Cloning and Structure of \( \delta \)-Latroinsectotoxin, a Novel Insect-specific Member of the Latrotoxin Family

**FUNCTIONAL EXPRESSION REQUIRES C-TERMINAL TRUNCATION**

(Received for publication, June 7, 1995, and in revised form, January 12, 1996)

Irina E. Dulubova‡§, Valery G. Krasnoperov‡§, Mikhail V. Khvotchev‡, Kirill A. Pluzhnikov‡, Tatyana M. Volkova‡, Eugene V. Grishin‡, Horia Vaisš†, David R. Bellís‡‡, and Peter N. R. Usherwood§§

From the ‡Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya, 16/10, 117871 GSP-7 Moscow V-437, Russia and §Department of Life Science, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom

The venom of the black widow spider (BWSV) \( \delta \)-Latrotoxin contains several potent, high molecular mass (>110 kDa) neurotoxins that cause neurotransmitter release in a phylum-specific manner. The molecular mechanism of action of these proteins is poorly understood because their structures are largely unknown, and they have not been functionally expressed. This study reports on the primary structure of \( \delta \)-latroinsectotoxin (\( \delta \)-LIT), a novel insect-specific toxin from BWSV, that contains 1214 amino acids. \( \delta \)-LIT comprises four structural domains: a signal peptide followed by an N-terminal domain that exhibits the highest degree of identity with other latrotoxins, a central region composed of 15 ankyrin-like repeats, and a C-terminal domain. The domain organization of \( \delta \)-LIT is similar to that of other latrotoxins, suggesting that these toxins are a family of related proteins. The predicted molecular mass and apparent mobility of the protein (\( \sim 130 \) kDa) encoded in the \( \delta \)-LIT gene differs from that of native \( \delta \)-LIT purified from BWSV (\( \sim 110 \) kDa), suggesting that the toxin is produced by proteolytic processing of a precursor. MALDI-MS of purified native \( \delta \)-LIT revealed a molecular ion with \( m/z \) of \( 110916 \pm 100 \), indicating that the native \( \delta \)-LIT is 991 amino acids in length. When the full-length \( \delta \)-LIT cDNA was expressed in bacteria the protein product was inactive, but expression of a C-terminally truncated protein containing 991 residues produced a protein that caused massive neurotransmitter release at the locust neuromuscular junction at nanomolar concentrations. Channels formed in locust muscle membrane and artificial lipid bilayers by the native \( \delta \)-LIT have a high \( \text{Ca}^{2+} \) permeability, whereas those formed by truncated, recombinant protein do not.

The work described in this study is the subject matter of a UK Patent Application (UKPA 9408446). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The authors thank Dr. S. W. Hallday and Dr. J. D. Saull for helpful correspondence. Tel.: 44-115-951-3210; Fax: 44-115-951-3251; E-mail: David.Bell@nottingham.ac.uk.

The abbreviations used are: BWSV, black widow spider venom; LTX, latrotoxin; LIT, latroinsectotoxin; DTT, dithiotretiol; mEPSP, miniature excitatory postsynaptic potential; LMWP, low molecular weight protein; ALR, ankyrin-like repeat; MALDI, matrix-assisted laser desorption ionization.

*The primary structures of \( \delta \)-latroinsectotoxin and the recombinant protein. The full-length, recombinant protein is larger than the native toxin and, unlike the latter, has no insect toxicity. However, when the C-terminal domain of \( \delta \)-LIT was removed by site-directed mutagenesis, the recombinant protein was highly toxic, causing characteristic neurotransmitter release from locust motor nerve terminals. We also show that the native and truncated, recombinant

† Supported by a grant from the Royal Society. Present address: Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Ave, New York, NY 10021.
‡ Supported by a grant from the Royal Society. Present address: Nelson Institute of Environmental Medicine, Long Meadow Road, Tuxedo, NY 10987.
§ Supported by the Royal Society and the Wellcome Trust. To whom correspondence should be addressed. Tel.: 44-115-951-3210; Fax: 44-115-951-3251; E-mail: David.Bell@nottingham.ac.uk.
** Supported by the Royal Society and the Welcome Trust. To whom correspondence should be addressed.
toxins form channels in lipid bilayers and locust muscle membrane, but that the channels differ greatly in terms of their Ca	extsuperscript{2+} selectivities.

