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Spider neurotoxins and their neuronal receptors

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Seven high molecular weight neurotoxins which stimulate secretion of various neurotransmitters were isolated from the venom of black widow spider Latrodectus mactans tredecimguttatus. Among them α-latrotoxin is very specific for vertebrate while latroinsectotoxins are active only for insects, α-latrocrustatoxin affects crustacean nerve endings. Latrotoxin preparation consists of 130 and 8 kD proteins. The cDNAs encoding α-latrotoxin and α-latroinsectotoxin were cloned and sequenced. Membrane receptors for all neurotoxins were studied in nerve tissue preparations of appropriate animals. α-Latrotoxin was shown to open the ion channels in the membrane of Xenopus oocytes injected by brain mRNA fraction.

INTRODUCTION

The venom from the poisonous glands of the black widow spider Latrodectus mactans tredecimguttatus affects a transmitter release from the nerve endings of vertebrates, insect and crustaceans (refs. 1, 2). The best studied neurotoxin from this venom is α-latrotoxin (α-LTX) which possesses its effect only on vertebrates by inducing massive release of numerous transmitters. The toxin stimulates the release of various kinds of transmitter but only from the nerve endings of vertebrate animals. Even high doses of α-LTX were ineffective in experiments on invertebrate preparations. The α-LTX molecular mass is about 130 kD, and its isoelectric point is 5.5. It is not a glycoprotein and shows no enzymic activity. Specialized membrane receptors of α-LTX are identified in brain preparations in some animals and in the PC12 cell line by radioligand analysis. Apparently, interaction of the neurotoxin with a presynaptic receptor leads to increase of the Ca^{2+} concentration inside the cell. At the same time α-LTX enhances cation conductivity of the bilayer lipid membrane due to the incorporation of the toxin molecule into the lipid layer with the formation of the cation-selective ion channel (ref. 3). Results which evidence fusogenic properties of the toxin were also obtained (ref. 4). This paper deals with isolation and characterization of other toxins from black widow spider venom, sequence determination of α-latroinsectotoxin (α-LIT), as well as investigation of membrane receptors for isolated toxins and expression of the α-LTX receptor in Xenopus oocyte membrane.

RESULTS AND DISCUSSION

First attempts of α-latrotoxin isolation indicated the presence in the venom of some other toxins stimulating neuro- transmitter secretion from insect and crustacea nerve terminals (ref. 5). To separate individual neurotoxins the venom of the black widow spider was chromatographed on Mono Q column (Fig. 1). All the fractions were tested on crustacea Procambarus cubensis, on the larvae of Galleria mellonella and on mice. Analysis of the toxic activity of purified fractions showed that the crustacea-specific activity was eluted as the single peak. The insecto-specific toxic activity was eluted as two

