The EMBO Journal Vol.17 No.21 pp.6188–6199, 1998

α-Latrotoxin action probed with recombinant toxin: receptors recruit α-latrotoxin but do not transduce an exocytotic signal

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α-Latrotoxin stimulates neurotransmitter release probably by binding to two receptors, CIRL/latrophilin 1 (CL1) and neurexin Iα. We have now produced recombinant α-latrotoxin (Ltx^{WT}) that is as active as native α-latrotoxin in triggering synaptic release of glutamate, GABA and norepinephrine. We have also generated three α-latrotoxin mutants with substitutions in conserved cysteine residues, and a fourth mutant with a four-residue insertion. All four α-latrotoxin mutants were found to be unable to trigger release. Interestingly, the insertion mutant Ltx^{N4C} exhibited receptor-binding affinities identical to wild-type Ltx^{WT}, bound to CL1 and neurexin Iα as well as Ltx^{WT}, and similarly stimulated synaptic hydrolysis of phosphatidylinositolphosphates. Therefore, receptor binding by α-latrotoxin and stimulation of phospholipase C are insufficient to trigger exocytosis. This conclusion was confirmed in experiments with La^{3+} and Cd^{2+}. La^{3+} blocked release triggered by Ltx^{WT}, whereas Cd^{2+} enhanced it. Both cations, however, had no effect on the stimulation by Ltx^{WT} of phosphatidylinositolphosphate hydrolysis. Our data show that receptor binding by α-latrotoxin and activation of phospholipase C do not by themselves trigger exocytosis. Thus receptors recruit α-latrotoxin to its point of action without activating exocytosis. Exocytosis probably requires an additional receptor-independent activity of α-latrotoxin that is selectively inhibited by the Ltx^{N4C} mutation and by La^{3+}.

Keywords: α-latrotoxin/CIRL/latrophilin/neurexins/neurotransmitter release

Introduction

The venom from black widow spider contains at least three related latrotoxins: vertebrate-specific α-latrotoxin and invertebrate-specific α- and δ-latroinsectotoxins (Frontali and Grasso, 1964; Dulubova *et al*., 1996). The three latrotoxins are potent neurotoxins that trigger neurotransmitter release from presynaptic nerve terminals (Frontali *et al*., 1976; Knipper *et al*., 1986; Magazanik *et al*., 1992). All latrotoxins are large proteins composed of four domains (Figure 1; Kiyatkin *et al*., 1990, 1993; Dulubova *et al*., 1996): (i) a cleaved signal peptide (domain I); (ii) a conserved N-terminal domain (of 425–434 residues) with three invariant cysteine residues (domain II); (iii) a domain composed of 15–22 ankyrin-like repeats covering 557–745 residues (domain III); and (iv) a relatively short (194–206 residues) C-terminal domain that is less conserved between latrotoxins (domain IV). The similar domain structures of different latrotoxins and their high degree of sequence identity suggest that the vertebrate-specific α-latrotoxin and the invertebrate-specific α- and δ-latroinsectotoxins use the same mechanisms of action. Since all latrotoxins stimulate neurotransmitter release, it is likely that the different toxins act on the same components of the release machinery, with their species specificity caused by differences in receptors.

Mature latrotoxins isolated from black widow spider venom are smaller than is predicted from their primary structures, indicating that latrotoxins are cleaved post-translationally during venom maturation (Kiyatkin *et al*., 1990, 1993; Dulubova *et al*., 1996). Mass spectroscopy of α-latrotoxin has suggested that it lacks domain IV but contains all ankyrin repeats (Dulubova *et al*., 1996). Expression of δ-latroinsectotoxin has shown that full-length recombinant toxin is inactive but that toxin lacking the C-terminal domain triggers neurotransmitter release (Dulubova *et al*., 1996). This supports the notion that the C-terminal domain of δ-latroinsectotoxin is removed during toxin maturation to activate the toxin. Expression experiments with full-length α-latrotoxin were also unable to detect activity (Kiyatkin *et al*., 1995; N.Kiyatkin, personal communication). To date, no expression of recombinant active α-latrotoxin has been reported. It is possible that recombinant α-latrotoxin was inactive because full-length toxin was expressed. An alternative possibility is that a low-molecular-weight peptide which co-purifies with α-latrotoxin (Kiyatkin *et al*., 1992) is required for activity (Grishin *et al*., 1993).

Among latrotoxins, α-latrotoxin is probably the best-studied toxin because it is active in vertebrates. Results from a variety of systems have demonstrated that α-latrotoxin has two modes of action. First, α-latrotoxin inserts into membranes and serves as a non-selective cation channel (Finkelstein *et al*., 1976; Rosenthal *et al*., 1990; Hurlbut *et al*., 1994). This activity potentially explains the ability of α-latrotoxin to cause neurotransmitter release in the presence of Ca^{2+}, since α-latrotoxin could function as a Ca^{2+} ionophore. Secondly, α-latrotoxin stimulates neurotransmitter release in the absence of Ca^{2+} (Gorio *et al*., 1978; Ceccarelli and

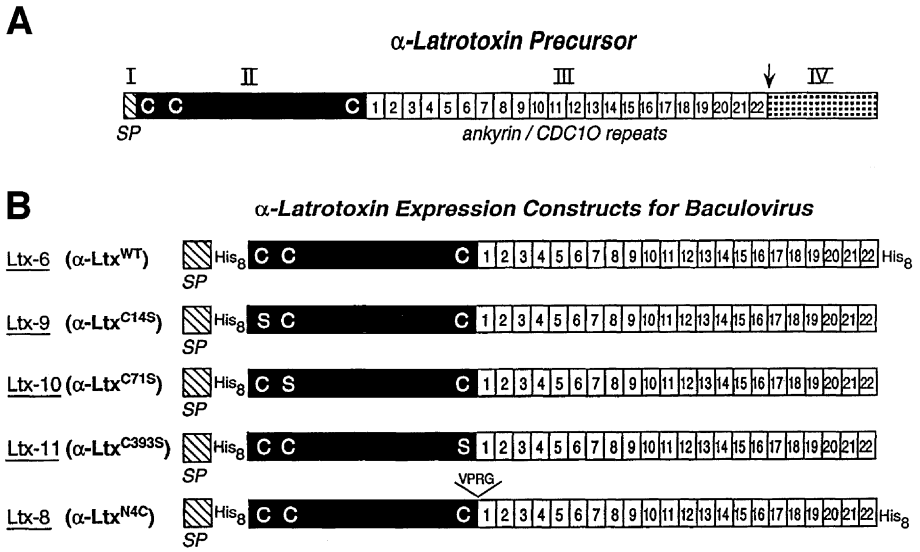

Fig. 1. Structure of α-latrotoxin and of recombinant latrotoxins expressed via baculovirus. (**A**) Composition of α-latrotoxin of four domains. (I) Signal peptide (SP). (II) Conserved N-terminal domain. Locations of the only three cysteine residues that are conserved in all latrotoxins are shown as 'C's. (III) Central region, composed of 22 ankyrin/CDC10 repeats. (IV) C-terminal region. Comparison of the size of the mature protein with the predicted size from the cDNA sequence suggests that α-latrotoxin is processed by endoproteolytic cleavage after the last ankyrin repeat (arrow). (**B**) Structures of recombinant α-latrotoxins. The names of the constructs are shown on the left. All recombinant α-latrotoxins are preceded by a signal peptide from baculovirus gp67 and are truncated after the ankyrin repeats at the putative site of endoproteolytic cleavage. Ltx-6 encodes wild-type latrotoxin (Ltx^{WT}). The recombinant toxin is flanked by eight histidine residues (His_8) for purification. Ltx-9, -10 and -11 encode Ltx^{WT} with three different cysteine-to-serine substitutions (Ltx^{C14S}, Ltx^{C71S} and Ltx^{C393S}). Ltx-8 encodes a mutant of Ltx^{WT} in which four amino acids (VPRG) were inserted between domains II and III (Ltx^{N4C}).

Hurlbut, 1980; Capogna *et al*., 1996). Under the same conditions, neurotransmitter release is not triggered by a Ca^{2+} ionophore, indicating that α-latrotoxin has Ca^{2+}-independent synaptic actions which are distinct from its ionophore properties. Although the latroinsectotoxins have not been investigated in detail, preliminary studies suggest a similar dual mode of action (Magazanik *et al*., 1992; Dulubova *et al*., 1996). The mechanism by which α-latrotoxin elicits neurotransmitter release in the absence of extracellular Ca^{2+} is unclear.

α-Latrotoxin binds to synaptic receptors to stimulate exocytosis (Tzeng and Siekevitz, 1979). Initially, a high-affinity receptor for α-latrotoxin was identified in neurexin Iα, a neuronal cell-surface protein that is part of a large family of cell-adhesion molecules (Ushkaryov *et al*., 1992; reviewed in Missler and Südhof, 1998). However, neurexin Iα binds α-latrotoxin only in the presence of Ca^{2+}, suggesting that it does not mediate the Ca^{2+}-independent actions of α-latrotoxin (Davletov *et al*., 1995). In agreement with this conclusion, studies on knockout mice lacking neurexin Iα showed that neurexin Iα is not essential for Ca^{2+}-independent α-latrotoxin action (Geppert *et al*., 1998). However, release triggered by α-latrotoxin in the presence of Ca^{2+} was impaired in mice lacking neurexin Iα. This result suggests that neurexin Iα acts as a co-receptor for α-latrotoxin.

An exciting recent discovery was the identification of a second α-latrotoxin receptor called CIRL (Krasnoperov *et al*., 1997) or latrophilin (Davletov *et al*., 1996), and abbreviated here as CL1. CL1 binds α-latrotoxin in the absence of Ca^{2+} and belongs to the family of G-protein-coupled receptors. Thus activation of a G-protein-linked receptor may directly trigger neurotransmitter release by a novel Ca^{2+}-independent pathway (Krasnoperov *et al*., 1997; Lelianova *et al*., 1997).