**Experimental Procedures**

Protein and Peptide Sequencing—2.5 nmol of δ-LIT isolated from BWSV (3) were subjected to tryptic digestion after reduction by DTT and carboxymethylation by iodoacetic acid according to Volkova et al. (14), at a protein to enzyme ratio of 30:1. Resulting peptides were fractionated by reverse-phase high performance liquid chromatography on an UltraspHERE ODS C18 column (Beckman) and rechromatography. For direct N-terminal sequence determination, 100 pmol of native δ-LIT were immobilized on a poly(vinylidene fluoride) membrane (Immobilon-P, Millipore). Amino acid sequences of δ-LIT and its tryptic fragments were determined by automatic sequencing on a gas-phase Sequenator 470A (Applied Biosystems) with identification of phenylthiohydantoin amino acids.

cDNA Cloning and Sequencing—Poly(A)	extsuperscript{+} RNA was isolated from venom glands of the black widow spider (L. m. tredecimguttatus) and a cDNA library was constructed in the plasmid vector pSP65 (10). A library of 60,000 clones was screened (15) with an oligonucleotide probe based on the N-terminal sequence of δ-LIT (5'-GA(C/T) GA(A/G) GA(A/G) CA/GT) GGA(AT) GAA ATG AC-3). Five independent clones were identified as the longest clone containing 2 kilobase pairs of open reading frame (pDT-1), which was sequenced across the library. Overlapping clone, pDT-17, was isolated and the clones were shown to be contiguous by reverse transcriptase-polymerase chain reaction on open reading frame RNA, both across the area of overlap, and across the entire open reading frame (data not shown). DNA sequences were determined at least twice on both strands using the Sequencing 2.0 kit (U.S. Biochemical Corp.).

Construction of Expression Vectors—First strand cDNA from venom gland was used for polymerase chain reaction with oligo(dT) primers (TTGAGATCCGATGAAAGTAGGAGA) plus P3 (TGGAGATCCGATGAAAGTAGGAGA) and P9 (GGTTGCAATCTCAGTGG), plus P8 (CAATGGTTGCAACAGAAAGTTGGA). The oligonucleotides contain the sites (underlined) and encode amino acids 1–991. Constructs of the N-terminal sequence of δ-LIT (5, 8-12) were isolated and the longest clone, containing 2 kilobase pairs of open reading frame (pDT-1), was used to rescreen the library. An overlapping clone, pDT-17, was isolated and the clones were shown to be contiguous by reverse transcriptase-polymerase chain reaction on open reading frame RNA, both across the area of overlap, and across the entire open reading frame (data not shown). DNA sequences were determined at least twice on both strands using the Sequencing 2.0 kit (U.S. Biochemical Corp.).

**RESULTS**

Cloning of δ-LIT cDNA and Predicted Polypeptide Structure—δ-LIT was purified to homogeneity from BWSV by three rounds of column chromatography (3). 23 amino acid residues of the N-terminal sequence of δ-LIT were determined, giving the sequence DEEGDCTXEREQQAXEYXN. An oligonucleotide probe based on the sequence of δ-LIT was used, giving cloned frequency data from α-LTX and δ-LIT DNAs (9, 10) and employed to screen a cDNA library from BWSV gland. Five different clones were isolated and the longest clone, containing more than 2 kilobase pairs of the δ-LIT coding region, was used to rescreen the library. An additional cDNA clone was isolated that covered the C-terminal coding region of δ-LIT. The two clones were shown to be part of a single, continuous RNA from venom glands by sequence analysis and polymerase chain reaction across the overlapping region, using three distinct sets of primers (data not shown). The composite clones encode a 4581-base pair cDNA with an opening reading frame of 3642 base pairs starting from the first in-frame ATG codon and ending with a TAA stop codon (accession no. X92679).

The N terminus of the mature protein was determined by amino acid sequencing and was found to be preceded by a short sequence, viz. MKSHEKOLITISAARKVQNTMVIRK, in the δ-LIT cDNA. This sequence contains two in-frame Met residues (−7 and −28) that can serve as translation initiation sites. The nucleotide sequence surrounding Met (−7) (actagtg) agrees with the classical Kozak consensus (27), but the nucleotide arrangement for Met (−28) (gaatg) resembles the starting points for at least three other arachnid proteins (12, 28, 29). This Met residue is preceded by an in-frame stop codon and is likely to be the initiation codon. Although the resulting 28 amino acid stretch does not fit a classical signal peptide consensus very well, the fact that it is cleaved during synthesis of...
δ-LIT, and that δ-LIT is a secreted protein, indicates that this sequence might serve as a signal peptide. According to direct N-terminal sequence determination, the mature protein starts from Asp (1) in Fig. 1 (lane 1). The identity of this protein as δ-LIT was confirmed by showing that it contains seven peptide sequences that are present in native δ-LIT (determined by amino acid sequencing of tryptic peptides (Fig. 1)). This δ-LIT cDNA encodes a protein with 1186 amino acid residues and a predicted molecular mass of 132671 Da. Although δ-LIT is a soluble protein, and hydrophobicity plots confirm its hydrophilicity, it contains two hydrophobic regions (Fig. 1) in its N-terminal region (residues 36–64 and 222–241).

