The α-Latrotoxin Mutant LTX^{N4C} Enhances Spontaneous and Evoked Transmitter Release in CA3 Pyramidal Neurons

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α-Latrotoxin (LTX) stimulates vesicular exocytosis by at least two mechanisms that include (1) receptor binding–stimulation and (2) membrane pore formation. Here, we use the toxin mutant LTX^{N4C} to selectively study the receptor-mediated actions of LTX. LTX^{N4C} binds to both LTX receptors (latrophilin and neurexin) and greatly enhances the frequency of spontaneous and miniature EPSCs recorded from CA3 pyramidal neurons in hippocampal slice cultures. The effect of LTX^{N4C} is reversible and is not attenuated by La^{3+} that is known to block LTX pores. On the other hand, LTX^{N4C} action, which requires extracellular Ca^{2+}, is inhibited by thapsigargin, a drug depleting intracellular Ca^{2+} stores, by 2-aminoethoxydiphenyl borate, a blocker of inositol(1,4,5)-trisphosphate-induced Ca^{2+} release, and by U73122, a phospholipase C inhibitor. Furthermore, measurements using a fluorescent Ca^{2+} indicator directly demonstrate that LTX^{N4C} increases presynaptic, but not dendritic, free Ca^{2+} concentration; this Ca^{2+} rise is blocked by thapsigargin, suggesting, together with electrophysiological data, that the receptor-mediated action of LTX^{N4C} involves mobilization of Ca^{2+} from intracellular stores. Finally, in contrast to wild-type LTX, which inhibits evoked synaptic transmission probably attributable to pore formation, LTX^{N4C} actually potentiates synaptic currents elicited by electrical stimulation of afferent fibers. We suggest that the mutant LTX^{N4C}, lacking the ionophore-like activity of wild-type LTX, activates a presynaptic receptor and stimulates Ca^{2+} release from intracellular stores, leading to the enhancement of synaptic vesicle exocytosis.

Key words: **α-latrotoxin; mutant; hippocampal slice culture; spontaneous synaptic transmission; evoked synaptic transmission; transmitter release; receptor; latrophilin; neurexin; intracellular Ca^{2+} stores**

Introduction

α-Latrotoxin (LTX) from the black widow spider venom acts on presynaptic nerve terminals and neurosecretory cells. The toxin strongly enhances spontaneous neurotransmitter release at the neuromuscular junction (Longenecker et al., 1970) and central synapses (Capogna et al., 1996b) and triggers secretion of transmitters and hormones in chromaffin, pancreatic, and PC12 cells (Grasso et al., 1980; Lang et al., 1998; Liu and Misler, 1998). In contrast, LTX reduces the amplitude of evoked synaptic responses (Ceccarelli and Hurlbut, 1980; Capogna et al., 1996b). This indicates that native LTX exerts distinct effects on the secretory apparatus.

To stimulate exocytosis, LTX needs to bind specific cell-surface receptors (Tzeng and Siekevitz, 1979). Two distinct high-affinity receptors for LTX have been identified: a one-transmembrane-domain neurexin Iα (NRX) (Ushkaryov et al., 1992) and a heptahelical, G-protein-coupled latrophilin (LPH) (Lelianova et al., 1997), or CIRL (Krasnoperov et al., 1997). NRX needs Ca^{2+} to bind LTX, whereas LPH binds the toxin regardless of divalent cations (Davletov et al., 1998).

Because LTX affects all types of neurotransmitters (Rosenthal and Meldolesi, 1989) and is likely to target an essential and ubiquitous component of the release machinery, the mechanism of the action of the toxin has been intensely investigated but proved difficult to elucidate (for review, see Südhof, 2001). The main problem is the complex mode of LTX action: as a result of binding to any receptor, toxin inserts into the plasma membrane and forms stable cation channels (Wanke et al., 1986; Van Renterghem et al., 2000; Volynski et al., 2000), which complicate the interpretation of results (for review, see Ushkaryov, 2002).