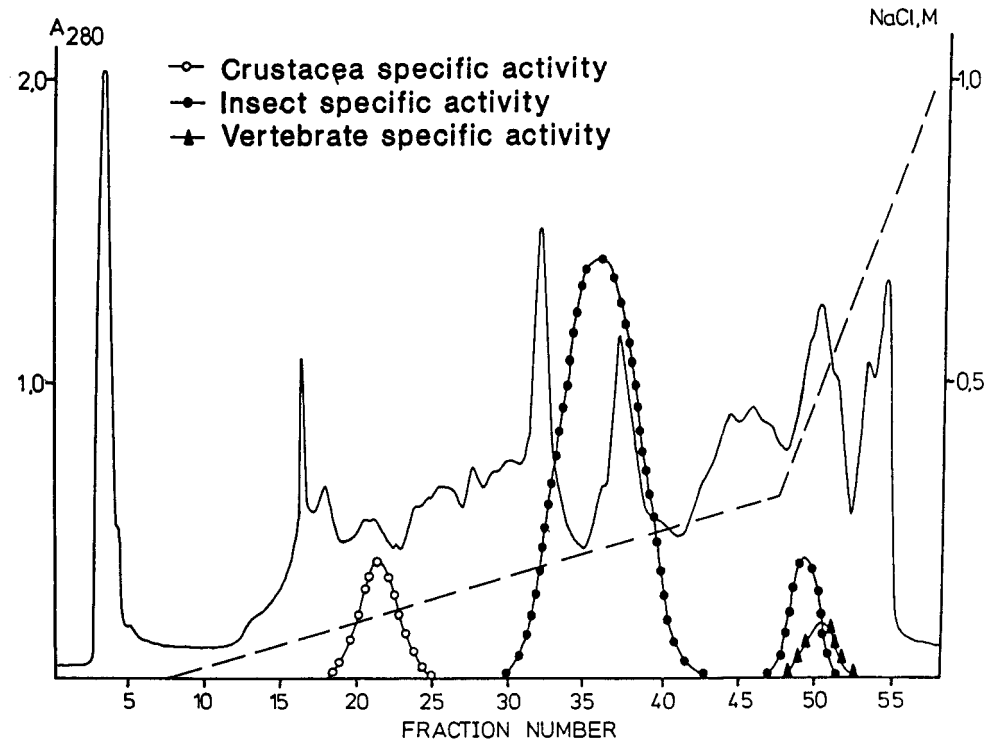

Fig.1. Separation of the black widow spider venom by ion exchange chromatography on the Mono Q column with a two-stage gradient of increasing molarity of sodium chloride. The volume of one fraction is 0.3 ml.

separate peaks. The second insecto-specific peak overlapped with the vertebrate-specific toxin peak. So, the total venom extract was separated into three groups of fractions by ion exchange chromatography, the first contained only the crustacea-specific toxic activity, the second - only the insecto-specific toxic activity, and the third possessed the toxic activities for insect and vertebrates. Further purification of the individual toxins was achieved by combination of ion exchange and hydrophobic chromatography. It resulted in seven different neurotoxins. To distinguish the toxins affecting various animal groups we proposed the following names: α-latrocrustatoxin (α-LCT) for the crustacea-specific neurotoxin, α-, β-, γ-, δ-, ε-latroinsectotoxins (LITs) for insectospecific neurotoxins. The toxic activity of the toxins was tested on cocroaches Periplaneta americana and on house flies Musca domestica as well. The toxins were lethal to cocroaches and flies and displayed no narrow species specificity. All latroinsectotoxins manifested no toxic activity in mouse and crayfishe at doses more than 5 mg/kg. Molecular masses of the toxins were about 120 kD. The biological activity of α-LIT was also tested by intracellular recording of miniature excitatory potentials (MEPSPs) in blowfly larvae muscle fibres (ref. 6). This toxin greatly increases the frequence of MEPSPs ($4.2 \cdot 10^{-10}$ M), its lethal dose for fly larvae is about 20 ng/species. At the same time α-LIT does not influence the MEPSPs being applied in dose $5 \cdot 10^{-8}$ M to the neuromuscular junction of the frog. No potentiation or antagonism could be observed when α-LIT is applied together with large amounts of α-LTX on insect preparations. Likewise , α-LIT does not affect the interaction of α-LTX with frog preparations. The observed data were strongly supported by the results of binding experiments showing the

highly specific binding of labelled α-LIT only to insect membrane preparations. The α-LTX could not affect the specific binding. In the same manner unlabelled α-LIT did not influence the specific binding of labelled α-LTX to bovine brain membranes. The selectivity of the toxin effects occurs from differences in the structure of toxin receptors in vertebrate and insect presynaptic membranes, which specify their binding. The α-LCT is a rather toxic principle with LD of 100 μg/kg for crayfishes. It has no visible activity on insects and mice in dose about 5mg/kg. Furthermore the α-LTX is absolutely inactive to crustasea. So, one neurotoxin specific only for the vertebrate, five neurotoxins selective for the insect and one neurotoxin active for the crustacea were isolated from the black widow spider venom.