Domain Structure of δ-LIT and the Ankyrin-like Repeat (ALR) Motif—Sequence comparisons revealed that the primary structure of δ-LIT is similar to those of α-LTX and α-LIT (9, 10). Alignment of the sequences of these three latrotoxins in Fig. 1 shows that there are conserved regions extending over the entire length of the proteins. This is also evident from dot matrix comparisons between the three proteins (Fig. 2A). The displacement of the central diagonals on the middle and bottom panels reflects the difference in the lengths of the proteins. The deduced amino acid sequence of δ-LIT is about 190 amino acids shorter than that of α-LIT and α-LTX. δ-LIT has 37% sequence identity with α-LTX and 38% identity with α-LIT, whereas α-LIT has 34% identity with α-LTX. 16% of amino acid residues are conserved in the three proteins (Fig. 1). Thus, δ-LIT is as similar to α-LIT as it is to α-LTX, yet α-LTX is a vertebrate-specific toxin, whereas the other two proteins are insect-specific toxins. The dot matrix analysis also shows that the middle region of δ-LIT is composed of repeated units, a property that it shares with other latrotoxins. These regions contain tandemly arranged imperfect copies of ALRs (30). There are at least 15 such repeats in δ-LIT, some of which are rather distant from the consensus sequence of ALR, but which are, nevertheless, clearly identifiable (aligned in Fig. 2B). The ALR domain of δ-LIT is shorter than that of α-LIT and α-LTX, a difference that is reflected in the asymmetric distribution of conserved segments in these regions (Fig. 2A, middle and bottom panels).

Four domains can be readily identified in the δ-LIT sequence.
and those of other latrotoxins. The first is a presumptive signal sequence, the second the N-terminal region, the third comprises the ALRs, and the fourth is the C-terminal region. The N-terminal region is the best conserved of these domains. Data base searches failed to detect significant sequence similarities between the N- and C-terminal domains of d-LIT (and those of the other two latrotoxins) and other polypeptide structures. However, as expected, similarities to the ALRs were detected in many proteins. The ALRs are relatively poorly conserved between the three latrotoxins, although their positional conservation is obvious (Fig. 3). By reanalyzing our previous data as suggested in Bork (31), we have identified at least 22 ALRs in d-LIT, a-LIT, a-LTX, and brain ankyrin. Gaps marked by periods were introduced to facilitate alignment.

**Fig. 2. Structural motifs of d-LIT.** A, dot matrix analysis. Dot matrix plots comparing the deduced amino acid sequence of d-LIT with itself (top panel), α-LIT (middle panel), and α-LTX (bottom panel). The axes refer to the numbering of amino acid residues. Each dot represents a match (40% minimum identity) between windows of 20 residues. B, ALRs in latrotoxins. I, optimal alignment of d-LIT ALRs (R1-R15). Amino acids present in at least half of ALRs are in bold type and summarized in a consensus shown below the alignment. A dash represents an amino acid residue where there is no consensus. Three amino acids of R1 are removed and placed outside in brackets to improve the alignment. II, comparison of the consensus sequences derived from ALRs in d-LIT, a-LIT, a-LTX, and brain ankyrin. Gaps marked by periods were introduced to facilitate alignment.

for d-LIT, a cDNA corresponding to residues 1 to 1186 was inserted into the bacterial vector, pT7-7, and designated pT7-d-LIT. When this cDNA was expressed in E. coli BL21(DE3)LysS cells, recombinant protein constituted 1% of the total bacterial lysate protein. A polyclonal antibody raised to d-LIT (purified from BWSV glands) specifically detected a protein of approximately 23 kDa larger (Fig. 4A, lanes 6 and 7). The expressed protein with that of d-LIT protein was compared to the bacterially expressed d-LIT protein (Fig. 4A, lane 2), thus confirming that the cDNA encodes the d-LIT protein. Comparison of the molecular mass of the expressed protein with that of d-LIT purified from BWSV glands revealed that the recombinant protein was approximately 23 kDa larger (Fig. 4A, lanes 2 and 7). This result is in agreement with the calculated molecular mass of the protein. Unlike native d-LIT, the full-length, recombinant d-LIT was not toxic to insects (data not shown). These data raise the possibility that the full-length protein is a precursor of d-LIT and that the latter is truncated during synthesis of the toxin.