Therefore, it is very important to separate the receptor- and pore-mediated LTX effects. One approach is to selectively block LTX pores with La^{3+} (Ashton et al., 2001); however, La^{3+} also blocks Ca^{2+} channels and does not allow studying evoked transmitter release. A much better tool has been found serendipitously: a mutant toxin, termed LTX^{N4C}, was designed (Ichtchenko et al., 1998) containing a small insert within the domain responsible for the formation of ring-like tetramers and pores (Orlova et al., 2000). As a result, LTX^{N4C} is unable to form pores (Ashton et al., 2001) (K. E. Volynski, M. Capogna, A. C. Ashton, D. Thomson, E. V. Orlova, C. Manser, R. R. Ribchester, and Y. A. Ushkaryov, unpublished observations). Lacking the most robust LTX activity, this mutant was originally thought to be inactive, despite its binding to the LTX receptors (Ichtchenko et al., 1998; Khvotchev and Südhof, 2000). However, we found recently that LTX^{N4C} still triggers neurotransmitter release (Ashton et al., 2001) (Volynski et al., unpublished observations).

In the current paper, for the first time, we reveal the mechanism of the LTX^{N4C} action on central synaptic transmission and thereby the mechanism of the receptor-dependent action of native LTX. Our experiments indicate that, in the absence of pore

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formation, LTX^{N4C} action leads to mobilization of Ca^{2+} from intracellular stores. Drugs that deplete Ca^{2+} stores, or inhibit their stimulation at various points, block both the rise in cytosolic Ca^{2+} and the action of LTX^{N4C} on synaptic transmission, suggesting that mobilization of Ca^{2+} from internal stores is required for the action of LTX^{N4C}. The properties of LTX^{N4C}-triggered transmitter secretion indicate that the receptor-mediated mechanism constitutes an important part of LTX action, which is usually masked by the strong effects of LTX pores.

Materials and Methods

Slice cultures and electrophysiology. Organotypic hippocampal slice cultures were prepared as described by Stoppini et al. (1991). In brief, hippocampi were dissected from isolated brains of 7-d-old rat pups. Slices of 400 μm thickness were prepared with a tissue chopper in minimal essential medium (MEM), placed on the membrane of cell culture inserts (Millicell-CM; Millipore, Watford, UK), and maintained in culture at the interface with a medium containing 50% MEM, 25% horse serum, 25% HBSS, and 7.5% $NaHCO_3$, pH 7.3 with Tris.

For electrophysiological recordings, performed after 10 and 21 d *in vitro*, the slice cultures were transferred under a nylon grid in a 0.5 ml perfusion chamber mounted on the stage of an upright microscope (AxioSkop; Zeiss, Jena, Germany) and superfused at room temperature at a flow rate of 1 ml/min with an extracellular solution containing the following: 130 mM NaCl, 3.5 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 48 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 10 mM glucose, and 1 mg/ml bovine serum albumin (BSA), pH 7.4, saturated with 95% O_2 and 5% CO_2. Whole-cell patch-clamp recordings from visually identified CA3 pyramidal cells were performed using borosilicate glass capillaries, pulled to a resistance of 2–5 MΩ and filled with the following (in mM): 126 K-gluconate, 10 HEPES, 10 Na_2-phosphocreatine, 4 KCl, 4 ATP-Mg, and 0.3 GTP-Na_2, pH 7.3 with KOH.