It is unclear what roles are played by α-latrotoxin receptors in release. At present, the dominant notion is that binding of α-latrotoxin to receptors directly activates exocytosis. This could involve the phospholipids PIP and PIP_2, since PIP and PIP_2 have been implicated in membrane traffic (De Camilli *et al*., 1995), their synthesis and hydrolysis are tightly regulated by G-protein-linked pathways, and α-latrotoxin activates PIP and PIP_2 hydrolysis (Vicentini and Meldolesi, 1984). Alternatively, the interaction of neurexin Iα with synaptotagmin I, the putative Ca^{2+} sensor in exocytosis, may transduce an exocytotic signal (Petrenko *et al*., 1991). A further possibility is that α-latrotoxin binding to its receptors activates an as yet unknown exocytotic pathway, perhaps via G-proteins and syntaxin. Both syntaxin and G-proteins have been reported to bind to CL1 (Krasnoperov *et al*., 1997; Lelianova *et al*., 1997). In contrast to these hypotheses, another very different mechanism of action is possible but has rarely been considered: the notion that receptors only recruit α-latrotoxin to the synapse but do not activate exocytosis. This notion implies, analogous to the mechanism of action of clostridial neurotoxins, that α-latrotoxin first binds to its receptors and then performs a second function, possibly by translocation into the cytosol or by activating yet another protein which cannot be directly activated.

In the current study, we have used recombinant toxins to investigate how α-latrotoxin stimulates neurotransmitter release. Our results demonstrate that α-latrotoxin expressed in a truncated form is fully active without addition of the low-molecular-weight peptide. We observed a mutant form of α-latrotoxin that still binds to both receptors (CL1 and neurexin Iα) and induces PIP and PIP_2 hydrolysis but triggers no neurotransmitter release. Our data demonstrate that receptor binding and/or PIP and PIP_2 hydrolysis are not sufficient to explain α-latrotoxin

action. We confirmed this conclusion by an independent approach using La^{3+} and Cd^{2+}, which inhibit or enhance α-latrotoxin action but have no effect on PIP and PIP_2 hydrolysis. Our findings imply that the receptors for α-latrotoxin function as conduits for toxin action without transducing an exocytotic signal.

Results

Production of recombinant α-latrotoxin

Black widow spiders synthesize α-latrotoxin and other latrotoxins as large precursor proteins. The precursors are processed to yield mature toxins, most likely by endoproteolytic cleavage after the ankyrin repeats (Figure 1). Previous attempts to express active recombinant α-latrotoxin may have failed because full-length toxin was synthesized. An alternative explanation is that recombinant α-latrotoxin was inactive because it lacked the low-molecular-weight component which co-purifies with native α-latrotoxin (Kiyatkin *et al*., 1992). To differentiate between these possibilities, we expressed a recombinant form of α-latrotoxin that is truncated after the ankyrin repeats (referred to as Ltx^{WT} because it contains the wild-type sequences; Figure 1). We chose a baculovirus system to produce the toxin, and added a signal peptide from baculovirus gp67 to the N-terminus of the recombinant toxin to enhance its secretion. In addition, we inserted eight histidine residues at the N- and C-terminal ends of the toxin to allow efficient purification of the recombinant protein on Ni^{2+}-agarose (Figure 1). Baculovirus-infected insect cells expressed Ltx^{WT} at high levels and secreted it into the medium. We purified Ltx^{WT} from the medium to apparent homogeneity by two chromatography steps (Figure 2), and confirmed its identity by immunoblotting with an antibody raised against native α-latrotoxin (data not shown).

Recombinant α-latrotoxin is active

We used release measurements from synaptosomes to investigate whether Ltx^{WT} triggers neurotransmitter release similarly to native α-latrotoxin. Synaptosomes were purified from mouse brain and loaded with various [^{3}H]-labeled neurotransmitters (Khvotchev and Südhof, 1998). The synaptosomes were then employed in release measurements in which we stimulated release with recombinant Ltx^{WT}, native α-latrotoxin purified from black widow spider venom and KCl depolarization. Our data show that, similar to native α-latrotoxin, recombinant Ltx^{WT} is biologically active at nanomolar toxin concentrations, triggers release with or without Ca^{2+}, and does not require the low-molecular-weight component for activity (Figures 3 and 4).

We first studied ^{3}H-glutamate release induced by Ltx^{WT} (Figure 3). Recombinant Ltx^{WT} triggered glutamate release with a concentration dependence that was similar in the presence and absence of Ca^{2+}. We observed half-maximal release at ~3 nM Ltx^{WT}, suggesting that Ltx^{WT} acts by binding to high-affinity sites (Figure 3). The concentration dependence of release obtained with recombinant Ltx^{WT} is similar to that reported for native α-latrotoxin purified from black widow spider venom (Frontali *et al*., 1976). Purified native α-latrotoxin contains a low-molecular-weight component in addition to the 120 kDa toxin

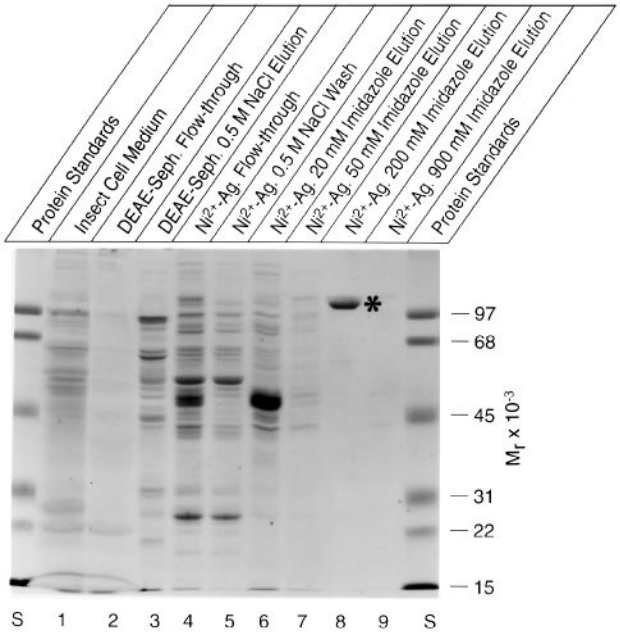

Fig. 2. Purification of α-latrotoxin from the medium of insect cells infected with recombinant baculovirus encoding Ltx^{WT}. Tissue-culture medium from 'high-five cells' infected with baculovirus Ltx-6 (lane 1) was chromatographed on DEAE–Sepharose (DEAE–Seph.; lanes 2 and 3) and nickel agarose (Ni^{2+}-Ag., lanes 4–9) with the indicated elution conditions. Equivalent amounts of the eluates were analyzed by SDS–PAGE and Coomassie Blue staining. Recombinant α-latrotoxin was eluted by 0.2 M imidazole from the Ni^{2+}–agarose column (asterisk). Protein standards were loaded on the left and right lanes (labeled S); their sizes are given on the right.

(Kiyatkin *et al*., 1992). To ensure that the recombinant α-latrotoxin is in fact comparable in activity to native spider toxin, we compared Ltx^{WT} with native α-latrotoxin in the same preparation of synaptosomes. The concentrations of the toxins were standardized on Coomassie Blue-stained SDS–polyacrylamide gels (data not shown). Identical amounts of toxins were then used to measure GABA, glutamate and norepinephrine release from synaptosomes (Figure 4). We found that the magnitude of release and its dependence on the α-latrotoxin concentration were very similar between native α-latrotoxin purified from black widow spider venom and recombinant Ltx^{WT}. This was true for all three neurotransmitters analyzed, suggesting that recombinant Ltx^{WT} precisely reproduces the effects of native toxin.

Cysteines are essential for α-latrotoxin activity

Using immobilized toxin as an affinity matrix, we next tested whether Ltx^{WT} binds to neurexin Iα and CL1. Since disulfide bond reduction abolishes the activity of α-latrotoxin and its binding to receptors (Davletov *et al*., 1996), we used reduced Ltx^{WT} as a negative control for these experiments. Neurexin Iα and CL1 bound only to non-reduced Ltx^{WT} but not to reduced Ltx^{WT} (Figure 5 and data not shown). These results affirm the activity of the recombinant α-latrotoxin and support the notion that α-latrotoxin contains essential disulfide bonds. Only three cysteine residues are conserved in all latrotoxins (Dulubova *et al*., 1995), suggesting that some or all of these cysteines may participate in disulfide bonding. All three cysteines are in the N-terminal domain. To investigate

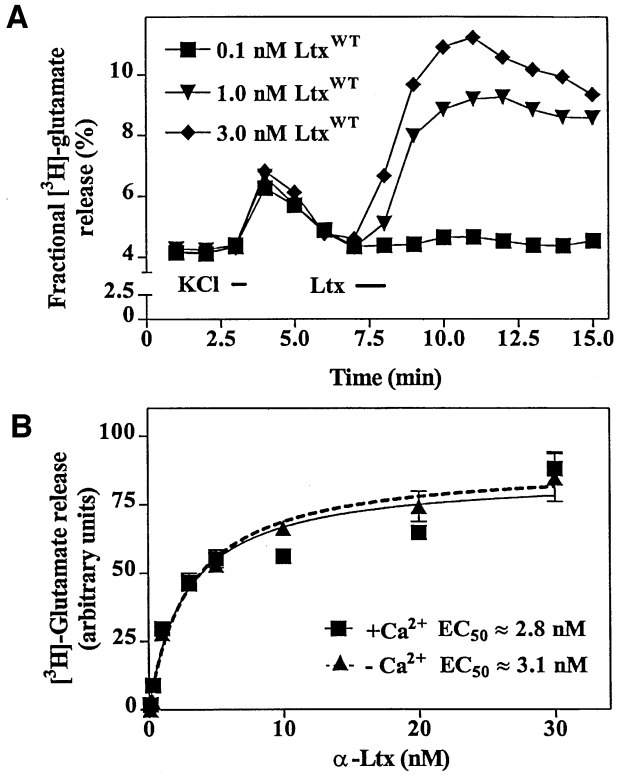

Fig. 3. Recombinant α-latrotoxin triggers ^{3}H-glutamate release from synaptosomes in the presence and absence of Ca^{2+}. (**A**) Representative glutamate release experiment. Synaptosomes preloaded with ^{3}H-glutamate were superfused under baseline conditions and stimulated with a 30 s pulse of KCl (25 mM) followed by a 1 min pulse of recombinant α-latrotoxin (Ltx^{WT}) at the indicated concentrations. Released ^{3}H-glutamate was measured continuously. (**B**) Analysis of stimulated ^{3}H-glutamate release as a function of α-latrotoxin concentration in the presence or absence of Ca^{2+}. Release was stimulated by recombinant α-latrotoxin (Ltx^{WT}) at various concentrations, as shown in (A). The total evoked release of ^{3}H-glutamate was calculated for each concentration of α-latrotoxin by integrating the amount of release over baseline release. Data were fitted to a receptor-binding model with a single binding site, with the indicated half-maximal concentrations of α-latrotoxin (EC_{50}s). Data are from multiple experiments carried out in duplicate; error bars show SEMs.

