and toxin α display similar affinities toward Tα128–142, suggesting that they recognize the fragment with more comparable determinants. That both toxins display lower affinity for the individual fragment as compared to AcChoR or even the isolated α subunit (K_d (αBgtx) = 3 nM with 0.02% SDS, [60]) agree with the current view that curaremimetic toxins recognize multiple regions of the α-subunit

That the fragment Tα185–199 is implicated in binding of long-chain curaremimetic toxins is in agreement with the literature [20,21,23,24,26,27,34,36] We now show that a short-chain toxin also recognizes this region but with lower affinity This finding contrasts with data from Ruan et al [33] who reported that 'region α182–198 was not a binding region for short-neurotoxins' However, these authors used a different binding protocol and the peptide used in their study had no disulfide, a factor that may greatly influence the toxin binding

In agreement with previous observations [20,21,33] we found that the fragment Tα128–142 is capable of binding to both long-chain and short-chain toxins The loop Tα128–142 may therefore be part of the site by which AcChoR from *Torpedo marmorata* is recognized by a curaremimetic toxin Strikingly, this loop is highly conserved in all AcChoR subunits (approximately 70%). Nevertheless, only the α subunits display a stable binding with the toxins [60] suggesting that the presence of the 128–142 fragment is not sufficient for a toxin to firmly bind to a receptor subunit As we have seen above, the more variable fragment Tα185–199 may be one of the additional elements that contribute to the toxin binding domain.

3 3 *Comparison of the binding affinities of various long-chain toxins*

It is tempting to conclude that the differential behaviour of αCobt and toxin α toward the two fragments (Table 2) reflects a general difference between long-chain and short-chain toxins However, it is also possible that it simply reflects variabilities between two toxins, irrespective of their size and their classification to either toxin group To examine this question, we determined