The recorded neurons were voltage clamped to −70 mV, unless stated otherwise, and membrane currents were amplified (10 mV/pA), filtered at 2.9 KHz, and digitized at 5 KHz. The currents were acquired online using Pulse software (Heka, Lambrecht/Pfalz, Germany) and analyzed offline with MiniAnalysis (Synaptosoft, Decatur, GA) and Pulsfit (Heka) software. After bath application of glutamate receptor antagonists 6,7-dinitroquinoxaline-2,3-dione (DNQX) (20 μM) and D(−)-2-amino-5-phosphonopentanoic acid (D-AP-5) (50 μM), no synaptic currents were detected, indicating that all currents observed in the absence of the drugs were mediated by AMPA–kainate–NMDA-preferring receptors. Analysis of spontaneous synaptic events was done at the same current threshold that resulted in detection of no events in the presence of DNQX–D-AP-5 (usually corresponding to three times the root mean square of the noise, 5–10 pA, and kept constant in each experiment). Evoked EPSCs were elicited with monopolar stimuli (0.1 msec, −10 to −30 μA) delivered through an isolation unit to a second patch pipette filled with the extracellular saline and placed in the dentate gyrus, with the cell voltage clamped at −70 mV in the absence of bicuculline. The mean amplitudes of evoked EPSCs were determined by averaging 30 consecutive EPSCs before and after LTX application. Paired-pulse ratio was calculated as the mean peak amplitude of responses to the second stimulus divided by the mean peak amplitude of responses to the first stimulus. The inverse square power of the coefficient of variation (CV) of each EPSC was calculated with the following formula: $1/CV^2 = M^2/\sigma_r^2$, where M is the mean amplitude and σ_r^2 is the variance of the EPSCs. Statistical comparisons of miniature and evoked currents were performed with a two-tailed paired t test using the original values of frequency and amplitude. The amplitude of miniature EPSCs (mEPSCs) before and after drug application was compared using the Kolmogorov–Smirnov test. Numerical values are given as the mean ± SEM.

In all electrophysiological experiments, LTXs were focally applied to the recording chamber as a 10–15 μl drop of the 10–50 nM stock solution, after stopping the superfusion. Perfusion was resumed when the frequency of spontaneous events began to increase (0.5–20 min after toxin application). Such brief interruptions in perfusion had no effect in control recordings. The actual concentration of LTXs reaching the cells was estimated to be ~1 nM (Capogna et al., 1996b). When the frequency of mEPSCs or the amplitude of evoked EPSCs remained constant for >20 min after a drug–toxin application, the effect was considered nonexistent.

Biochemical methods. Recombinant latrotoxins, LTX^{WT} and LTX^{N4C} (Ichtchenko et al., 1998), were expressed in a baculovirus system as described previously (Volynski et al., 1999). The toxins were purified from the expression medium by affinity chromatography on an immobilized anti-LTX monoclonal antibody (a gift from E. V. Grishin, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia). The column was washed with 150 mM NaCl and 50 mM Tris-HCl, pH 8.2, and eluted with 1 M $MgCl_2$ and 50 mM Tris-HCl, pH 8.2. The recombinant LTXs were dialyzed against TBSM (in mM: 150 NaCl, 2.0 $MgCl_2$, and 50 Tris-HCl, pH 8.2) and concentrated in a Vivaspin 6 U (Sartorious, Epsom, UK) to ~50 nM.

For binding studies, the toxins were labeled with ^{125}I as outlined by Ushkarev and Grishin (1986). Hippocampal slices cultured for 2 weeks were dislodged from the filters, homogenized in ice-cold TBSM, and incubated for 10 min on ice with iodinated LTX^{WT} or LTX^{N4C} in TBSM supplemented with 0.5 mg/ml BSA and 2 mM $CaCl_2$ or 2 mM EGTA. Total LTX binding was determined by quickly passing the reaction mixtures through GF/F filters (Whatman, Springfield Mill, UK) and measuring the radioactivity of the filters. The specific binding was calculated by subtracting the nonspecific binding (determined in the presence of a 100-fold excess of unlabelled LTX) from the total binding; the Ca^{2+}-dependent binding was established as the difference between the binding in Ca^{2+} and that in EGTA.

To detect the LTX receptors, hippocampal slices or brain membranes (P2) were solubilized in 1 ml of ice-cold TBSM supplemented with 2 mM $CaCl_2$ and 0.7% Thesit. After a 2 hr incubation with LTX-Sepharose (Davletov et al., 1996) and washing, the bound receptors were eluted with SDS-electrophoresis sample buffer. The starting materials and eluted proteins were incubated in the sample buffer for 30 min at 37°C, separated in SDS-polyacrylamide gels, and transferred onto Immobilon membrane (Millipore); proteins were visualized using respective primary and secondary antibodies and chemiluminescent substrate (Pierce, Rockford, IL), followed by exposure to x-ray film.