Of much value might be information on the α-LIT structure when elucidating the detailed mechanism of its action. The cDNA library was obtained on the basis of poly(A)$^+$ RNA from venom glands of the black widow spider in the plasmid vector pSP65. The cDNA library was screened by hybridization with the labelled synthetic oligonucleotide probes, based on partial amino acid sequences of the toxin tryptic peptides. The screening of the cDNA library revealed several clones positive to the probes. Structural analysis of overlapping cDNA clones revealed a continuous nucleotide sequence of 5205 bp. The sequence contains an open reading frame of 4236 bp starting with the first nucleotide residue and ending at the termination TAA codon. This potentially codes for a 1411 amino acid protein with calculated molecular mass of 157826 Da and pI of 6.38. Corresponding α-LIT mRNA includes a 3′-noncoding region of 972 nucleotides. The potential polyadenylation signal and poly(A)-tail were not found here, although an AATAAA sequence occurs three times in position 4556-4561, 4881-4886 and 4948-4953. According to Northern-blot analysis, the size of the α-LIT transcript is larger than that deduced from the combined sequence of the overlapping cDNA clones (apparent size of α-LIT RNA was estimated by comparison with 18S and 28S ,bovine ribosomal RNA standards). Therefore part of the 5′- and/or 3 - untranslated region is apparently missing from the estimated structure. The deduced polypeptide chain contains all partial amino acid sequences determined earlier by peptide analysis. The amino-terminal sequence of the mature α-LIT determined by protein analysis (EMSRADQCKLLAY) (ref. 7) was found at residues 1-13. Upstream there is Met residue (-10), which is a candidate for initiating amino acid residue. As in case of the α-LTX structural gene there is uncertainty about the true initiation translation site because the open reading frame extends to the 5′-end of the cDNA. A possibility of additional coding sequence cannot be completely ruled out. Two possibilities should thus be considered. First, Met in position -10 is the true initiation translation site. In this case the signal peptide is absent and post-translational modification of α-LIT N-terminus is limited to removal of ten amino acid residues (a pro sequence that precedes the N-terminus of the mature protein). A second possibility is that α-LIT is synthesized as nonactive pre-pro-neurotoxin. In this case overlapping cDNA clones do not cover the entire α-LIT precursor mRNA and the initiating methionine residue is encoded by sequence beyond the 5 -terminus of α-LIT cDNA. The existence of a potential endopeptidase-cleavage site Lys-Arg in position -2 - -1 supports a hypothesis that post-translational processing occurs in the N-terminus. The calculated molecular mass of the deduced polypeptide starting with the Glu1 residue (153957 kD) differs from that earlier determined for α-LIT by SDS gel electrophoresis (about 130kD). Thus α-LIT has either abnormal electrophoretic mobility or can be coded as a double precursor with an additional processing step in the C-terminal region of the polypeptide chain during maturation. Identified tryptic peptides of α-LIT support double processing in that all were found in the polypeptide fragment with coordinates 1-1133 despite numerous cleavable regions which occur after this fragment. We consider that as in case of α-LTX the potential cleaving C-terminal fragment should be no less than

200 amino acid residues. The detailed analysis of the α-LIT polypeptide amino acid sequence reveals that the region extending from residues 464 to 1176 is almost entirely composed of a series imperfect repeats which represent a motif also found in α-LTX (ref. 8). As a first approximation, these can be viewed as 7 amino acid repeats with consensus sequence TPLH(L/I)A(A/I). These repeats occur in the α-LIT structure 20 times with highly conserved space length of 26-27 amino acid residues except for the interval between repeats 16 and 17. These 7 amino acid repeats represent a constant part of a repetitive 33 amino acid motif found in membrane-binding domains of human ankyrins (ref. 9). α-LIT repeats can be viewed as 33-34 amino acid ankyrin-like repeats containing N-terminal conserved and C-terminal variable parts. The ankyrin-like repeat motif has been observed in several other proteins involved in cell differentiation, cell cycle control and transcription (ref. 9).

The alignment of C-terminal fragments of α-LIT and α-LTX beginning from the phenylalanine 838 of mature proteins is shown in Fig. 2. Gaps were introduced to enhance the comparison. Overall homology is 34.1%. Hydropathy profiles of the neurotoxins indicate a very similar distribution of hydrophobic and hydrophilic fragments. A number of hydrophobic regions can be identified of unsufficient length to constitute a conventional membrane-spanning α-helix, but might provide membrane interaction. The structural organization of the neurotoxin precursors should be very similar. Both neurotoxins can be hypothetically divided into three structural domains: an N-terminal domain (M_r about 51 kD) including 450 amino acid residues essentially free of internal repeats; an ~81 kD domain extending from residue 450 to about 1180, comprised almost entirely of 20 imperfect ankyrin-like repeats and likely assembled as an integrated unit; a C-terminal domain (about 200 amino acids) with M_r about 22 kD which is likely released during toxin maturation., Since α-LIT and α-LTX have an open reading frame extending to the 5 -end mRNAs, the possibility of an additional N-terminal domain of α-neurotoxin precursors cannot be completely ruled out. Functional significance of such structural organization of neurotoxin molecules is still unknown. Identification of ankyrin-like repeats in the neurotoxin molecules suggests a structural basis of neurotoxin-membrane interaction. Ankyrins constitute a family of proteins that coordinate interactions between various integral membrane proteins and cytoskeletal elements (ref. 9). Recent studies indicate that the repetitive motif domain of the ankyrin molecule is responsible for high affinity binding with membrane proteins . Thus we propose that the structural domains of α-LIT and α-LTX containing ankyrin-like repeats also take part in binding to presynaptic receptors from insects and vertebrates, respectively.