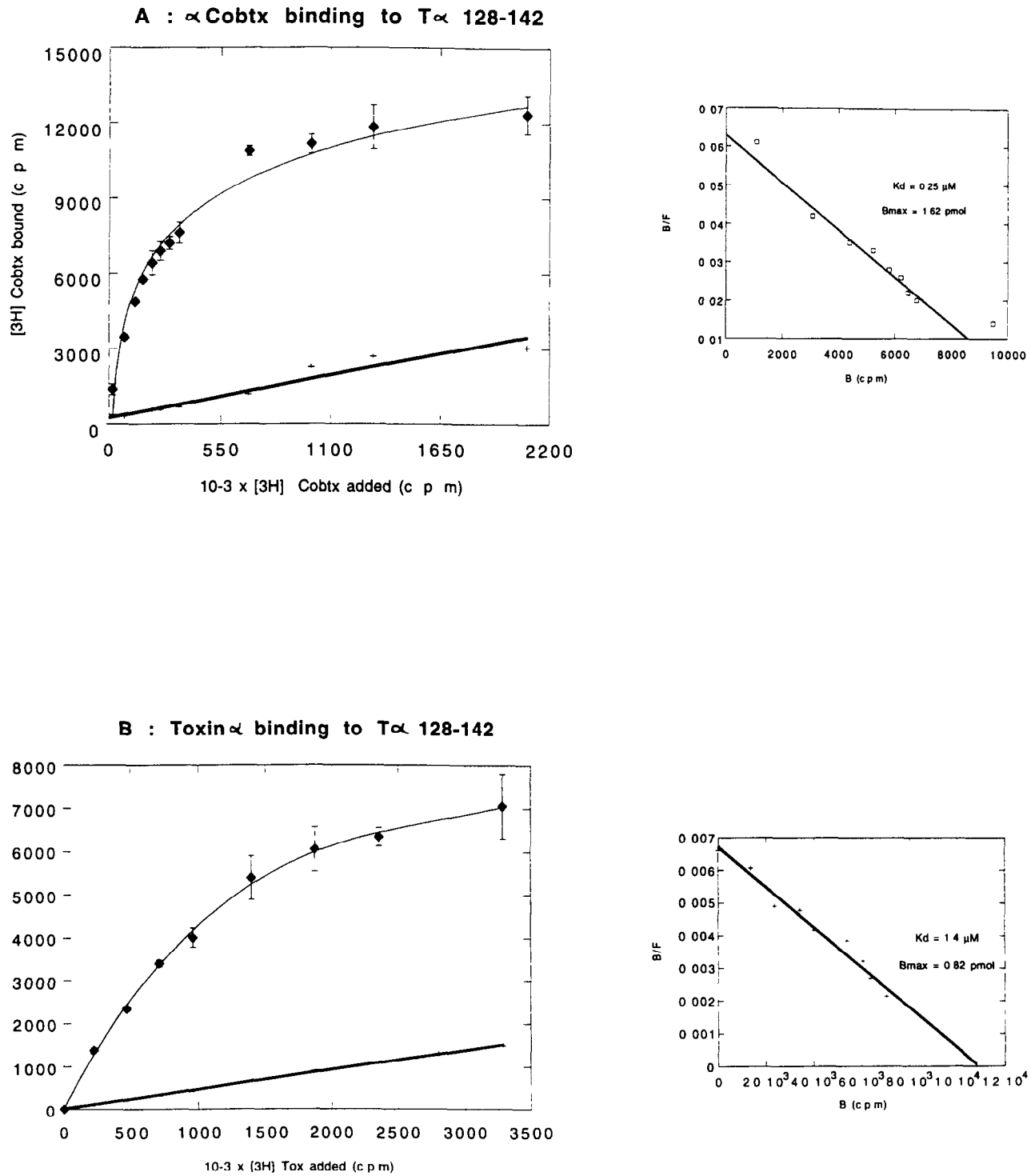

Fig 1 Specific binding of [^{3}H]αCobtx and Toxin α to synthetic peptide Tα128–142 Wells coated with 1 μM peptides were incubated 4 h at room temperature with different concentrations of [^{3}H]αCobtx (11 2 Ci/mmol) or [^{3}H]toxin α (14 2 Ci/mmol) Similar results were obtained when incubation was maintained overnight The wells were washed with 0 25 N NaOH and the radioactivity present in the supernatant was counted A and B represent respectively the binding of [^{3}H]αCobtx and [^{3}H]Toxin α to Tα128–142 Total binding (◆) and nonspecific binding (+) as obtained in the presence of an excess of unlabelled αCobtx (100 μM) Each experiment was done in triplicate Figures on the right show Scatchard analyses derived from saturation binding curves on the left Data are represented as bound/free cpm vs bound cpm αCobtx was characterized by K_d = 0 25 μM and B_{max} = 1 62 pmol and toxin a by K_d = 1 4 μM and B_{max} = 0 82 pmol

the binding affinity of several long-chain curaremimetic toxins for both fragments, on the basis of their capacity to inhibit the binding of [^{3}H]αCobtx to Tα185–199 and to Tα128–142 (Fig 2) We also determined their affinity

Table 2
Apparent equilibrium dissociation constants values of different neurotoxins for peptides Tα185–199 and Tα128–142 Data are from Scatchard analysis derived from binding data

	Tα185–199 K_d (μM)	Tα128–142 K_d (μM)
α-Cobratoxin	0 4 ± 0 2	0 25 ± 0 14
Toxin α	40 ± 20	1 4 ± 0 7

for the complete receptor (AcChoR). The apparent dissociation constants were derived from IC_{50} values according to Ishikawa et al. [61] (Table 3) The most striking observation resides in the great behavioural variability among the toxins Thus, LSIII from venom of the sea snake *L semifasciata* does not recognize any of the two fragments This may explain why this toxin has a low affinity for the complete receptor (see Table 3) Other toxins do inhibit the binding of [^{3}H]αCobtx toward either peptides, however marked differences are seen from one toxin to another Thus, αCobtx binds to both fragments with dissociation constants equal to 0 3–0.4 μM for both peptides whereas α-bungarotoxin (αBgtx) from the krait *Bungarus multicinctus* binds with a similar affinity to Tα185–199 but with a 40-fold lower affinity to Tα128–142 Presumably, the determinants by which αCobtx and αBgtx recognize Tα128–142 are different in the two toxins whereas those by which they recognize Tα185–199 may be more similar Toxin I from *Naja naja oxiana* and Toxin III from *Naja naja annulifera* have both a low affinity toward the two peptides and in particular the affinity of toxin I for Tα128–142 is nearly 50 times lower as compared to that of αCobtx. Clearly, each long-chain toxin has its own specific pattern of interactions toward each peptide This may explain why, by using one toxin or another, different results have been reported in the literature regarding the ability of toxins to recognize a given α subunit fragment. The differential behaviour seen above between αCobtx and the short-chain toxin α falls into the wide range of binding diversity observed between two long-chain toxins. By no means the differences shown in Table 2 reflects differences between short-chain and long-chain toxins. This variability is somewhat intriguing. Clearly, it is a consequence of amino acid differences between toxins [38], suggesting that residues other than those that are strictly common to all toxins also contribute to the binding of a toxin to the receptor fragments