The influx of $^{45}Ca^{2+}$ in hippocampal slices was determined as described by Davletov et al. (1998) and Volynski et al. (2000). Briefly, slices were incubated with 2 mM Ca^{2+} and 4 μM $^{45}Ca^{2+}$ and stimulated for 5 min with 1 nM LTX^{N4C} or LTX^{WT}, quickly washed, and transferred into scintillation fluid for measuring radioactivity uptake.

Optical methods. Slice cultures were placed in a recording chamber mounted on an Olympus Optical (Tokyo, Japan) BX50 upright microscope equipped with a confocal scanhead (Radiance 2000; Bio-Rad, Hemel Hempstead, UK). CA3 pyramidal cells were impaled with glass microelectrodes and iontophoretically injected with the calcium indicator Oregon Green 488 BAPTA-1 (Molecular Probes, Cambridge Bioscience, Cambridge, UK) as described by Emptage et al. (1999). Filled axons were visualized by confocal imaging (488 nm excitation), and sample images were taken of lengths of axon both before and 15 min after the addition of LTX^{N4C} to the recording chamber. At this time, 1 μM tetrodotoxin (TTX) was also added to the chamber to suppress action potentials. In experiments in which thapsigargin (Th) was used, the slices were treated with the drug for 20 min before the collection of control (pre-LTX^{N4C}) images and remained in the bath after the addition of LTX^{N4C}.

Source of chemicals. The following commercially available compounds were used: 2-aminoethoxydiphenylborate (2-APB), ryanodine, TTX, DNQX, D-AP-5, 1–2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1H-imidazole (SKF 96365), thapsigargin, and bicuculline (Tocris Cookson, Bristol, UK); and $CdCl_2$, $LaCl_3$, BSA (B-4287), U73122, and U73343 (Sigma-Aldrich, Dorset, UK). Antibodies against NRX and the termini of LPH were described previously (Volynski et al., 2000); rabbit polyclonal antibodies to synaptobrevin were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse serum against synaptophysin and monoclonal antibodies against SNAP-25 (synaptosome-associated protein of 25 kDa) were a kind gift from G. Lawrence (Imperial College London, London, UK); anti-rabbit and anti-mouse peroxidase-conjugated IgGs were from Sigma-Aldrich.