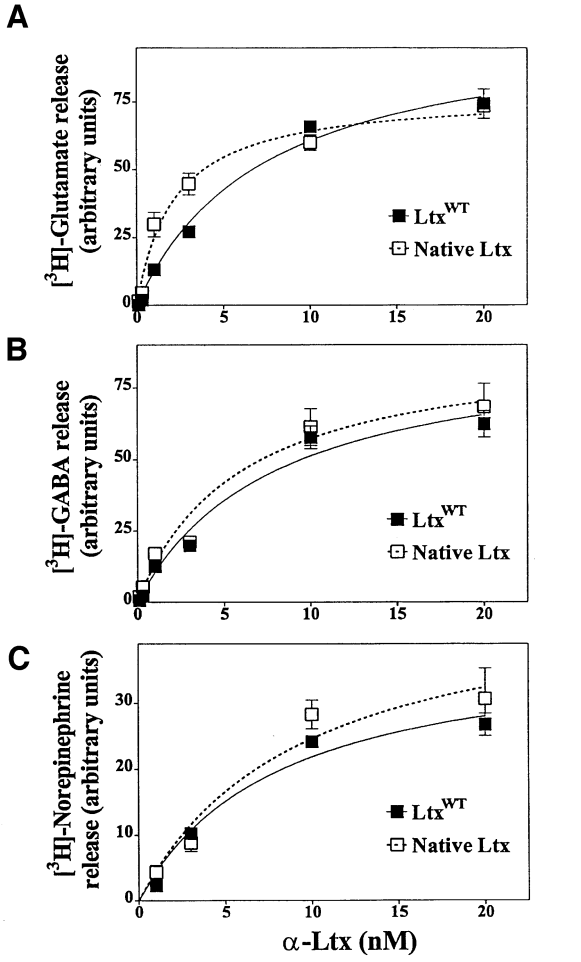

Fig. 4. Recombinant α-latrotoxin (Ltx^{WT}) and native α-latrotoxin purified from black widow spider venom (Native Ltx) are equally active. (**A**) Analysis of glutamate release, (**B**) GABA release and (**C**) norepinephrine release. Synaptosomes were preloaded with the respective ^{3}H-labeled neurotransmitters, and release was measured in superfused synaptosomes as shown in Figure 3A. The same set of synaptosomes with either glutamate, GABA or norepinephrine was used for parallel experiments with recombinant (Ltx^{WT}) and native α-latrotoxins which had been quantified on the same gel by Coomassie Blue staining. The total amount of evoked release was quantified for each neurotransmitter and α-latrotoxin concentration as described in Figure 3B. Data shown are from a representative experiment performed in duplicate.

whether the three conserved cysteine residues are required for α-latrotoxin activity, we individually substituted the three cysteines for serine in Ltx^{WT} (Figure 1). We expressed the mutant proteins (referred to as Ltx^{C14S}, Ltx^{C71S} and Ltx^{C393S}; Figure 1) in insect cells and purified them from the medium. The mutant α-latrotoxins were employed for glutamate release assays with synaptosomes (Figure 6). All three mutants (Ltx^{C14S}, Ltx^{C71S} and Ltx^{C393S}) were unable to elicit glutamate release even at high concentrations (10 nM), suggesting that the cysteine residues are essential for α-latrotoxin action. Since disulfide bond reduction abolishes α-latrotoxin binding to its receptors, these results indicate that the N-terminal domain of α-latrotoxin is involved in receptor binding.

Ltx^{N4C} is unable to trigger glutamate release from synaptosomes

Mature native α-latrotoxin and our recombinant α-latrotoxin (Ltx^{WT}) are composed of two clearly distinguishable domains: an N-terminal conserved domain with three invariant cysteines (domain II, Figure 1), and a C-terminal domain composed of 22 ankyrin-like repeats (domain III). This division suggests that the two domains form separate units. To facilitate the functional analysis of these domains, we generated an α-latrotoxin mutant with a four-amino-acid insertion between the two domains (Ltx^{N4C}; Figure 1). The insertion creates a thrombin-recognition site to allow cleavage of the mutant toxin into two parts. In this mutagenesis experiment, we were guided by the idea that the connection between two well-delineated domains in a protein is often composed of a flexible linker sequence whose exact size and composition are not critical.

Ltx^{N4C} was expressed in insect cells at levels similar to those of Ltx^{WT}, suggesting that the mutant toxin was well folded (data not shown). Surprisingly, however, Ltx^{N4C} did not stimulate ^{3}H-norepinephrine release (Figure 7A) or ^{3}H-glutamate release (Figure 7B and 7C) from synaptosomes at concentrations of up to 10 nM. Even prolonged

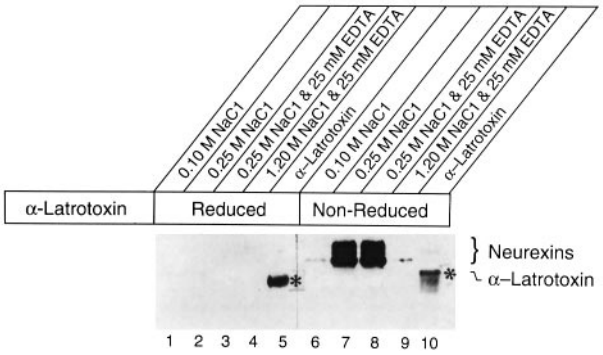

Fig. 5. Affinity purification of neurexin Iα on immobilized recombinant α-latrotoxin. Recombinant wild-type α-latrotoxin (Ltx^{WT}) was immobilized on CNBr-activated Sepharose with or without sulfhydryl reduction and used as an affinity matrix for protein purification from rat brain. Column eluents obtained with the indicated buffers were immunoblotted with a neurexin antibody which weakly cross-reacts with α-latrotoxin loaded in lanes 5 and 10 to illustrate its position on the blot.

application of Ltx^{N4C} was without effect (Figure 7D). Thus, the connection between the N-terminal domain and the ankyrin repeat domain must be functionally important for α-latrotoxin activity. Further support for this conclusion was derived from the finding that Ltx^{N4C} could not be cleaved by thrombin, suggesting that the thrombin cleavage site in the linker between the two halves of the toxin is not readily accessible (data not shown).

Ltx^{WT} and Ltx^{N4C} bind CL1 and neurexin Iα

One explanation for the inability of Ltx^{N4C} to trigger release is that Ltx^{N4C} does not bind to α-latrotoxin receptors. To test this, we investigated the binding of CL1 and neurexin Iα to Ltx^{WT} and Ltx^{N4C} by affinity chromatography. Equivalent amounts of Ltx^{WT} and Ltx^{N4C} were immobilized on a column matrix and used for affinity purification of rat brain proteins, and bound proteins were evaluated by immunoblotting and Coomassie Blue staining (Figure 8 and data not shown). Ltx^{WT} and Ltx^{N4C} retained CL1 and neurexin Iα equally well. The fact that both receptors are efficiently purified on Ltx^{WT} and Ltx^{N4C} demonstrates that the mutant toxin is fully capable of binding to both receptors.

Several proteins have been shown to interact with α-latrotoxin receptors (Petrenko *et al.*, 1991; O'Connor *et al.*, 1993; Krasnoperov *et al.*, 1997; Lelianova *et al.*, 1997). It is possible that these interactions are selectively disturbed upon receptor binding by Ltx^{N4C}. To investigate this, we immunoblotted the eluents from the Ltx^{WT} and Ltx^{N4C} affinity chromatography with antibodies against interacting proteins (Figure 9 and data not shown). As reported previously, synaptotagmin I and CASK were efficiently co-purified with the receptors on both Ltx^{WT} and Ltx^{N4C} (Petrenko *et al.*, 1991; Hata *et al.*, 1996). However, neither syntaxin nor G-proteins were enriched with the receptors under our chromatography conditions (Figure 9).

Ltx^{WT} and Ltx^{N4C} exhibit similar receptor-binding affinities

The affinity chromatography data do not rule out the possibility that Ltx^{N4C} is inactive in neurotransmitter release because its receptor-binding affinity is lower than that of Ltx^{WT}. An additional possibility to explain the inability of Ltx^{N4C} to trigger release is that a third, as yet unidentified, receptor exists which is the 'real' receptor for release and which binds only Ltx^{WT}, and not Ltx^{N4C}. To test these possibilities, we measured the relative binding affinities of Ltx^{WT} and Ltx^{N4C}. Our data show that Ltx^{WT} and Ltx^{N4C} have similar affinities and bind to the same sites in brain membranes (Figure 10).

We labeled Ltx^{WT} and Ltx^{N4C} with ^{125}I, and monitored competition for binding between the iodinated toxins and unlabeled Ltx^{WT} and Ltx^{N4C}. Ltx^{WT} and Ltx^{N4C} exhibited nanomolar binding affinities with no significant differences between wild-type and mutant toxins. Both Ltx^{WT} and Ltx^{N4C} fully displaced each other from their respective binding sites. This result shows that if there are binding sites that interact only with Ltx^{WT}, and not Ltx^{N4C}, these must be very rare (Figure 10).