**Synthesis and Functional Expression**—When native d-LIT was analyzed by MALDI-MS it yielded a prominent molecular ion with a m/z + ratio of 110916 ± 100 (Fig. 4B); peaks with lower m/z + ratio were associated with contaminants and multiply charged ions. In comparison, the bacterially expressed protein yielded a molecular ion with a m/z + ratio of 133631 ± 100 (data not shown), which is within 100 Da of its calculated value. Site-directed mutagenesis was used to create a d-LIT cDNA clone (pT7.6M), that was truncated after amino acid 991 of the d-LIT sequence, in the anticipation that this would code for a protein of similar size to that of native d-LIT. When this protein was expressed in bacteria, it yielded a protein of similar molecular mass to that of native d-LIT (Fig. 4A, lanes 6 and 7). In addition, unlike the full-length, recombinant protein, the
truncated protein was toxic to locusts and house fly larvae. For the toxicity studies, bacteria containing plasmids pT7-7, pT7.δFL and pT7.δM were induced, lysed, and the proteins were separated by ammonium sulfate fractionation. The pelleted material was resuspended in buffer, and 5-15 μl were injected into locusts. Alternatively, the recombinant protein was purified by ion-exchange chromatography followed by dialysis, and 2-4 μl of the protein-containing fraction were injected into house fly larvae. Extracts from bacteria carrying pT7-7 and pT7.δFL were inactive, whereas those from bacteria carrying pT7.δM, or the purified recombinant toxin, caused death within 5 min. The fast protein liquid chromatography purified recombinant δ-LIT had an LD_{50} of 10-50 μg kg^{-1} of body weight in house fly larvae.

*Physiological Studies*—The truncated, recombinant protein and native δ-LIT had qualitatively similar effects on spontaneous transmitter release from terminals of locust motor neurons. In standard locust saline containing 2 mM Ca^{2+}, 0 mM Mg^{2+} the control mEPSP frequency was 1-10 s^{-1} (Fig. 5A) (24). This was dramatically, albeit temporarily, increased by 10^{-9} M recombinant protein (n = 11 experiments) (Fig. 5, B-D) and 10^{-9} M native δ-LIT (n = 6; data not shown). The change in mEPSP frequency occurred with a delay (Fig. 6A), the duration of which was inversely proportional to the protein concentration (e.g., -15-45 min for 10^{-9} M protein (n = 3) and -1 min for 10^{-7} M protein (n = 5)) (Fig. 6B). The change in miniature discharge was characterized initially by the appearance of bursts of mEPSPs, but it later became catastrophic (Fig. 5, C and D), with mEPSPs occurring at frequencies in excess of 1000 min^{-1}. After 40-60 min of exposure to either 10^{-9} M recombinant protein or 10^{-8} M native δ-LIT, the miniature activity declined to levels at or below those of controls (Fig. SE). When a muscle was bathed in saline containing 2-100 mM MgCl_{2} (either as an addition to standard saline or as a substitute for NaCl), the recombinant protein (and native δ-LIT) still increased mEPSP frequency, but the onset of this change was further delayed (the delay was proportional to the MgCl_{2} concentration) (Fig. 6, B and C). The control mEPSP frequency was very low when preparations were perfused (10 ml min^{-1}) with saline containing no divalent cation (33), but 10^{-6} M recombinant protein (and native δ-LIT) induced an increase in mEPSP frequency without much delay, although the magnitude of the change was small (Fig. 6D).