3 4 *Competition experiments between [^{3}H]α-cobratoxin and chemically modified neurotoxins on peptide Tα185–199 and Tα128–142*

Monoderivatization and site-directed mutagenesis are well-suited approaches for identifying residues involved in protein–protein interacting surfaces Previously, some residues that contribute to the binding of αCobtx to AcChoR have been pointed out using various monomodified toxin derivatives [38,51,59]. Table 4 (column 4) recalls that acetylation of Lys-27 or Lys-53 as well as nitrophenylsulfenylation of Trp-29 provoke affinity decreases in the nanomolar range, as deduced from competition experiments, using [^{3}H]αCobtx as a tracer. These results together with other data [52,59] suggested that these side chains belong to the contact area between αCobtx and AcChoR In contrast, nitration of Tyr-25 or reduction and carboxymethylation of Cys-30 and Cys-34 with iodoacetamide did not alter the toxin affinity for

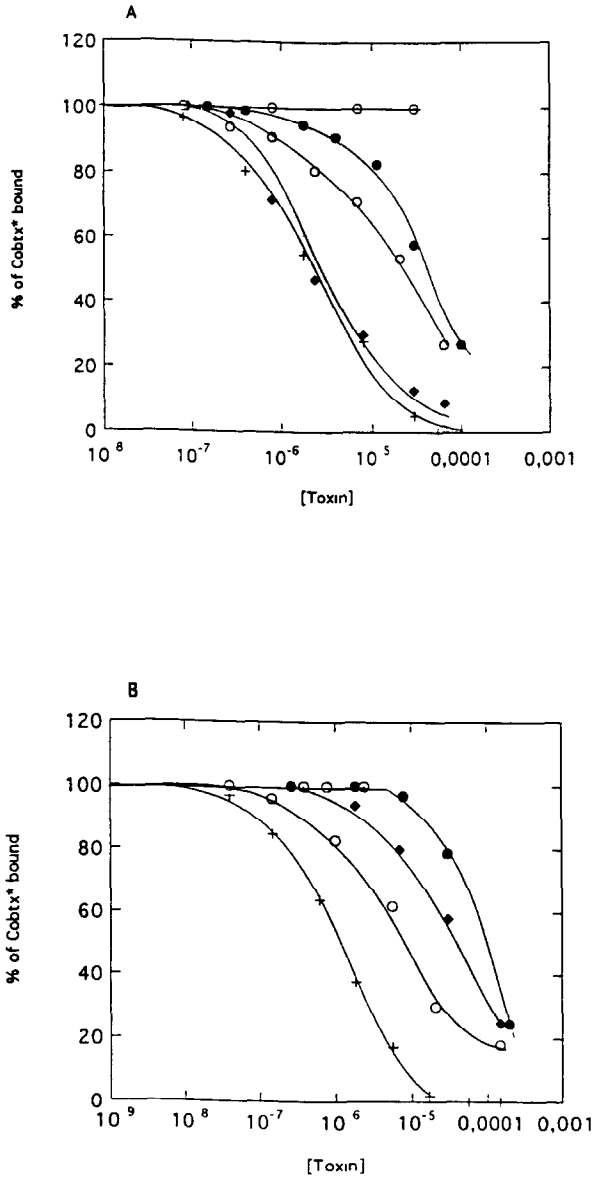

Fig 2 Competition between [^{3}H]αCobtx and long-chain toxins for binding to Tα185–199 and Tα128–142 Wells coated with 0 1 μg of peptide were incubated for 4 h at room temperature with [^{3}H]αCobtx (1 1 μM) and in the presence of various concentrations of long-chain toxins (10^{-8} M to 10^{-3} M) The radioactivity in each well was measured Each experiment was done in triplicate Panels A and B indicate respectively the binding to Tα185–199 and Tα128–142 (+), αCobtx, (♦), αBgtx, (●), Toxin I, (○), Toxin III, (○), LSIII

Table 3
Apparent dissociation constants values of different neurotoxins for peptides Tα185–199, Tα128–142 and for AcChoR Data are from competition experiments shown in Fig 2