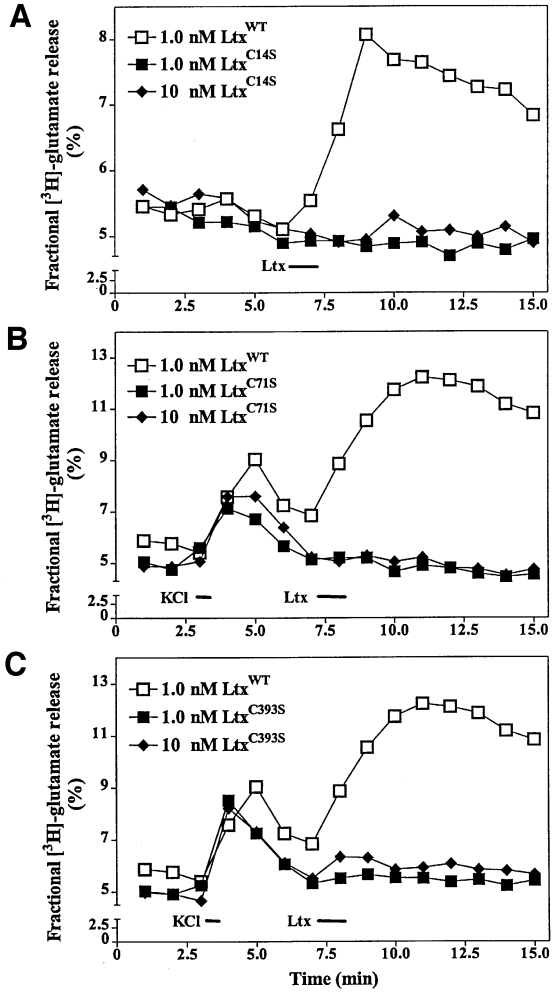

Fig. 6. Substitutions in three conserved cysteines in the N-terminal domain of α-latrotoxin abolish activity. Recombinant wild-type (Ltx^{WT}) and mutant α-latrotoxin with cysteine-to-serine substitutions in the three conserved cysteine residues (Ltx^{C14S}, Ltx^{C71S} and Ltx^{C393S}) were used for release studies with synaptosomes preloaded with ^{3}H-glutamate. Release was measured in superfused synaptosomes triggered by a 30 s pulse of 25 mM KCl or a 60 s pulse of the indicated concentrations of the different α-latrotoxins (see Figure 3A). Experiments were performed in duplicate and repeated multiple times with identical results.

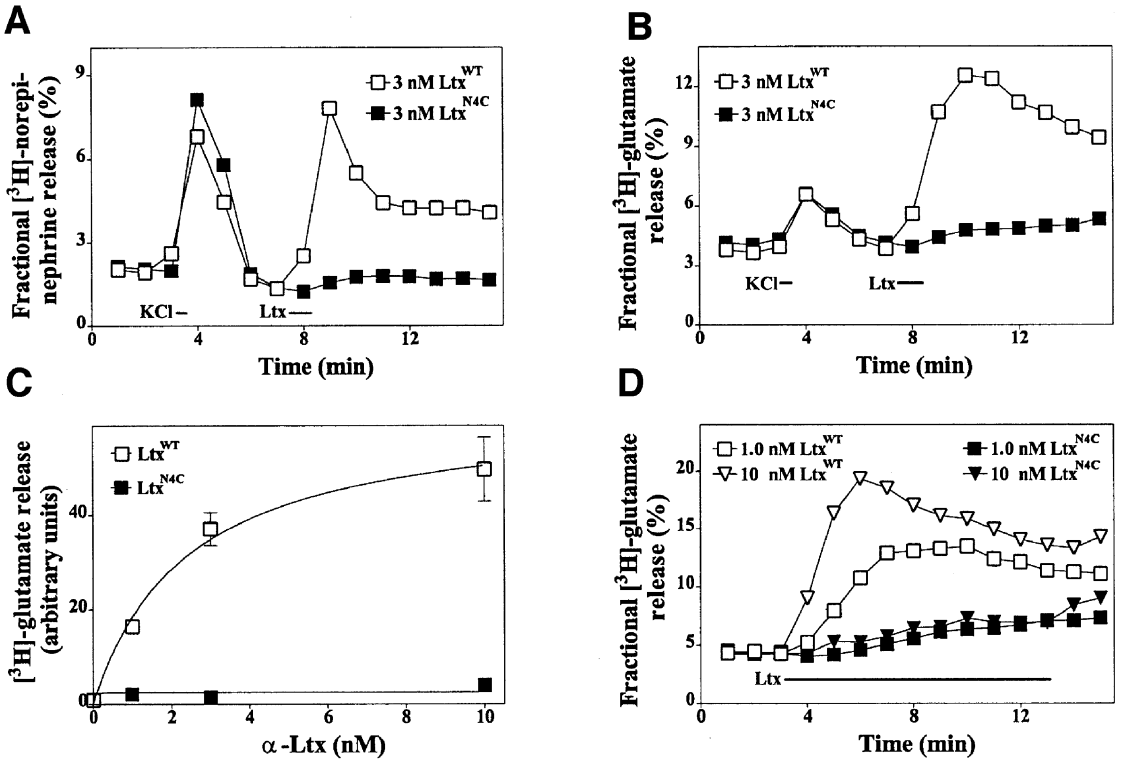

Fig. 7. A four-amino-acid insertion between the N-terminal domain and the ankyrin-repeat region of α-latrotoxin (Ltx^{N4C}) abolishes activity. (**A**) Representative experiment of norepinephrine release stimulated by Ltx^{WT} and Ltx^{N4C}. Synaptosomes were loaded with ^{3}H-norepinephrine and stimulated under continuous superfusion with a pulse of KCl (25 mM for 30 s) followed by a pulse of Ltx^{WT} or Ltx^{N4C} (3.0 nM for 60 s). (**B**) Representative experiment of glutamate release stimulated by Ltx^{WT} and Ltx^{N4C}. The experiment was carried out as in (A), except that synaptosomes were loaded with ^{3}H-glutamate. (**C**) Summary graph of experiments in which glutamate release was stimulated by various concentrations of Ltx^{WT} amd Ltx^{N4C}. Data shown are means ± SEMs from multiple experiments carried out as in (B). (**D**) Representative experiment in which Ltx^{WT} and Ltx^{N4C} were applied at 1 and 10 nM to synaptosomes for 10 min instead of 1 min to test if release could be evoked by long exposures to Ltx^{N4C}. The experiment was carried out as in (B).

Stimulation of PIP and PIP_2 hydrolysis by Ltx^{WT} and Ltx^{N4C}

α-latrotoxin induces PIP_2 hydrolysis in PC12 cells, suggesting that lipid-derived second messengers may mediate the ability of α-latrotoxin to trigger exocytosis (Vicentini and Meldolesi, 1984). To test whether α-latrotoxin has a similar effect in nerve terminals, we studied PIP and PIP_2 hydrolysis in synaptosomes. For this purpose we incubated synaptosomes with $^{32}P_i$ and determined the levels of ^{32}P-labeled phospholipids by thin-layer chromatography. Under the conditions used, $^{32}P_i$ was preferentially incorporated into phosphoinositides (data not shown). After ^{32}P-labeling, we exchanged the medium of the synaptosomes for Ca^{2+}-containing and Ca^{2+}-free buffers identical to those employed in the release assays. We then exposed the synaptosomes to Ltx^{WT}, extracted the phospholipids, and quantified their PIP and PIP_2 content by thin-layer chromatography and PhosphorImager detection (Khvotchev and Südhof, 1998).

Ltx^{WT} caused significant PIP and PIP_2 hydrolysis in the presence of Ca^{2+}. Ltx^{WT} had no effect on PIP levels in the absence of Ca^{2+}, and caused only a small decrease in PIP_2 (Figure 11). Under identical conditions, Ltx^{WT} caused similar amounts of glutamate release from synaptosomes in the presence and absence of Ca^{2+} (Figure 3). These data show that in the presence of Ca^{2+}, α-latrotoxin activates a signal transduction cascade that results in PIP and PIP_2 breakdown. Although PIP and PIP_2 hydrolysis can be used to monitor α-latrotoxin activity, hydrolysis appears to be associated only peripherally with neurotransmitter release, since release and hydrolysis do not correlate. This conclusion was confirmed when we tested whether mutant α-latrotoxin, Ltx^{N4C}, activates PIP and PIP_2 hydrolysis in nerve terminals. Direct comparisons of the activities of Ltx^{WT} and Ltx^{N4C} applied at the same concentrations revealed that they both stimulate PIP and PIP_2 hydrolysis (Figure 11). Under the same conditions, only Ltx^{WT}, and not Ltx^{N4C}, elicits neurotransmitter release (Figure 7).

Cd^{2+} and La^{3+} exert opposite effects on α-latrotoxin

To obtain insight into the mechanism of α-latrotoxin action by a second, independent approach, we studied the effects of Cd^{2+} and La^{3+} on release stimulated by α-latrotoxin (Figure 12A). Cd^{2+} greatly enhanced the amount of release induced by α-latrotoxin, whereas La^{3+} almost completely inhibited it (Figure 12B). The effects of Cd^{2+} and La^{3+} on α-latrotoxin-stimulated release could be caused by a modulation of the release machinery or by interference with the mechanism of α-latrotoxin action. We explored these two hypotheses by studying the effect of Cd^{2+} and La^{3+} on two other secretagogs, KCl and hypertonic sucrose (Figure 12C). Similar to α-latrotoxin, hypertonic sucrose triggers glutamate release from nerve terminals by a Ca^{2+}-independent mechanism that does not

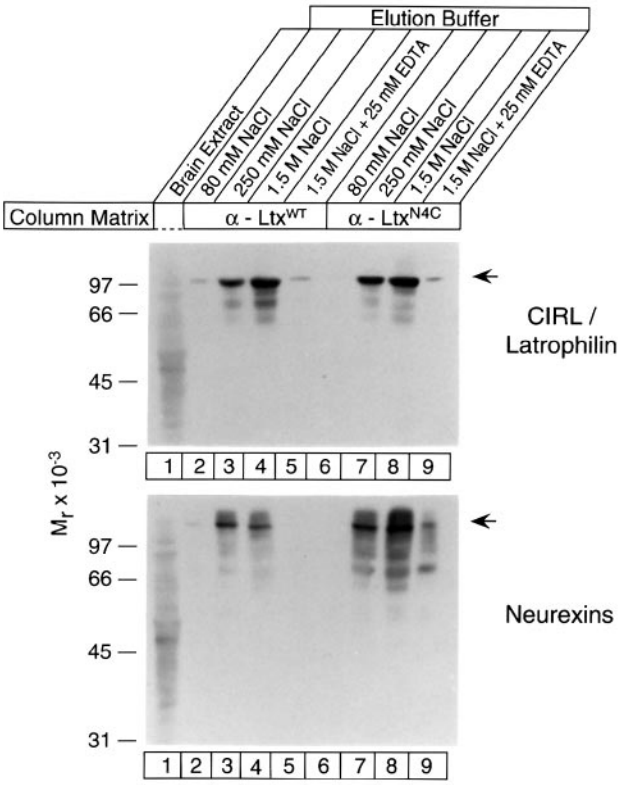

Fig. 8. Binding of CIRL/latrophilin (CL1) and α-neurexin to Ltx^{WT} and Ltx^{N4C}. Affinity columns with equal amounts of immobilized wild-type α-latrotoxin (Ltx^{WT}) or mutant α-latrotoxin (Ltx^{N4C}) were used to purify proteins from total rat brain homogenates. Eluents with the indicated buffers were analyzed by immunoblotting for CL1 (top) and neurexins (bottom). Cross-reactive bands in lane 1 arise because large amounts of brain protein or brain proteins were loaded to visualize the low-abundance CL1 and neurexins. The identities of CL1 and neurexins in the eluates were verified by direct amino acid sequencing. Numbers on the left indicate positions of molecular weight markers.