A further comparison of the truncated, recombinant protein and native δ-LIT was made by investigating their channel-forming properties. Previous studies have shown that latrotoxins are channel-forming proteins (e.g., see Ref. 34). The recombinant protein formed channels of ~20 pS in artificial bilayers (n = 15 experiments) (Fig. 7A). The bilayers were exposed on both sides to 190 mM NaCl. The saline on the cis side contained 2 mM CaCl_{2}; the trans CaCl_{2} concentration was 0.2 mM. When 10^{-11} M truncated, recombinant protein was applied to the cis side, channel openings appeared 5-30 min later. The channels exhibited a slight inward rectification with a conductance ratio of ~0.8. The rectification was reversed when recombinant protein was applied to the trans side. These observations suggest that δ-LIT inserts into an artificial bilayer and that its insertion is polarized and Ca^{2+}-insensitive. Changes in Ca^{2+} concentration (n = 19) did not affect either the rectification or the reversal potential (V_{rev} = 0 mV) of the single channel current.
thus suggesting that the channel formed by recombinant δ-LIT does not select for Ca\(^{2+}\) (Fig. 7A). Also, \(V_{rev}\) was unchanged when SO\(^{4}\)\(^{-}\) was substituted for Cl\(^{-}\) on one side of a bilayer. Although not tested systematically, the probability of occurrence of channels formed by the recombinant protein did not appear to be affected by membrane potential. How do the results for the recombinant toxin compare with those for native δ-LIT? In lipid bilayers, native δ-LIT formed channels with a maximal slope conductance of \(-40\) pS (\(n = 33\)) (Fig. 7B). This compares with a conductance of \(-5\) pS for channels induced in bilayers by α-LIT (34), but the lipids and saline solutions were different. The channels formed by native δ-LIT also inwardly rectified (the conductance ratio was 0.5–0.6). The rectification was insensitive to changes in Ca\(^{2+}\) concentration (Fig. 7B).

However, \(V_{rev}\) for the single channel current varied with the Ca\(^{2+}\) concentration gradient across the bilayer (\(n = 5\)). Analysis of the Ca\(^{2+}\) concentration data using a modified Goldman equation for divalent ions gave a permeability (P) ratio for P_{Ca}\(^{2+}\)/P_{Na}\(^{+}\) of \(-60:1\). In other words, the channels formed by native δ-LIT were Ca\(^{2+}\)-selective. Shatsky et al. (34) have shown that native α-LIT forms channels that are selective for divalent cations. Channels also formed in inside-out patches of membrane excised from locust extensor tibiae muscle fibers using patch pipettes containing 10^{-9} M δ-LIT (\(n = 17\)) or 10^{-9} M protein (\(n = 12\)) (Fig. 8). The conductances and ion selectivities of these channels were similar to those observed in the bilayer experiments, except that channels formed by the recombinant protein inwardly rectified (Fig. 8A), whereas those formed by native δ-LIT exhibited outward rectification (Fig. 8B). In terms of mechanism, it is important to know whether channels are formed when a latrotoxin is applied to the cytoplasmic face of a natural membrane, but we have not yet developed a suitable protocol to test this. Despite this, it is clear from the results of the studies with artificial membranes and locust muscle membrane patches that if recombinant δ-LIT inserts to form channels, then its insertion is not influenced by net surface charge, lipid content of membrane, and presence of surface sugars etc. However, this does not seem to be true for the native toxin, because the channels that it forms exhibit opposite rectification properties in artificial and natural membranes.

**DISCUSSION**

In this study, we have used amino acid sequences obtained from a novel insect-specific toxin, δ-LIT, to clone its cDNA, and we have raised a specific antibody to native δ-LIT to confirm the identity of the clone as δ-LIT. The data described herein demonstrate that the primary structure of δ-LIT exhibits features in common with those of other cloned latrotoxins (9, 10), indicating that the latrotoxins have evolved from a single ancestral gene. The greatest similarity between these toxins is to be found in their N-terminal domains, where the most noticeable structural feature is two hydrophobic segments that are
positionally well conserved (Fig. 1). The second hydrophobic segment, which is shorter and has better defined borders, is one of the most conserved regions between the latrotoxins and may, therefore, be involved in an important aspect of latrotoxin function. Sequences preceding the N-terminal domain, and which are removed during maturation, differ greatly among $d$-LIT, $a$-LTX, and $a$-LIT. These sequences are identified as separate domains in Fig. 3 and are probably signal sequences. The only structural feature common to these sequences is a cluster of basic amino acid residues at positions 1 to 4 that represents a potential endopeptidase cleavage site, and is followed by an acidic N terminus of Glu and Asp residues. The central domain of $\delta$-LIT is comprised of $cdc10/ALR$, but the ALR domain is shorter in $d$-LIT than in other latrotoxins and the number of the repeated units is reduced. The presence of ALRs in a large number of functionally different proteins (e.g. cell cycle proteins, enzymes, and transcription factors), which are widespread from prokaryotes to human (31), suggests that they do not have a unique function but rather serve as a general structural domain. The data on several of these proteins, particularly ankyrin itself, indicate that they could mediate protein-protein interactions (35, 36). Although individual members of an ALR may have distinct functions (37), it seems
likely that an ALR domain acts as an integral structural unit and that the spatial organization of its repeats is a functional determinant. Interestingly, the vast majority of other proteins containing ALRs are either intracellular or membranous (where the ALR domain is intracellular), but the ALRs of δ-LIT are extracellular. The role, if any, of the ALRs in latrotoxin toxicity has not yet been established, although they may be involved in the binding of toxin to presynaptic receptors (6, 38).