The structural basis of the selectivity of α-LIT and α-LTX for insect and vertebrate receptors, respectively, is still a question of considerable interest. The comparative amino acid sequences alone provide some reasonable basis for speculation. Despite a high level of structural similarity, strong divergence is observed in analogous regions of the neurotoxins extending from residues 920 to 1030 (underlined on Fig. 2), which contain both an unusual interval between two ankyrin-like repeats and a clustering of cysteine residues. This region is supposed differentially cross-linked within itself in α-LIT compared with α-LTX and its differential spatial arrangements can account for differential selectivity for invertebrate and vertebrate receptors.

Iodine-125 α-LCT specifically binds to the plasmatic membranes from the ganglia nerve tissue of the Paralithodes camtshatica crab with K_d 3×10^{-10} M. The density of binding sites for this preparation is about 60 fmoles of bound toxin per mg of membrane protein. The optimal pH value for the toxin specific binding to the receptor sites is 6.4.

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          v       850v       860v       870v       880v       890v
ITX       PFYLAVEKRYKDIFDYFVSKDANVNEVDHNGNTLLHLFSSTGELEVVQFLMQNGANFRLKN
          P: LA.:.:. D:.YF:.:A::N. :.:G : L FS..G:L::V::L.:.AN : :
LTX       PLNLAAQNSHIDVIKYFIDQGADINTRNKKGLAPLLAFSKKGNLDMVKYLFDKNANVYIAD
           v         910v         920v         930v         940v
ITX       NERKTFFDLAIENGRLNIVAFAV-EKNK---VNLQAAHRG------KTILYHAICDSAKYD
          N: ..FF A::NG:LNIV :A: EK:K N :..:R: :I . A:CD:.:D
LTX       NDGMNFFYYAVQNGHLNIVKYAMSEKDKFEWSNTDNNRRDECPNEECAISHFAVCDAVQFD
          v      960v       970v       980v       990v      1000v
ITX       KIEIVKYFIEKLNE-SECNPLHEAAAYAHLDLVKYFVQERGINPAEFNEENQASPFCITIH
          :IEIVKYF:..L.: : C.PLH:AA Y:HLD:VKY:V:E EF . : :.. :
LTX       RIEIVKYFVGTLGNFAICGPLHQAARYGHLDIVKYLVEE------EFLSVDGSKT-----D
          v     1020v      1030v      1040v      1050v      1060v      1070v
ITX       GAPCGYSLDCDTPDRLEVVEYLSDKIPDINGKCDVQENTPITVAIFANKVSILNYLVGIGA
          .: C Y: .. :::.VV:YL .: :.:N .C: :. T:I. AI N.: ::::L.: G.
LTX       TPLC-YA---SENGHFTVVQYLVSNGAKVNHDCG-NGMTAIDKAITKNHLQVVQFLAANGV
           1080v      1090v      1100v      1110v      1120v
ITX       D-PNQQVDGDPPLYIAARQGRFEIVRCLI--EVHKVDINTRNKERFTALHAAARNDFMDVV
          D ..:: G..P: .A. :. ::I. .LI . :.::IN.:N :: TALH A. . ::::
LTX       DFRRKNSRGTTPFLTAVAENALDIAEYLIREKRQDININEQNVDKDTALHLAVYYKNLQMI
           v      1140v      1150v      1160v      1170v      1180v
ITX       KYLVRQGADVNAKGIDDLRPIDIAGEKAKAYLQSSRFLRS-GHSFQSNEIDSFGNTIHGIS
          K L:: G DV. :. D ::DIA : AK : : :L:: : .F:.: .S:G: :::
LTX       KLLIKYGIDVTIRNAYDKTALDIAID-AK-FSNIVEYLKTKSGKFRREYKSSYGE--RSLL
           v      1200v      1210v      1220v      1230v      1240v
ITX       MSARTNDKLTQQISSKGTRSDSNSTEGKMHSENVHVRSIDVNGALLLLDFMIRVFASKKTN
           :.:.:: :.:: ..: . N:.:.. : : ..:IDV G:LLL:D :IR F .K .
LTX       QTNQISNFIDRKNIEHDHPLFINADNESSELFSKTASNIDVIGTLLLIDVLIRYF--SKQG
          v      1260v      1270v      1280v      1290v      1300v      1310v
ITX       FAPYGSRIKTRSAAEAQAEALIMTERFENLLSGLIGDPIPDSIDFSNVHSKIYKAIMSGRR
          : : . .: S.: :QA.AL :TE:FE::L::L .:: : :D:::VH:K:Y A:.SGR.
LTX       YIS--KESDSASDGITQAAALSITEKFEDVLNSLHNESAKEQVDLAEVHGKVYAALKSGRN
               1320v     1330v      1340v      1350v      1360v      1370v
ITX       SVISEMLCSFAEEYSKLNHESIKQLLSEF-ETLTTTKASEIHIEESVPYAPFEICELKVNS
          S I ::LCS :. S.L:.E.:.:L S : :: ::.. :E: ..: :Y: .
LTX       SQIHQILCSSLNSISTLKPEDMEKLESVIMNSHSSVSLPEVTDSANEAYGETLHLFGESCL