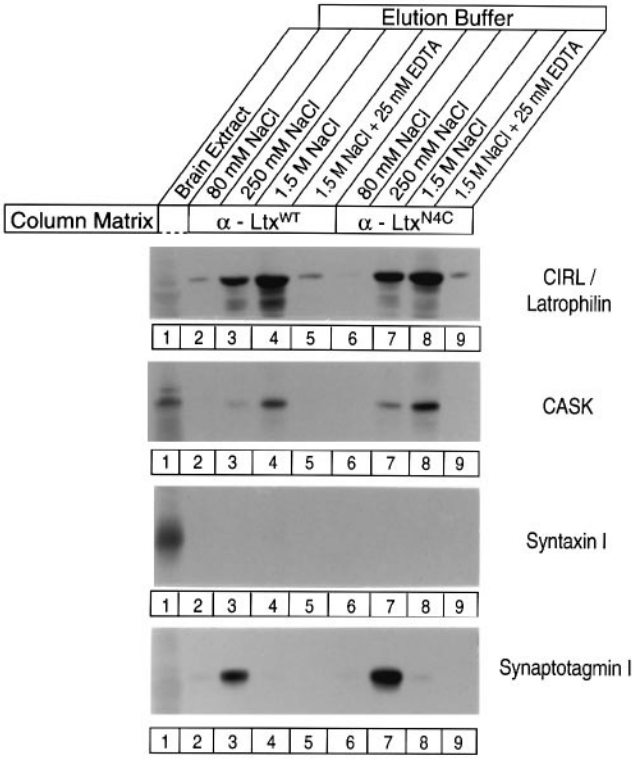

Fig. 9. Analysis of proteins co-purifying with α-latrotoxin receptors on immobilized Ltx^{WT} and Ltx^{N4C}. Eluates from the Ltx^{WT} and Ltx^{N4C} affinity columns in Figure 8 were analyzed by immunoblotting for CASK, syntaxin and synaptotagmin, as indicated. The CL1 immunoreactivity from Figure 8 is shown in the top panel to identify the fractions in which CL1 and neurexins elute.

require opening of ion channels (Rosenmund and Stevens, 1996). Cd^{2+} and La^{3+} had no significant effect on release stimulated by hypertonic sucrose, suggesting that Cd^{2+} and La^{3+} do not interfere with exocytosis as such (Figure 12C). In contrast to sucrose, KCl stimulates release by depolarizing the membrane and opening Ca^{2+} channels. Cd^{2+} and La^{3+} inhibited KCl-induced release, an effect that is not unexpected, since both cations are Ca^{2+} channel blockers. However, La^{3+} inhibited release only moderately and Cd^{2+} inhibited release strongly, whereas their effects on release stimulated by α-latrotoxin were the converse. Thus La^{3+} and Cd^{2+} have divergent effects on release that appear to be specific for the secretagog used. These effects do not seem to act directly on the secretory apparatus.

La^{3+} or Cd^{2+} do not change PIP and PIP_2 hydrolysis induced by α-latrotoxin

We next investigated whether the effects of Cd^{2+} and La^{3+} on neurotransmitter release triggered by α-latrotoxin correlate with PIP and PIP_2 hydrolysis. PIP and PIP_2 hydrolysis induced by α-latrotoxin in synaptosomes was not inhibited but was even enhanced slightly in the presence of either Cd^{2+} or La^{3+} (Figure 13). Since Cd^{2+} and La^{3+} have opposite effects on α-latrotoxin action, this result confirms the conclusion obtained from experiments with the α-latrotoxin mutant Ltx^{N4C}, that receptor binding and activation of PIP and PIP_2 hydrolysis by α-latrotoxin are not sufficient for stimulating exocytosis.

Discussion

α-Latrotoxin triggers neurotransmitter release in the presence and absence of Ca^{2+}. It bypasses the normal Ca^{2+}-dependent pathways for stimulating release (Gorio *et al.*, 1978; Ceccarelli and Hurlbut, 1980). Thus, α-latrotoxin represents a tool for unraveling the mechanisms of exocytosis; understanding how α-latrotoxin works would give fresh insight into synaptic neurotransmitter release. Two putative receptors for α-latrotoxin are known: neurexin Iα interacts with α-latrotoxin only in the presence of Ca^{2+} and is a neuronal cell-surface receptor (reviewed in Missler and Südhof, 1998). CIRL/latrophilin 1 (CL1) binds to α-latrotoxin Ca^{2+}-independently and is a G-protein-linked receptor (Krasnoperov *et al.*, 1997; Lelianova *et al.*, 1997). In neurexin Iα knockout mice, α-latrotoxin stimulates neurotransmitter release in the absence of Ca^{2+} but is only partially active in the presence of Ca^{2+} (Geppert *et al.*, 1998). Accordingly, neurexin Iα may serve as a co-receptor for α-latrotoxin which is not essential for neurotransmitter release triggered by α-latrotoxin but enhances the amount of release in the presence of Ca^{2+}.

The discovery of CL1 as a G-protein-coupled receptor for α-latrotoxin raised the exciting prospect that activation

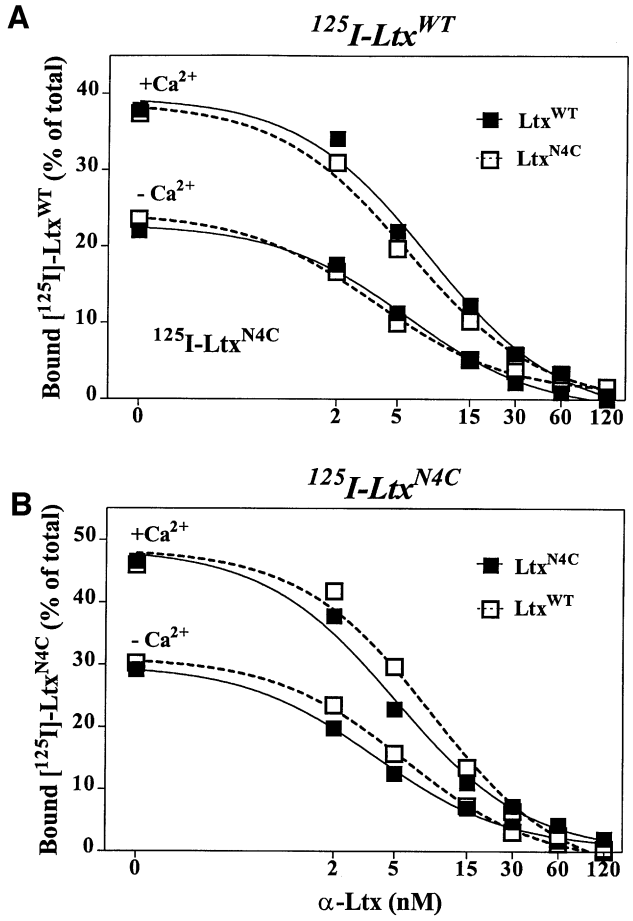

Fig. 10. High-affinity binding of Ltx^{WT} and Ltx^{N4C} to brain membranes. [^{125}I]-labeled Ltx^{WT} and Ltx^{N4C} (0.1 nM) were bound to mouse brain membranes. (**A**) Displacement of [^{125}I]-Ltx^{WT} or (**B**) of [^{125}I]-Ltx^{N4C} by unlabeled Ltx^{WT} and Ltx^{N4C} at the indicated concentrations was measured. Note that Ltx^{WT} and Ltx^{N4C} exhibit similar affinities and completely compete with each other in both combinations. The data are corrected for non-specific binding observed in the presence of 100 nM unlabeled α-latrotoxin.

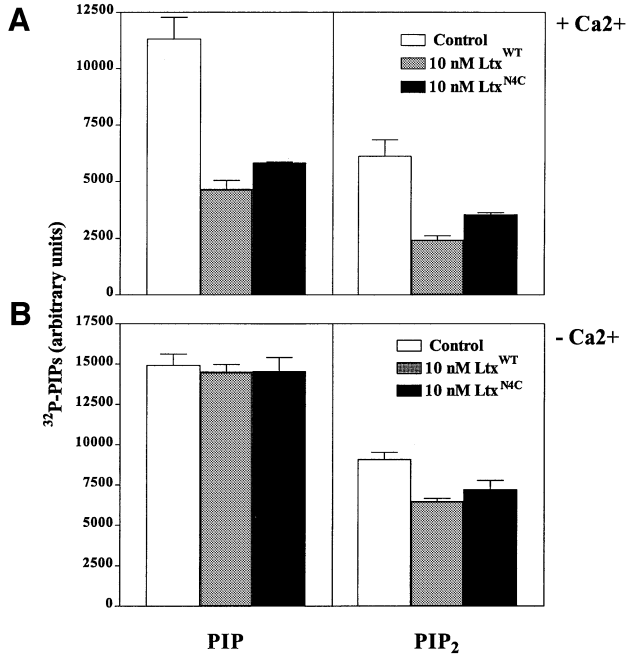

Fig. 11. Quantitation of phosphatidylinositolphosphates (PIP and PIP_2) in synaptosomes stimulated by wild-type (Ltx^{WT}) and mutant α-latrotoxin (Ltx^{N4C}), with and without Ca^{2+}. ^{32}P-labeled synaptosomes were stimulated with control buffer, Ltx^{WT} or Ltx^{N4C} prior to phospholipid extraction. Extracted phospholipids were analyzed by thin-layer chromatography and quantified using PhosphorImager detection (Khvotchev and Südhof, 1998). Experiments were performed in Ca^{2+}-containing buffer (top) or Ca^{2+}-free buffer (bottom). Data shown are from a single representative experiment performed in triplicate.

of a G-protein pathway may initiate exocytosis without Ca^{2+}. This would indicate a direct stimulation of release by G-protein-linked receptors and their ligands, opening up interesting new possibilities for synaptic function. As described here, however, the mechanism of action of α-latrotoxin is probably very different from what had been thought previously. We have used two independent approaches to show this, first with recombinant wild-type and mutant α-latrotoxins, and secondly, modulation of the α-latrotoxin response by Cd^{2+} and La^{3+}. Our data demonstrate that with one particular mutation (Ltx^{N4C}) or in the presence of La^{3+}, α-latrotoxin binds to receptors and stimulates nerve-terminal PIP and PIP_2 hydrolysis without inducing neurotransmitter release. This overall observation reveals that receptor binding is only a preliminary step in α-latrotoxin action.