The structure of the protein encoded in the δ-LIT gene predicts a molecule that is larger than the mature toxin isolated from the venom, i.e. that there is a disparity between the molecular weight of the toxin, as deduced from the cDNA sequence, and the relative mobility of δ-LIT purified from BWSV. While the N terminus of δ-LIT was identified unambiguously by protein sequencing, the precise position of the C terminus was difficult to determine, and it was unclear whether the apparent molecular mass of the purified, native toxin on SDS-PAGE reflected an electrophoretic artifact. Expression of the full-length δ-LIT cDNA in bacteria revealed that its calculated molecular mass is accurately reflected in the relative mobility of the protein on SDS-polyacrylamide gel electrophoresis and clearly showed for the first time that functionally active latrotoxin in BWSV derives from proteolytic, C-terminal processing. The full-length recombinant protein was not insectotoxic, whereas C-terminal truncation produced a protein that exhibited many of the properties of native δ-LIT, a result that may be relevant to other latrotoxins. Comparison of the primary structure of the C-terminal domain of δ-LIT, α-LTX, and δ-LIT shows that the three latrotoxins are similar in this region, which suggests that this domain may have an important biological function. Our prediction that the cDNA of δ-LIT encodes a protein that is substantially larger than the toxin purified from BWSV (3) is supported by the experimental data described herein. In view of the conservation of the C-terminal domain among latrotoxins, we propose that C-terminal processing is required for all members of this family. In support of this contention, it has recently been shown by MALDI-MS that native α-LTX is C-terminally truncated.2

Although it has been firmly established that latrotoxins cause a massive release of transmitter from axon terminals, the mechanism underlying this phenomenon is not well understood. A putative receptor for α-LTX has been purified (39, 40). This comprises two structurally similar protein subunits (200 and 160 kDa, respectively) (40). The subunits are thought to form a complex with others that do not bind α-LTX. Is this receptor involved in the formation by α-LTX of ion channels in axon terminal membrane? Support for this possibility comes from studies of channel formation by α-LTX in the surface membrane of Xenopus laevis oocytes. α-LTX channels were formed in oocytes injected with RNA extracted from rat brain, but not in uninjected oocytes (41). However, latrotoxins form channels in artificial membranes lacking other proteins (this study) (34), and in native membranes as well, such as locust muscle membrane, in which one might not expect to find latrotoxin receptors. Nevertheless, although in these cases latrotoxin receptors are not essential to the formation of channels by latrotoxins, their presence may greatly enhance this phenomenon. The involvement of receptors in latrotoxin-induced release of neurotransmitter is an attractive proposal because it provides a ready explanation for the phylum specificities of the latrotoxins. It is difficult to understand how a single δ-LIT molecule could form an ion channel because, according to hydrophobic profile analysis, it contains only two hydrophobic regions that are of a sufficient length to constitute a conventional membrane-spanning α-helix. Also, the primary structure of δ-LIT does not reveal direct sequence homology with proteins that form channels. However, it has been shown that α-LTX molecules aggregate in solution (42), so it is possible that a latrotoxin ion channel is formed from several toxin molecules.

Our initial hypothesis that the multifunctional activity of a latrotoxin requires the presence of a low molecular weight protein (LMWP) is clearly incorrect because the truncated, recombinant protein was toxic and induced transmitter release in the absence of a LMWP. However, the different permeability properties of the channels induced by native δ-LIT on the one hand and truncated, recombinant δ-LIT on the other hand require an explanation. Possibly, there are differences in post-translational modifications of the two toxins, although native α-LTX is neither glycosylated nor phosphorylated. When α-LTX is purified from BWSV by conventional biochemical methods it is always tightly associated with a LMWP (7947 Da) (12) called lactrotoxin (13). There are other LMWPs in BWSV, and, according to immunochemical studies, at least one of these (LMWP2) is present in venom fractions containing latroinsectotoxins (43). Although there is no direct evidence that a LMWP is associated with native δ-LIT, its presence could account for the Ca2+ selectivity of the channel formed by this protein. It could also account for the higher slope conductance of the channel that it forms in both artificial and natural membranes (almost twice that of channels formed by the recombinant protein) and for the outward rectification of the channel that it forms in locust muscle membrane patches. Although the presence of a LMWP is clearly not essential for the induction of transmitter release from locust motor nerve terminals by the recombinant protein, further comparative studies of native δ-LIT and the recombinant protein are required to determine whether there are subtle differences in their properties in this respect.