ITX       NVSQIK
          : . I
LTX       HSDGILTKKLM
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Fig. 2. Alignment of the C-terminal amino acid sequences of the α-LIT and α-LTX precursors. Gapes (indicated by dashes) were introduced for optimal alignment. Conserved amino acids replacements are indicated bycolons. The segment of neurotoxins with minimal linear similarity is underlined.

The binding increases by 20% in the presence of 2-5 mM calcium ions. Sodium chloride in over 0.075 M concentration inhibits the toxin binding. Wheat germ agglutinin and lenthil-lectin do not affect the α-LCT reception. A highly specific binding of the labelled toxin is also observed on plasmatic membranes from crayfish Astacus astacus nerve cell with K_d 0.7×10^{-10} and B_{max} 40 fmoles per mg of membrane protein (refs. 10,11).
Iodine labelled α-LIT specifically binds to the membrane preparations from heads of Gryllades suplicans crickets with K_d 3.8×10^{-10} M and B_{max} 90 fmoles per mg. The pretreatment of membrane preparation with 1 or 5 μM concanavalin A for 10 min decreases toxin binding by 45% or 66 %, respectively. An attempt to reveal cross-binding of α-LIT and α-LTX was made on bovine and insect brain membrane preparations. Excess of unlabelled α-LTX prevented binding of labelled α-LTX with bovine membranes but failed to protect insect preparation against labelled α-LIT binding. Changes in the calcium concentration with the range 1