In our study, we first produced recombinant α-latrotoxin to explore the mechanism of action of α-latrotoxin and the role of the toxin receptors in triggering release. Our recombinant α-latrotoxin (called Ltx^{WT}) was truncated after the ankyrin-like repeats (Figure 1) and was fully active in all tests used. Ltx^{WT} efficiently triggered glutamate, GABA and norepinephrine release from synaptosomes at nanomolar concentrations in a manner indistinguishable from that of native α-latrotoxin (Figures 3 and 4), making this the first description of active recombinant α-latrotoxin. Ltx^{WT} bound tightly to both receptors (CL1 and neurexins) with high affinity, and binding was sensitive to disulfide bond reduction (Figures 5, 9 and 10). Furthermore, Ltx^{WT} stimulated PIP and PIP_2 hydrolysis in synaptosomes (Figure 11), confirming in nerve terminals previous data from PC12 cells that α-latrotoxin stimulates phospholipase C (Vicentini and Meldolesi, 1984). A low-molecular-weight peptide was shown to co-purify with α-latrotoxin and speculated to be important for its biological activity (Kiyatkin *et al.*, 1992; Grishin *et al.*, 1993). Since recombinant α-latrotoxin stimulates neurotransmitter release without the low-molecular-weight peptide, the peptide is not required for this activity.

In a second step, we generated mutant α-latrotoxins in addition to the wild-type toxin Ltx^{WT}. The first three mutants studied, Ltx^{C14S}, Ltx^{C71S} and Ltx^{C393S}, contained cysteine-to-serine substitutions in the N-terminal domain of α-latrotoxin (Figure 1). These substitutions target the only three cysteine residues that are conserved in the other latrotoxins (Dulubova *et al.*, 1996). All cysteine substitutions abolished the ability of α-latrotoxin to trigger release (Figure 6). This result extends previous observations that α-latrotoxin requires disulfide bonds for activity and for receptor binding (Davletov *et al.*, 1996; Figure 5). It seems likely that at least some of the mutated cysteines are involved in disulfide bonds, and that the

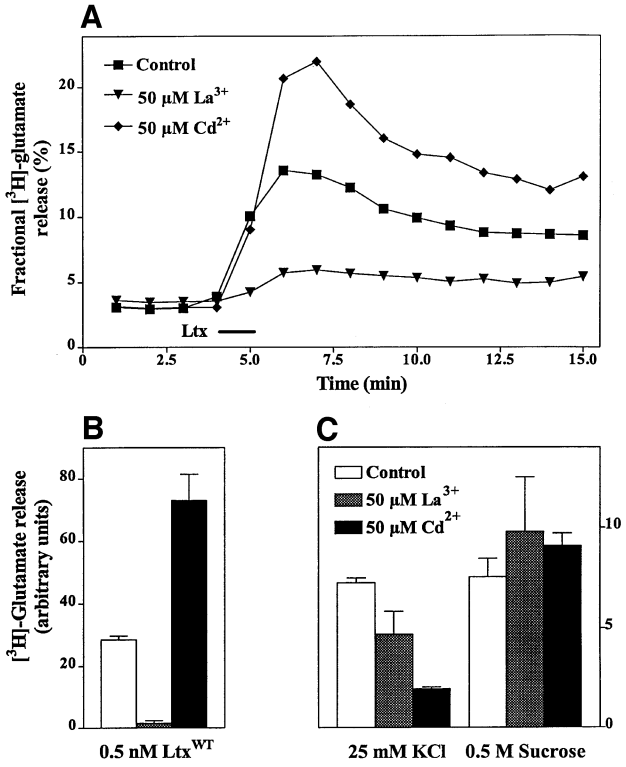

Fig. 12. La^{3+} inhibits and Cd^{2+} enhances the effects of α-latrotoxin. (**A**) Representative experiment. Synaptosomes loaded with ^{3}H-glutamate were stimulated with wild-type α-latrotoxin in control buffer or buffer containing 50 μM La^{3+} or Cd^{2+}. (**B**) Summary graph of α-latrotoxin experiments. ^{3}H-glutamate release was triggered by 0.5 nM α-latrotoxin with or without 50 μM La^{3+} or 50 μM Cd^{2+}, and total release was quantified as described in the legend to Figure 3. (**C**) Summary graph of control experiments. ^{3}H-glutamate release was induced by 25 mM KCl or 0.5 M sucrose in control buffer or buffers containing 50 μM La^{3+} or 50 μM Cd^{2+}, and total release was measured.

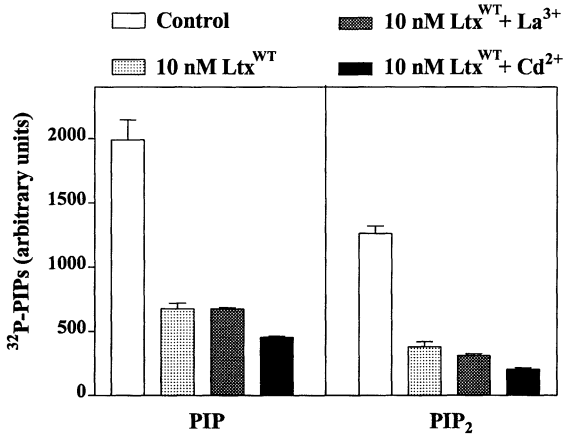

Fig. 13. Effect of La^{3+} or Cd^{2+} on PIP and PIP_2 hydrolysis stimulated by α-latrotoxin. ^{32}P-labeled synaptosomes were stimulated by α-latrotoxin or control buffer with or without 50 μM La^{3+} or 50 μM Cd^{2+}. After stimulation, PIP and PIP_2 were extracted from the synaptosomes and analyzed by thin-layer chromatography and PhosphorImager quantitation. Data shown are from a single representative experiment performed in triplicate.

cysteine mutants are inactive because they are unable to bind to receptors. This would suggest that the N-terminal domain of α-latrotoxin is involved in receptor binding.

We also studied a fourth α-latrotoxin mutant called Ltx^{N4C}. This mutant yielded unexpected results. Ltx^{N4C} contains an insertion of four amino acids between the N-terminal domain and the ankyrin repeat region to introduce a thrombin cleavage site into α-latrotoxin. The thrombin cleavage site was intended to allow separate evaluation of the N-terminal domain and the ankyrin repeat region. However, thrombin did not cleave the mutant toxin, presumably because the site is buried in the tertiary structure. Ltx^{N4C} was nevertheless very informative. Although Ltx^{N4C} was unable to trigger exocytosis in synaptosomes at concentrations of up to 10 nM, it was otherwise indistinguishable from Ltx^{WT}. Ltx^{WT} and Ltx^{N4C} bound to neurexins and CL1 with similar tightness, exhibited identical receptor-binding affinities and competed fully with each other for binding (Figures 8–10). Furthermore, Ltx^{N4C} evoked PIP and PIP_2 hydrolysis in nerve terminals to the same extent as Ltx^{WT} (Figure 11). These results show that binding of α-latrotoxin to its receptors and activation of signal transduction cascades do not directly cause exocytosis.

The fact that Ltx^{N4C} cannot trigger release but binds to CL1 and to neurexin Iα with a normal affinity, and activates PIP and PIP_2 hydrolysis, demonstrates that receptor binding and activation of phospholipase C are not sufficient to stimulate exocytosis. We confirmed this conclusion by a second, independent approach. We found that Cd^{2+} and La^{3+} have opposite effects on neurotransmitter release triggered by wild-type α-latrotoxin, with Cd^{2+} enhancing release and La^{3+} blocking it. Nevertheless, in agreement with the conclusion derived from the Ltx^{N4C} data, neither of La^{3+} and Cd^{2+} changed the stimulation of PIP and PIP_2 hydrolysis by α-latrotoxin. Our conclusion is in direct contradiction to a recent study (Davletov *et al.*, 1998), which found a critical role for phospholipase C in α-latrotoxin action. However, in that study only a single transmitter was investigated with a single drug of undefined specificity, making the study difficult to interpret.

Several hypotheses could be advanced to explain how α-latrotoxin acts in view of our result that α-latrotoxin binding to receptors and activation of PIP and PIP_2 hydrolysis are insufficient for initiating exocytosis. (i) The most plausible model is that the two α-latrotoxin receptors serve to recruit α-latrotoxin to its point of action, but are not directly involved in triggering exocytosis. In addition to our data, this model is supported by the finding that α-latrotoxin action is impaired but not abolished in neurexin Iα knockouts (Geppert *et al.*, 1998). Furthermore, this model would offer an attractive explanation for the puzzling identification of two high-affinity receptors for α-latrotoxin, since two independent receptors would increase the efficiency of toxin recruitment.

(ii) A second hypothesis is that there is a third 'real' α-latrotoxin receptor in brain which selectively binds Ltx^{WT} but not Ltx^{N4C}. This hypothesis predicts that neurexins and CL1 are unimportant for α-latrotoxin action. Three lines of evidence argue against this hypothesis: (a) Active Ltx^{WT} and inactive Ltx^{N4C} fully compete with each other for binding sites in brain and exhibit similar binding affinities. Thus any binding site that is specific for Ltx^{WT} would have to be of very low abundance, whereas in brain synapses are abundant and all synapses

are sensitive to α-latrotoxin. (b) The knockout study mentioned above shows that neurexin Iα contributes to α-latrotoxin action. (c) Transfection experiments have shown that CL1 acts as a functional receptor in PC12 cells (Krasnoperov *et al*., 1997). These three lines of evidence effectively rule out this hypothesis.