It has been noted previously that the presence of Ca2+ is not essential for the presynaptic action of latrotoxins (8, 44), although other divalent cations may be required if Ca2+ is absent. In the studies reported herein, δ-LIT and the truncated, recombinant protein increased mEPSP frequency in saline containing no divalent cations. However, chelators were not used, so we cannot be certain that the extracellular concentrations of Ca2+ and Mg2+ were indeed zero. Nevertheless, this result is essentially similar to that obtained by Magazanik et al. (8) in their studies of α-LIT in which divalent cation-free saline containing EGTA was used. Normally, extracellular Ca2+ plays a primary role in the action of the latrotoxins on motor nerve terminals; but Mg2+ can substitute for Ca2+. Perhaps entry of Mg2+ into an axon terminal through channels induced by a latrotoxin slowly exchanges with Ca2+ in intracellular stores and this leads to enhanced transmitter release. However, the concentration of Ca2+ in synaptosomes, as measured by Fura-2 fluorescence, does not rise when transmitter release is induced by α-LTX in Ca2+-free saline (44). In the present study, when the extracellular concentration of MgCl2 was raised while keeping the concentration of CaCl2 constant (2 mM), the increase in mEPSP frequency induced by δ-LIT was delayed. Competition between Mg2+ and Ca2+ for transport through the toxin-induced channels could account for this.

By virtue of their potent and specific action on transmitter release from nerve terminals, the latrotoxins have considerable potential as tools for investigating mechanisms of transmitter release in nervous systems. The ability to synthesize a functional, recombinant latrotoxin offers exciting opportunities for using site-directed mutagenesis to identify those structural features that contribute to the mode of action of δ-LIT. The structural and functional information on cloned and native

---

2I. E. Dulubova and B. T. Chait, personal communication.
δ-LIT presented herein has emphasized the common structural organization of the latrotoxins, although it is not yet clear from their sequence differences how they exhibit distinct phylum specific toxicities. It is clear, however, that the latrotoxins have a common domain organization and that only two domains participate in their biological function. Nevertheless, the parts of these proteins that are responsible for high affinity binding, multimerization and formation of channels remain to be determined. The important question of how the unique insect specificity of δ-LIT is achieved could be addressed in future experiments using hybrids between different latrotoxins.

Acknowledgments—We wish to acknowledge the help of R. Chabhra (Nottingham University, Nottingham, UK) with native δ-LIT MALDI-MS; T. G. Galkina (Shemyakin Institute, Moscow, Russia) for sequencing the native protein; N. S. Bystrov (Shemyakin Institute) and J. Keyte (Nottingham University, Nottingham, UK) for oligonucleotide synthesis; Dr. A. S. Petrenko (New York University, New York) for samples of α-LTX and δ-LIT; and Dr. B. Chait (Rockefeller University, New York) for unpublished data on α-LTX.