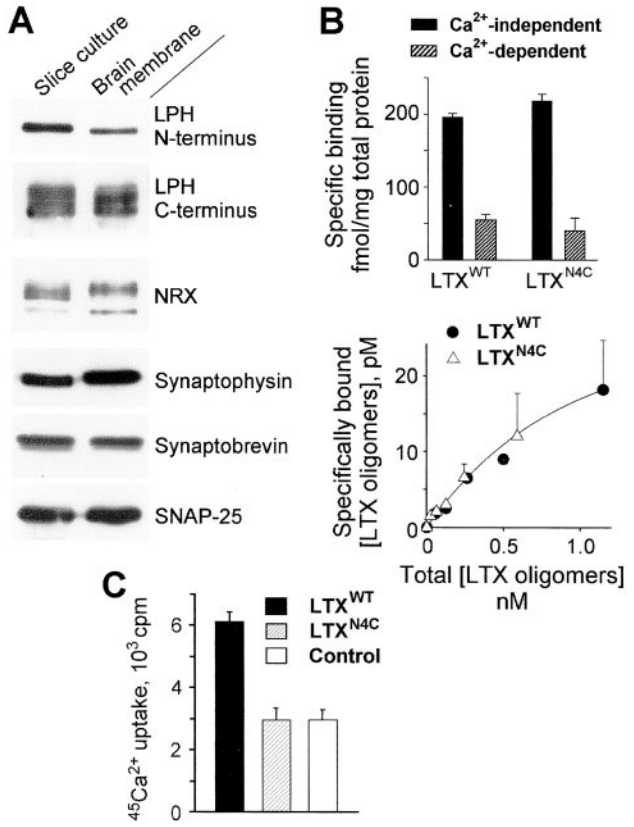

Figure 1. *A*, Immunodetection of LTX receptors in hippocampal slices. Crude brain membranes (P2) and slices were separated in 8% SDS-polyacrylamide gels either directly (0.05 mg of protein per lane) or after enrichment by LTX affinity chromatography (equivalent to 0.3 mg of starting protein per lane) and then blotted and immunostained with respective antibodies (shown on the right). *B*, Binding of LTX^{WT} and LTX^{N4C} to hippocampal slices. Top, the specific binding of 1 nM iodinated recombinant latrotoxins was determined (see Materials and Methods) in the presence of 2 mM $CaCl_2$ or 2 mM EGTA. Bottom, Dose dependence of the specific binding of LTX^{WT} and LTX^{N4C} to slices in the presence of Ca^{2+}. One homogenized slice was used per point. The data are from a typical experiment done in triplicate. *C*, LTX^{WT}, but not LTX^{N4C}, induces $^{45}Ca^{2+}$ influx in hippocampal slice cultures. One slice was used per experimental point (see Materials and Methods), and the data are the mean of two experiments done in duplicate.

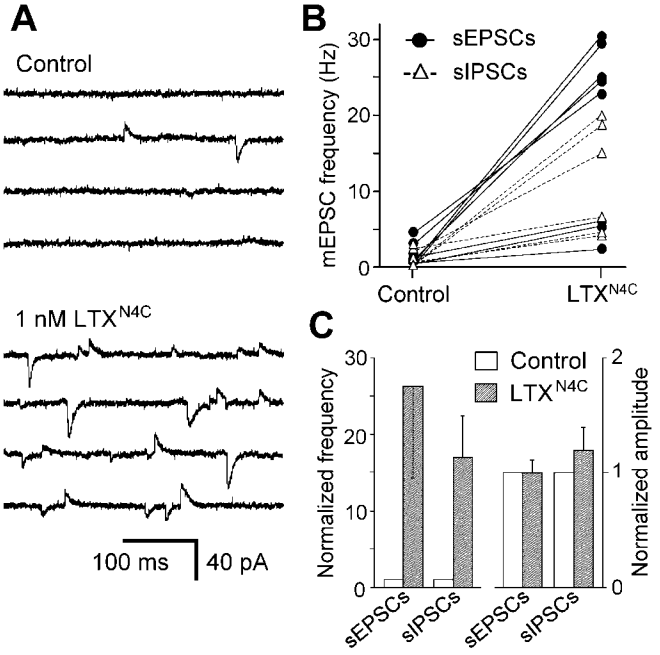

Figure 2. LTX^{N4C} increases the frequency of spontaneous synaptic currents. *A*, Continuous whole-cell recordings of sEPSCs (downward peaks) and sIPSCs (upward peaks) from a CA3 pyramidal cell in a hippocampal slice culture, before and after focal application of 1 nM LTX^{N4C}. *B*, Pooled data for all recorded cells: on average, sEPSC frequency rose from 1.4 ± 0.5 Hz in control to 18.3 ± 4.1 Hz in the presence of LTX^{N4C} ($n = 8$; $p < 0.004$), and sIPSC frequency increased from 0.8 ± 0.3 to 10.3 ± 3.8 Hz ($n = 6$; $p < 0.03$), respectively. *C*, The frequencies and amplitudes of sEPSCs and sIPSCs normalized to control values for each cell. Note that LTX^{N4C} causes no significant changes in the amplitudes.

Results

LTX^{N4C} and LTX^{WT} bind LTX receptors in hippocampal slice cultures

As a model to study the effects of mutant LTX on synaptic transmission, we chose organotypic hippocampal cultures, which combine the convenience of culture environment with accessibility of individual neurons for electrophysiological recordings but maintain the general hippocampal organization (Gähwiler et al., 1997).