(iii) A third potential explanation is that Ltx^{N4C} binding to neurexin Iα and CL1 activates only PIP and PIP_2 hydrolysis, and not the mythical receptor activity involved in exocytosis. This model cannot be excluded by our current data, but is biologically implausible. There is no precedent for a receptor that performs multiple activities which are selectively evoked by different ligands. For example, many G-protein linked receptors stimulate several downstream pathways, but in a given cell all ligands always stimulate the different pathways to similar extents. In addition, this model is difficult to reconcile with the finding of two distinct receptors with similar affinities, both of which appear to be important.

Viewed together, it is most likely that α-latrotoxin first binds to neurexins and CL1 as receptors and then stimulates exocytosis in a second, independent step. What is the nature of this second step? It is tempting to speculate that α-latrotoxin translocates into the cytosol after receptor binding and then activates exocytosis intracellularly. This speculation suggests that α-latrotoxin acts in a manner similar to those of botulinum and tetanus toxins, which are also internalized in nerve terminals before poisoning them by an intracellular action. An alternative speculation that is equally plausible is that α-latrotoxin, after being recruited to the synaptic plasma membrane, binds to a downstream effector protein. Similar mechanisms operate in receptors for TGFβ (Massague, 1996), so there is also a biological precedent for this model. Future experiments will have to address these interesting possibilities.

Materials and methods

Construction of α-latrotoxin baculovirus expression vectors

Resequencing of the α-latrotoxin cDNA from *Latrodectus mactans* (pRLtx4; Kiyatkin *et al*., 1990) revealed a series of changes resulting in multiple amino acid substitutions (submitted as revised sequence to DDBJ/EMBL/GenBank, accession No. AF069521). The following constructs were made.

(A) Ltx-6 (N- and C-terminally His_8-tagged α-latrotoxin [E^1-F^{1177}] with a signal peptide from baculovirus acidic glycoprotein gp67). PCR with pRLtx4 as template and oligonucleotides #73 and #69 (sequences: CACGTCGACGAAGGAGAAGATTTAACTTT AGAGGAA, and CCG-CTCGAGCTAATGGTGGTGGTGATGATGATGGTGAAATTTTCCG-CTTTTTGTTTTCAAATAT) was used to amplify the coding region of α-latrotoxin (residues 1–1177) with additions of a 5′ *Sal*I site and 3′ eight-histidine sequence followed by a stop codon and an *Xho*I site. The 3.56 kb PCR product was subcloned into the *Sal*I/*Xho*I sites of pFastBac1 (Gibco-BRL). The 40-residue signal sequence of baculovirus acidic glycoprotein gp67 was obtained by PCR from the plasmid pAcGP67B (Pharmingen) using oligonucleotides #71 and #74 (sequences: TCGCGGTCCGACCACCATGCTACTAGTAAATCAGTCA and CAC-GTCGACGCTAGCATCCGC CGCAAAGGCAGAATG) and cloned into the *Rsr*II (*Csp*I) and *Sal*I sites of the intermediate plasmid constructed in the first step via restriction sites incorporated into oligonucleotides #71 and #74. The resulting plasmid was cleaved by *Nhe*I and *Sal*I (unique sites located between the signal peptide and the N-terminus of α-latrotoxin), and an eight-histidine sequence was inserted at this position by ligation of an annealed oligonucleotide duplex (sequences #75 = CTAGCCACCATCA TCATCACCACCACCATG and #76 = TCGACA-TGGTGGTGGTGATGATGATGGTGG). The final plasmid, pFastBAC–Ltx6, encodes the GP67 signal peptide followed by α-latrotoxin residues 1–1177 flanked on both ends by octahistidine sequences.

(B) Ltx-8 [Ltx-6 with insertion of four amino acids (VPRG) between L^{428} and S^{429}]. pRLtx4 was amplified with two pairs of oligonucleotides (#97 = GGTTCCGGATCCACCCAGACATATGTC and #98 = TCCCCGGGGTACTAGGTATGGTGTGAT, to give a 0.63 kb fragment; and #99 = GTACCCCGGGGATCA AATTTCATCGAC and #100 = GTCCTTAAGCCTGCGCTGAGCAGCCAA, to give a 0.39 kb product). The combined PCR products were reamplified with oligonucleotides #97 and #100. The final PCR product (1.01 kb) was digested with *Nde*I and *Blp*I, which have unique sites in the α-latrotoxin cDNA sequence, and cloned into the same sites of pFastBAC–Ltx6 to give pFastBAC–Ltx8. This plasmid contains a unique *Sma*I site in the four residues introduced into the cDNA by oligonucleotides #98 and #99.

(C) Ltx-9, Ltx-10 and Ltx-11 (Ltx-6 with cysteine-to-serine substitutions in C^{13}, C^{71} and C^{393}, respectively). (i) Ltx9: PCR was performed on pFastBAC-Ltx6 with oligonucleotides #71 and #103 (sequence GAGTTCTAATTCTGAGCTTATTTCTGCTTTTTC) (153 pb product) and on pRLtx4 with oligonucleotides #104 (sequence GAAAAAGCA-GAAATAAGCTCAGAATTAGAACTC) and #102 (sequence GTCG-AATTCCTTAAAACCTACAGCTGAATCAGGATT) (1.37 kb product). PCR products were reamplified with oligonucleotides #71 and #102 to yield a 1.53 kb final product which was digested with *Rsr*II and *Eco*RI and cloned into the same sites of pFastBAC–Ltx6 to yield pFastBAC–Ltx9. (ii) Ltx10 was constructed in a similar manner to Ltx9 except that the initial PCRs were performed with oligonucleotides #71 versus #105 (sequence GAAAGGTAAGTCACTGCTACCCAACAA CGTCTG) (product 0.40 kb), and a second PCR with oligonucleotides #106 (sequence CAGACGTTGTTGGGTAGCAGTGACTTACCTTTC) versus #102 (product 1.18 kb). (iii) Ltx11: PCRs were performed on pRLtx4 with oligonucleotides #97 (see above) and #107 (sequence CATTCT-GAGAGTAGGACTTGCTTTCTCT CTCAC) (product 0.51 kb), and with #108 (sequence GTGAGAGAGAAAGCAAGTCCTACTCTCAG AATG) and #102 (product 0.215 kb). PCR products were reamplified with oligonucleotides #97 and #102, and the final product (0.69 kb) was digested with *Nde*I and *Eco*RI and cloned into the same site of pFastBAC–Ltx6, yielding pFastBAC–Ltx11.

Baculovirus expression of recombinant proteins

This was performed in Sf9 and High-five cells with the Bac-to-Bac Baculovirus Expression System (Gibco-BRL) according to the manufacturer's recommendations. α-latrotoxin expression was monitored by SDS–PAGE of TCA-precipitated supernatant from bacmid-transfected Sf9 cells 96 h after transfection, followed by immunoblotting with an antibody to α-latrotoxin (X751). The primary recombinant viral stock was amplified on Sf9 cells with a MOI ~1 and harvested 5 days after infection to yield secondary viral stock (stored at 4°C), which was amplified into tertiary viral stock in the same manner. Tertiary viral stock was used for recombinant protein expression using a shaking culture of High-five cells in ExCell 405 medium (JRH Biosciense) with a cell density of $\sim 1.2 \times 10^6$ at the moment of infection and an MOI ~0.1.

Purification of recombinant and native α-latrotoxin

For recombinant α-latrotoxin purification, insect cell cultures (typically 10 l) were chilled for 30 min to 4°C 72–75 h after infection, protease inhibitors were added (final approximate concentrations: 100 mg/l PMSF, 1 mg/l leupeptin, 1 mg/l pepstatin A, 2 mg/l aprotinin, 0.5 mM EDTA), cells were removed by centrifugation in a Sorvall RC5C centrifuge (5000 *g* for 15 min at 4°C), and the supernatant was filtered through 0.22 μm PS filters (Corning). The filtrate was concentrated and dialyzed against buffer A (10 mM NaCl, 5 mM EDTA, 25 mM Tris–HCl pH 8.0) to ~7% of the starting volume with a Millipore concentrating tangential flow system, and clarified by centrifugation (50 000 *g* for 30 min at 4°C). The supernatant was loaded onto a 50 ml Fast-Flow DEAE–Sepharose column (Pharmacia) preequilibrated with buffer A. The column was washed with 0.5 l buffer A without EDTA, and eluted with 250 ml of buffer B (0.5 M NaCl, 25 mM Tris–HCl pH 8.0). The eluent containing α-latrotoxin was loaded onto a 12 ml Ni^{2+}–NTA column (Qiagen) preequilibrated with buffer B. The column was washed with 120 ml buffer B and eluted with 50 ml of 20 mM imidazole, 0.5 M NaCl, 25 mM Tris–HCl pH 8.0, followed by the same buffer containing 50 mM imidazole, 0.2 M imidazole and 0.9 M imidazole. α-latrotoxin was eluted from the column by 0.2 M imidazole in virtually homogeneous form (Figure 2), concentrated to 20 ml under buffer exchange (new buffer = buffer C: 0.3 M NaCl, 0.1 M $NaHCO_3$ pH 8.5) in an Amicon ultrafiltration system. Approximately 1 mg Ltx^{WT} and Ltx^{N4C} were obtained per liter of culture. The cysteine mutants expressed at ~10-fold lower levels. For long-term storage at –80°C, glycerol was added to a final concentration of 10%. Reduced α-latrotoxin was obtained by

incubation in buffer C with 5% β-mercaptoethanol at room temperature for 1 h and subsequent extensive dialysis against buffer C in the Amicon ultrafiltration system at 4°C. Native α-latrotoxin purified from black widow spider venom was a gift from Dr A.G.Petrenko (New York University Medical Center). The concentrations of recombinant and native toxins were determined on the same gel by SDS–PAGE and Coomassie Blue staining or different amounts of toxins, with bovine serum albumin (BSA) as a standard.