REFERENCES

1. Longenecker, H. E., Jr., Hurlbut, W. P., Mauro, A., and Clark, A. W. (1970) Nature 225, 701–703
2. Cull-Candy, S. G., Neal, H., and Usherwood, P. N. R. (1973) Nature 241, 353–354
3. Krasnopover, V. G., Shamotienko, O. G., and Grishin, E. V. (1992) J. Nat. Toxins 1, 17–23
4. Grasso, A. (1988) in Neurotoxins in Neurochemistry (Doly, J., O., ed.) pp. 67–78, Ellis Horwood, London
5. Fritz, L. C., Tzeng, C., and Mauro, A. (1980) J. Biol. Chem. 255, 24770–24776
6. Kiyatkin, N. I., Dulubova, I. E., Chekhovskaya, I. A., and Grishin, E. V. (1990) FEBS Lett. 270, 127–131
7. Kiyatkin, N. I., Dulubova, I., and Grishin, E. (1993) Eur. J. Biochem. 213, 423–427
8. Kiyatkin, N. I., Kulikovskaya, I. M., Grishin, E. V., Beadle, D. J., and King, L. A. (1995) Eur. J. Biochem. 230, 854–859
9. Kiyatkin, N., Dulubova, I., Chekhovskaya, I., Lipkin, A., and Grishin, E. V. (1992) Toxicon 30, 771–774
10. Pescatori, M., Bradbury, A., Bouet, F., Gargano, N., Mastroiacovo, A., and Grasso, A. (1995) Eur. J. Biochem. 230, 322–328
11. Volkova, T. M., Galkina, T. G., Kudelin, A. B., and Grishin, E. V. (1991) Bioorg. Khim. 17, 437–441 (in Russian)
12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Tabor, S., and Richardson, C. C. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1074–1078
14. Volkova, T. M., Pluzhnikov, K. A., Woll, P. G., and Grishin, E. V. (1995) FEBS Lett. 378, 608–612
15. Coffey, J. P., and Usherwood, P. N. R. (1978) J. Physiol. 285, 113–128
16. Corronado, R., and Latorre, R. (1993) Biophys. J. 63, 231–240
17. Huddie, P. L., Ramsey, R. L., and Usherwood, P. N. R. (1986) J. Physiol. 378, 608–612
18. Kozak, M. (1989) J. Cell Biol. 106, 229–234
19. Laemmli, U. K. (1970) Nature 227, 680–685
20. Towbin, J., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
22. Hoyle, G. (1995) Proc. R. Soc. Lond. B 243, 343–367
23. Usherwood, P. N. R. (1963) Nature 191, 814–815
24. Usherwood, P. N. R. (1973) J. Physiol. 241, 127–129
25. Conlon, G., and Latorre, R. (1993) Biophys. J. 63, 231–240
26. Kiyatkin, N. I., Dulubova, I. E., Chekhovskaya, I. A., and Grishin, E. V. (1990) Biochim. Biophys. Acta 1233, 14–20
27. Bennett, V. (1990) J. Biol. Chem. 265, 8703–8706
28. Thompson, C. C., Brown, T. A., and McKnight, S. L. (1991) Science 253, 762–768
29. Davis, L. H., Otto, E., and Bennett, V. (1991) J. Biol. Chem. 266, 11163–11169
30. Kiyatkin, N. I., Dulubova, I., and Grishin, E. (1992) FEBS Lett. 294, 283–288
31. Kiyatkin, N. I., Dulubova, I., and Grishin, E. V. (1991) Proteins Struct. Funct. Genet. 12, 1–10
32. Huddie, P. L., Ramsey, R. L., and Usherwood, P. N. R. (1986) J. Physiol. 285, 113–128
33. Kiyatkin, N. I., Dulubova, I. E., Chekhovskaya, I. A., and Grishin, E. V. (1990) FEBS Lett. 270, 127–131
34. Kiyatkin, N., Dulubova, I., and Grishin, E. (1993) Eur. J. Biochem. 213, 423–427
35. Kiyatkin, N. I., Kulikovskaya, I. M., Grishin, E. V., Beadle, D. J., and King, L. A. (1995) Eur. J. Biochem. 230, 854–859
36. Kiyatkin, N., Dulubova, I., Chekhovskaya, I., Lipkin, A., and Grishin, E. V. (1992) Toxicon 30, 771–774
37. Pescatori, M., Bradbury, A., Bouet, F., Gargano, N., Mastroiacovo, A., and Grasso, A. (1995) Eur. J. Biochem. 230, 322–328
38. Volkova, T. M., Pluzhnikov, K. A., Woll, P. G., and Grishin, E. V. (1995) Toxicon 33, 483–489
39. Kiyatkin, N. I., Dulubova, I. E., Chekhovskaya, I. A., and Grishin, E. V. (1990) Biochim. Biophys. Acta 1074–1078
Cloning and Structure of -Latroinsectotoxin, a Novel Insect-specific Member of the Latrotoxin Family: FUNCTIONAL EXPRESSION REQUIRES C-TERMINAL TRUNCATION

Irina E. Dulubova, Valery G. Krasnoperov, Mikhail V. Khvotchev, Kirill A. Pluzhnikov, Tatyana M. Volkova, Éugene V. Grishin, Horia Vais, David R. Bell and Peter N. R. Usherwood

J. Biol. Chem. 1996, 271:7535-7543.
doi: 10.1074/jbc.271.13.7535

Access the most updated version of this article at http://www.jbc.org/content/271/13/7535

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 41 references, 11 of which can be accessed free at http://www.jbc.org/content/271/13/7535.full.html#ref-list-1