	Tα185–199 K_d (μM)	Tα128–142 K_d (μM)	AcChoR K_d (nM)
α-Cobratoxin	0 4	0 25	0 06
α-Bungarotoxin	0 4	12	0 4
Toxin I	10	14	n d
Toxin III	6	2	0 7
LS III	> 30	> 15	1 6

AcChoR, indicating that these side chains are functionally unimportant for the binding Similar studies carried out for various other long-chain toxins led the authors with comparable conclusions [38] More recently, Pillet et al [48] used a large set of site-directed mutants and defined the role of invariant residues in the curaremimetic function of short-chain toxins The site by which such a toxin recognizes AcChoR appears to involve several conserved residues located on the concave face of a toxin molecule and belonging to the central (Lys-27, Trp-29, Asp-31, Phe/Tyr/His-33, Arg-37, Gly-38, according to the numbering in Table 1) and third loops (Lys-53) In addition, we recently showed that the curaremimetic site of a short-chain toxin seems also to involve Ile-40 on the central loop and Gln-10 in the first loop (Ménez et al , unpublished results)

Do any of the residues forming a curaremimetic site also interact with Tα185–199 or Tα128–142? To approach this question we determined the affinities of various monoderivatives of αCobtx toward both fragments, as deduced from competition experiments (Fig 3 A and B) The binding of Tα128–142 was not affected by modification at Lys-27, Tyr-25, Trp-29, Cys-24 and Cys-30, suggesting that none of these side chains interact with this peptide In sharp contrast, abolition of the single positive charge of Lys-53 provoked a 200-fold decrease in affinity (Table 4) This effect was unlikely to reflect nonspecific electrostatically-based interactions between the peptide and toxin since abolition of the positive charge of Lys-27 was followed by a much smaller effect Therefore, the positive charge of Lys-53 is one of the elements that directly contribute to the binding of αCobtx to Tα128–142 Two lines of evidence indicate that this conclusion can be extended to other toxins First, using a series of derivatives of the short-chain toxin α monoderivatized at positions equivalent to those in αCobtx (Table 4, column 3), only acetylation of Lys-53 (Lys-46 in toxin α) provoked a substantial decrease in binding affinity Second, the long-chain toxin I (Table 4) has a low affinity for Tα128–142 and it is significant that among the numerous mutations that occur in its amino acid sequence, position 53 is occupied by a glutamate residue [38] Therefore, the positive charge located on the third loop at position 53 is of considerable importance for the binding of both long-chain and short-chain toxins to Tα128–142 It is of interest that residues located on the adjacent loop II of αCobtx can be modified without affecting the affinity of the toxin for Tα128–142 We also demonstrated that Tα128–142 does not interact with the tip of the central loop of a short-chain toxin by determining the affinity of a site-directed mutant of erabutoxin a in which Arg-33 was replaced by a Glu residue [48]. As recalled in Table 4, this substitution affects toxin binding to the whole receptor, but not to Tα128 142 Therefore the binding area of this fragment seems not to encompass the central loop and perhaps remains limited to the loop III where the important Lys-53 is located.

Binding of αCobtx to Tα185–199 is not affected by any of the modifications made on the toxin (Table 4, column 2) Only a slight decrease was seen upon acetylation of either lysine residues, however, in view of the data obtained previously with the other fragment (see column 3) we suspect that these small variations reflect some

Table 4
K_d on Tα185–199, Tα128–142 and AcChoR for native and monomodified derivatives of αCobtx and Toxin α

	K_d on Tα185–199 (μM)	K_d on Tα128–142 (μM)	K_d on AcChoR (nM)
α Cobtx	0 4	0 25	0 06
Acetylated Lys-27	1 5	0 90	0 30
Acetylated Lys-53	1 5	**> 60**	0 25
Nitrated Tyr-25	0 4	1	0 06
NPS Trp-29	0 4	0 25	1 7
RCM Cys-30–Cys-34	0 4	0 15	n d
Tox α	50	1 4	0 02
Acetylated Lys-27		1 10	0 30
Acetylated Lys-46		**28**	0 50
Nitrated Tyr-25		0 70	0 10
NSP Trp-29		0 45	0 60
Erabutoxin α (Eα)		1 40	0 10
Eα Arg-33 → Glu		2 40	18