μM-1 mM did not affect binding of α-LIT to membrane preparations from cricket heads (ref. 6).
Iodinated α-LTX specifically binds to the membrane preparations from bovine brain with K_d 1.6×10^{-10} M and B_{max} 0.3 pmoles per mg. The purified brain receptor consists of two toxin-binding glicoproteins of 200 kD and 160 kD that have high homology in their primary structure and form a noncovalent complex with several nontoxin-binding proteins of 79, 65 and 43 kD.
The stability of the solubilized toxin-receptor complex in the absence of Ca^{2+} depends on the salt concentration. Affinity chromatography made possible purification of the toxin receptor with a high specific binding activity (1.6 nmol of [^{125}I]α-LTX per mg of protein). According to analysis of the functional properties of other subunits, 79 and 43 kD proteins were in vitro substrates for protein kinase C, while toxin-binding glycoproteins were found to inhibit phosphorylation. The presence of 65 kD protein (p65) in receptor preparations might be explained by specific interactions with 200 and 160 kD glycoproteins during their isolation by affinity chromatography. Furthermore 200 and 160 K components of the toxin receptor specifically suppressed phosphorylation of the p65 molecule. So, p65 initially associated with or having affinity to toxin-binding proteins formed the complex irresistant to ionic strength increased during isolation. One can presume that p65 forms a dynamic complex with receptor components of the presynaptic membrane regulating the polyfunctional activity of the protein related to fusion of the synaptic vesicle with the presynaptic membrane. The complex can be formed at approach of the synaptic vesicle to the presynaptic membrane, receptor components of 200 and 160 kD have then an additional function of "doc"-proteins. Thus, the interaction of p65 and α-LTX-binding components implies an involvement of these proteins in the final step of secretion of neuromediators - fusion of vesicular and presynaptic membranes (refs. 12-15).
One might conclude that similar toxin-binding protein components are situated in many kinds of presynaptic membranes, being important for neuromediator secretion. The neurotoxin family from the black widow spider venom disturbs a normal function of the presynaptic components that evoke massive stimulation followed by complete blockade of the neuromediator release. Structural analysis indicates high homology between different neurotoxins, but at the same time reveals certain peculiarities in the amino acid sequence of each toxin molecule, presumably caused by the vertabrate- or insectospecific activity of neurotoxins. This suggests the presence of some distinguishable regions in presynaptic proteins, which can be implicated in specific binding with different neurotoxins in nerve terminals of varied animals.
According to electrophoretic analysis purified α-LTX preparations contained two polypeptides: α-LTX (M_r~130 kD) and a low molecular weight protein (LMWP) with molecular mass about 8 kD. The fact that highly purified latrotoxin preparation consists of two polypeptides raises the question about specificity of their interaction. We suppose that the copurification of the α-LTX and LMWP is not an artifact, since biochemical properties of these proteins are distinguished (pI of α-LTX ~5,9; calculated pI of LMWP ~3,95). Presumably, LMWP tightly associates with the α-LTX and can be designated as the β-subunit of neurotoxin. Clones carrying cDNA sequence for a LMWP copurified with α-LTX were isolated from spider venom glands. Nucleotide sequence analysis of the cloned cDNA revealed the primary structure of the polypeptide of 18 amino acids signal peptide and 70 amino acids protein chain with molecular mass of 7947 and pI of 3,952. The protein exhibits certain structural homology with erabutoxin A from the sea snake (ref. 16).
The α-LTX receptor can be expressed in the membrane of Xenopus laevis oocytes (refs. 17,18). On oocytes injected with rat brain poly (A^+)-RNA, α-LTX induced a slow increasing transmembrane inward current usually unobserved on uninjected oocytes. Some rat brain mRNA preparations obtained by sucrose density gradient fractionation could

also evoke an expression of the toxin receptor in oocyte membrane. The most prominent toxin-induced inward current arose from the use of mRNA larger than 6kB. The high molecular mass toxin-binding components of the α-LTX receptor probably 200 and 160 glycoproteins) are related to the toxin-induced ion channel. The main question to be answered in these experiments is: what is the nature of channel forming molecule. To clarify this point further, patch clamp experiments on oocytes were carried out. Very long single openings (from several hundred msec to several seconds open time) were observed in approximately 30% of both cell-attached and inside-out patches on oocytes injected with rat brain mRNA fraction of 7-8 kB when α-LTX was in a pipette. The amplitude of the current going inward the cell decreased when the membrane was depolarized. These regular long single channel openings were not observed both on uninjected oocytes and on mRNA injected oocytes without α-LTX in the pipette. The channel conductance determined from the slope of the current-voltage relation was 7 pS. Single channel openings assembled into groups of bursts. Long intervals of channel "silence" were often observed between the groups of burst openings when toxin in a pipette was dissolved in the solution with normal 1.8 mM Ca^{2+}. These intervals of "silence" were much shorter when Ca^{2+} concentration was increased. The reversal potential of single channel currents was near to 0 and did not change significantly when Ca ions were substituted for Na ions. This indicates that the channel produced by α-LTX does not discriminate between Ca and Na and can conduct both the cations. Preliminary results showed that channel openings were completely blocked by 2 mM Cd^{2+}.

Today there is no evidence that α-LTX receptor is a channel. Moreover the structure of neurexin 1α, the presynaptic protein cloned form the brain and representing most likely the variant of α-LTX receptor (ref. 19) does not remind in any sense the ion channel structure. Does α-LTX receptor play any role in the mechanism of toxin action? Certainly, it does. We did not observe any channel produced by α-LTX on uninjected oocytes, where the toxin receptor was not expressed. In contrary, α-LTX produced the channel even at 0.1 nM on brain mRNA fraction injected oocytes, the concentration comparable to that effective on mammalian synapses. It seems that the binding of α-LTX to the receptor increases greatly the toxin insertion into the cell membrane. One might conclude that the α-LTX binds specifically to the receptor and inserts the membrane forming ion channels exactly in the very active zones of synaptic transmission where docking and fusion of synaptic vesicles occurs. As a result a Ca^{2+} influx through this permanently open cation channel triggers vesicle exocytosis and massive neurotransmitter release.

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