Affinity chromatography on immobilized α-latrotoxins

Ten milligrams of purified Ltx^{WT} or Ltx^{N4C} (reduced or non-reduced) were crosslinked to 1 ml of activated CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's protocol. Rat brains (100 g, Pel-Freeze) were homogenized in 0.4 l buffer D (80 mM NaCl, 4 mM KCl, 5 mM $CaCl_2$, 2.5 mM EDTA, 20 mM $NaHCO_3$, 20 mM Tris–HCl pH 7.5, 0.1 g/l PMSF, 1 mg/l leupeptin, 1 mg/l pepstatin A, 2 mg/l aprotinin). CHAPS was added to a final concentration of 2% (w/v) and dissolved under stirring at 4°C for 1 h. Insoluble material was removed by low-speed (5000 *g*) and high-speed (80 000 *g*) centrifugations. The supernatant (~380 ml) was diluted with an equal volume of buffer D and chromatographed on the affinity columns (Ltx-6, Ltx-6 reduced, Ltx-8). Columns were washed with buffer D containing 1% CHAPS and sequentially eluted with: (i) 250 mM NaCl, 4 mM KCl, 5 mM $CaCl_2$, 2.5 mM EDTA, 20 mM $NaHCO_3$, 20 mM Tris–HCl pH 7.5, 1% CHAPS; (ii) the same buffer containing 1.5 M NaCl; (iii) the same buffer containing 1.5 M NaCl and 25 mM EDTA. Samples were analyzed by SDS–PAGE, Coomassie R-250 Blue staining and immunoblotting. For amino acid sequencing, CL1 was separated on SDS gel, blotted and digested with trypsin (Hata *et al*., 1993). Sequences were obtained after high-pressure liquid chromatography purification of tryptic fragments on an ABI amino acid sequencer.

Binding measurements to brain membranes

Fresh forebrains from adult mice were homogenized in ice-cold binding buffer (0.1 M Tris–HCl pH 7.5, 2 mM $MgSO_4$, 1 mM EDTA) containing 10 mM EDTA and 1 mM PMSF. Homogenates were cleared by centrifugation (1000 *g* for 10 min), and membranes were prepared by centrifugation at 14 000 *g* for 15 min. Membranes were washed once in binding buffer and resuspended at 1.5 mg/ml protein in binding buffer containing 0.1% BSA. Ltx^{WT} and Ltx^{N4C} were labeled with ^{125}I as described in Petrenko *et al*. (1990). For binding experiments, a 0.15 ml reaction contained 0.15 mg brain membrane protein, 0.1% BSA, 0.1 nM ^{125}I-Ltx^{WT} or ^{125}I-Ltx^{N4C}, and the indicated concentrations of unlabeled Ltx^{WT} or Ltx^{N4C}. All reactions were carried out in binding buffer containing 1.33 mM Ca^{2+} for Ca^{2+}-dependent binding or 1.33 mM EGTA for Ca^{2+}-independent binding. Reactions were incubated in triplicate for 20 min at room temperature and terminated by 5 min centrifugation at 12 000 *g* in a table-top centrifuge, and the pellet was washed once with binding buffers. Pellets were counted in an LKB 1272 γ-counter. Data were corrected for non-specific binding determined in the presence of a 1000-fold excess of unlabeled α-latrotoxin and expressed as a percentage of total radioactivity employed.

Antibodies and immunoblot analysis

Most antibodies have been described previously (Rosahl *et al*., 1995; Geppert *et al*., 1998) and used without further purification. To raise antibodies against CL1, we first amplified the full-length CL1 cDNA by PCR using oligonucleotide #117 (sequence TTGACGCGTACCAC-CATGACCAACTTCGCAGTGCTCATGGC TCAC) and #118 (sequence TTGACGCGTACCACCATGACCAACTTCGCAGTGCTCATGGCT-CAC) with 3 ng Quick-Clone rat-brain cDNA (Clontech) as a template and a mixture of Taq-polymerase (Perkin-Elmer) and Taq Extender (Strata-gene). The 4.45 kb product was cloned into the *Mlu*I and *Cla*I sites of pCMV5 to yield pCMVCIRL, and was used as a template for PCR with oligonucleotides #124 (GCTCTCGAGGAGAATGCA-AACTATGGGCGC) and #125 (AGCAAGCTTCTAATTGGAGGCTG-AGCGCTTGTC) that amplify the extracellular CL1 sequences corresponding to residues E^{59}–N^{313} (Krasnoperov *et al*., 1997). The resulting 0.76 kb product was cloned into the *Xho*I and *Hin*dIII sites of pGEX–KG to yield pGEXCIRLABS1, and into the *Sal*I and *Hin*dIII sites of pMAL-C2 to yield pMALCIRLABS1. GST-CL1 fusion protein was purified from SURE cells transformed with pBEXCIRLABS1 and used to raise antibodies (numbers U047 and U048).

Preparation of synaptosomes

The neocortex was dissected from adult mouse brain and homogenized at 900 r.p.m. in 0.32 M sucrose, 5 mM HEPES, 0.1 mM EDTA at pH 7.4. Homogenate was cleared by low-speed centrifugation at 1000 *g* for 10 min and centrifuged at 14 500 *g* for 20 min to obtain the crude synaptosome fraction (P_2). For glutamate release measurements, the P_2 pellet from one neocortex was resuspended in 3 ml ice-cold gassed (95% O_2/5% CO_2) Krebs' bicarbonate (composition in mM: 118 NaCl, 3.5 KCl, 1.25 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 5 HEPES–NaOH pH 7.4, 11.5 glucose), and used within 2–3 h. For phospholipid measurements, the P_2 pellet was resuspended in 8.5% (v/v) Percoll solution containing 0.25 M sucrose, 5 mM HEPES, 0.1 mM EDTA pH 7.4, and layered on the top of 12%/20% Percoll step gradient in the same buffer. After centrifugation at 18 000 *g* for 30 min, synaptosomes were recovered from the 12%/20% Percoll interface. Percoll was removed by addition of 30 vol. of homogenization buffer and centrifugation at 18 000 *g* for 20 min. Pelleted synaptosomes were resuspended in 3 ml phosphate-free Krebs' bicarbonate buffer and used immediately. In some experiments $CaCl_2$ was substituted with 0.1 mM EGTA.

Measurements of PIP and PIP_2 in ^{32}P-labeled synaptosomes

Synaptosomes were incubated at 35°C in a 95% O_2/5% CO_2 atmosphere with ^{32}P-orthophosphate (1 mCi/ml) for 1 h (Khvotchev and Südhof, 1998). After incubation, labeled synaptosomes were diluted with an equal volume of Krebs' bicarbonate buffer containing wild-type or mutant recombinant α-latrotoxin with or without La^{3+} or Cd^{2+} ions, and incubated for additional 10 min under the same conditions. Reactions were stopped by chilling on ice and lipids were extracted by addition of 3.75 vol. of chloroform:methanol:concentrated HCl (100:200:1). After 10 min on ice, 10 μg of carrier phosphatidyl ethanolamine were added and phase partitioning was induced by addition of 1.25 vol. of chloroform and of 0.1 N HCl. The organic phase containing phospholipids was washed twice with cold acidified 50% methanol. Equal volumes of lipid extracts (up to 50 μl) were loaded onto TLC plates (6 nm, Whatman) with phospholipid standards (10–20 μg per lane) and developed in a solvent system of *n*-propanol, water and concentrated NH_4OH (65:20:15). Labeled phospholipids were visualized by autoradiography and marker lipids were stained with iodine vapors. ^{32}P incorporation was quantified using a PhosphorImager (Molecular Dynamics). Each treatment was performed in triplicate in several independent experiments.

Measurements of ^3H-glutamate, ^3H-GABA and ^3H-norepinephrine release from synaptosomes

These were carried out essentially as described previously (Geppert *et al*., 1998; Khvotchev and Südhof, 1998). Synaptosomes were incubated with 140 nM ^3H-glutamate (specific activity 15 Ci/mmol), 130 nM ^3H-norepinephrine (46.8 Ci/mmol) or 66 nM ^3H-GABA (90 Ci/mmol) for 5 min. In buffers for the norepinephrine experiments, 0.4 mM ascorbic acid, 30 μM EDTA and 10 μM pargyline were added to prevent metabolism of norepinephrine. Synaptosomes (0.1 ml) loaded with [^3H]-labeled neurotransmitters were trapped on glass-fiber filters (GF/B, Whatman), covered with 50 μl of a 50% Sephadex G-25 slurry and superfused at 0.8 ml/min with Kreb's bicarbonate buffer at 33°C under continuous aeration with 95% O_2/5% CO_2. For release experiments under Ca^{2+}-free conditions, synaptosomes were superfused with Ca^{2+}-free Kreb's bicarbonate buffer containing 0.1 mM EGTA instead of 1.25 mM $CaCl_2$. In some experiments, 50 μM La^{2+} or Cd^{2+} were added to the superfusion buffer. After 12 min of washing, two 1 min fractions were obtained to determine baseline release, and neurotransmitter release was stimulated by applications of the following agents in Ca^{2+}-containing and Ca^{2+}-free buffers: (i) 25 mM KCl for 30 s (to standardize the response of the synaptosomes); (ii) various concentrations of recombinant α-latrotoxins (Ltx^{WT}, Ltx^{C14S}, Ltx^{C71S}, Ltx^{C393S} and Ltx^{N4C}) or native α-latrotoxin purified from black widow spider venom applied for 1 min or 10 min as indicated; and (iii) 0.5 M sucrose for 30 s (in Ca^{2+}-free Kreb's bicarbonate buffer only). Stimulations were performed by rapid switching of the superfusion lines between regular and stimulation buffers. The amounts of ^3H-glutamate, ^3H-norepinephrine or ^3H-GABA secreted into the superfusate and retained in the synaptosomes at the end of the experiment were quantified by liquid scintillation counting. Fractional neurotransmitter release was calculated by dividing the amount of neurotransmitter released during a time interval by the amount of transmitter remaining in the synaptosomes at that time. To obtain the total GABA, glutamate and norepinephrine release induced by a given stimulus, the evoked release above baseline was integrated over the time of the experiment.

Acknowledgements

We wish to thank Dr C.Slaughter, Mr S.Afendis and Ms C.Moomaw for protein sequencing; Dr A.G.Petrenko and Dr V.Krasnoperov for the gift

of α-latrotoxin purified from black widow spider venom; and Drs M.S.Brown and J.L.Goldstein for invaluable discussions. This study was supported by NIH grant MH50824 (to T.C.S.).

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Received June 1, 1998; revised August 19, 1998; accepted September 1, 1998