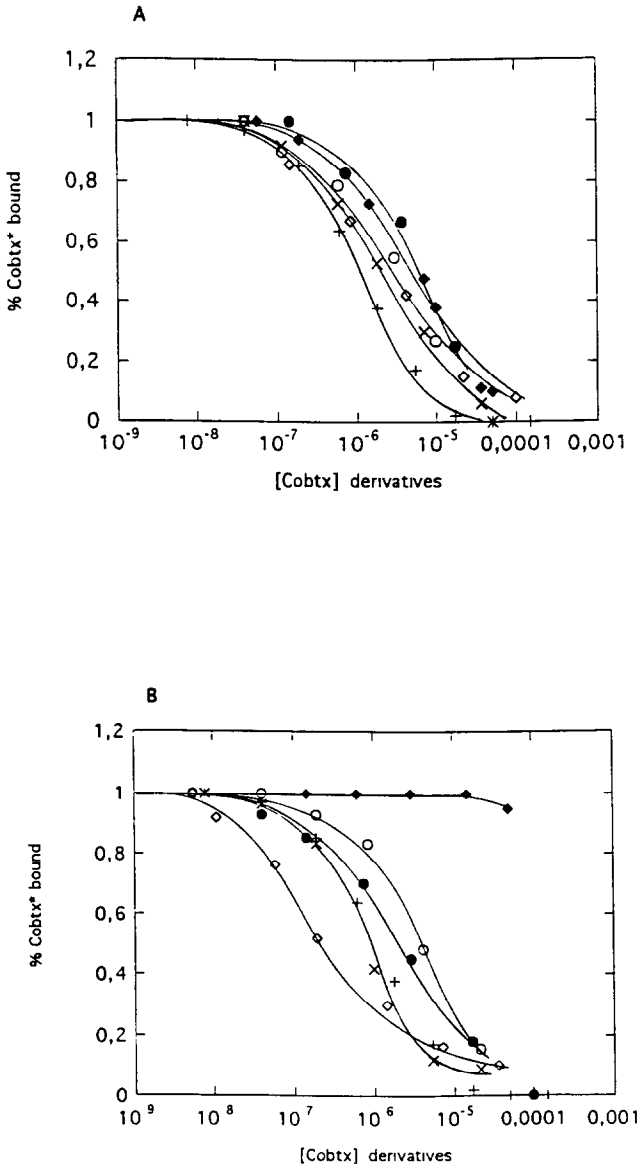

Fig 3 Competition between [^{3}H]αCobtx and chemically modified derivatives for binding to Tα185–199 and Tα128–142 Wells coated with 0 1 μg of peptide were incubated for 4 h at room temperature with [^{3}H]αCobtx (1 4 μM) and in the presence of various concentrations of the indicated monomodified toxin derivatives (10^{-8} M to 10^{-3} M) The radioactivity in each well was measured Each experiment was done in triplicate Panels A and B indicate respectively the competition for binding to Tα185–199 and Tα128–142 (+), native αCobtx, (♦), αCobtx modified on Lys-53, (●), αCobtx modified on Lys-27, (○), αCobtx modified on Tyr-25, (×), αCobtx modified on Trp-29, (◇) αCobtx modified on Cys-30–34

electrostatically-based non specific interactions Furthermore, experiments made with the longer fragment Tα174–199 led us with similar results as compared to those obtained with Tα185–199 (not shown). Therefore, we conclude that none of the modified residues contributes to the recognition by the toxin of receptor regions Tα185–199 or Tα174–199 In other words, the receptor area around its disulfide 192–193 is not recognized by residues that are highly conserved among curaremimetic toxins. This conclusion is somewhat surprising since this area is often considered as a predominant region for the binding of cholinergic ligands.

The toxin region recognized by Tα185–199 remains obscure. Clearly, it does not bind to Lys-53 nor to toxin residues that are located on the β-sheet strand of the second loop. Possibly, therefore, it could bind either to residues that are on the other strand of the second loop and/or to residues of the first loop which were recently shown to be important for formation of toxin-AcChoR complex (Ménez et al., unpublished data)

Presumably, snake toxins bind to AcChoR by recognizing several parts of the α subunit, including the two regions that have been studied in the present paper Other regions, however, remain to be found. In particular, the receptor region that is recognized by the conserved residues of the toxin central loop remains to be identified A number of other receptor fragments bind curaremimetic toxins [20,21,33] Using such fragments together with toxin derivatives or mutants and the approach described in this paper, one can anticipate that identification of the toxin-receptor contacts will be achieved in the near future

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