Although primary hippocampal neurons contain large amounts of the LTX receptors (Malgaroli et al., 1989; Davletov et al., 1998), it was not known whether both LPH and NRX remain in these cells during long-term culture. The presence of functionally active receptors in hippocampal slice cultures was shown by Western blotting (after LTX-affinity chromatography) and compared with known presynaptic protein markers (Fig. 1*A*). All of these proteins, including both LTX receptors, were found in slice cultures, although, based on the quantification of all bands (data not shown), NRX was relatively less abundant than LPH.

To demonstrate that both LTX^{WT} and LTX^{N4C} bind similarly to hippocampal neurons under the conditions of electrophysiological recordings (see below), the recombinant toxins were iodinated and then incubated with slice cultures in the presence or absence of Ca^{2+}. As shown in Figure 1*B* (top), the two toxins displayed similar receptor affinities and numbers of binding sites. Both Ca^{2+}-dependent and Ca^{2+}-independent binding were found (Fig. 1*B*, bottom), corresponding to NRX and LPH, respectively.

We showed previously that, under the conditions in which LTX^{WT} formed massive numbers of ionic pores, LTX^{N4C} failed to cause any cation fluxes (Ashton et al., 2001) (Volynski et al., unpublished observations). Here, we extended these observations to hippocampal cultures by studying the $^{45}Ca^{2+}$ influx stimulated by the recombinant toxins. As expected, LTX^{WT} caused considerable accumulation of the radioactive tracer, whereas LTX^{N4C} induced no detectable $^{45}Ca^{2+}$ influx (Fig. 1*C*).

Combined, our findings (1) confirm the published data that the mutation does not alter the binding of LTX^{N4C} to the receptors (Ichtchenko et al., 1998) and (2) illustrate that hippocampal slice cultures are a useful model for studying the involvement of receptors in the LTX action.

LTX^{N4C} stimulates spontaneous release

We then studied the action of LTX^{N4C} on synaptic transmission in hippocampal slice cultures. At the membrane potential of −60 mV, CA3 pyramidal neurons displayed spontaneous inward synaptic currents attributable to vesicular release of glutamate from mossy and associational-commissural fibers and spontaneous outward synaptic currents attributable to exocytosis of GABA from interneurons. These inward and outward currents were mediated by AMPA and $GABA_A$ receptors, respectively, because they were abolished by 20 μM DNQX and 30 μM bicuculline, respectively ($n = 3$; data not shown). Focal application of 1 nM

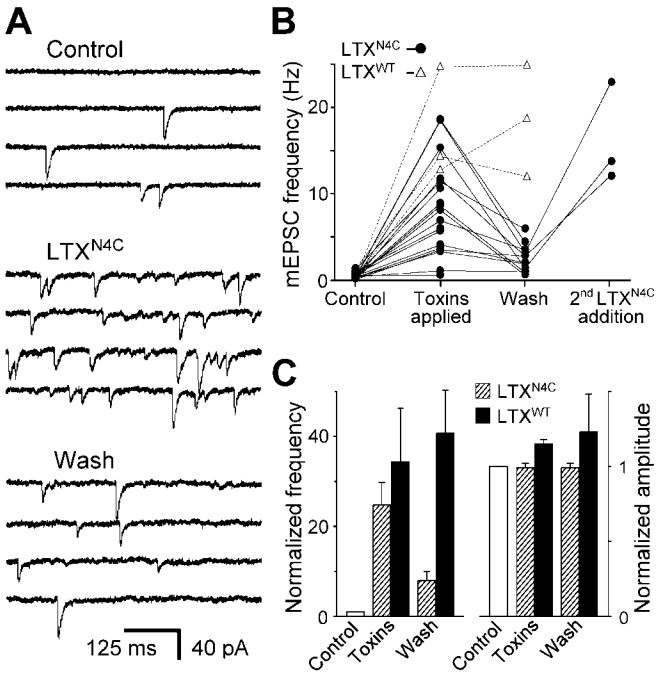

Figure 3. LTX^{N4C} increases mEPSC frequency in a reversible manner. *A*, Continuous whole-cell recordings of mEPSCs from a CA3 pyramidal cell in a slice culture in the presence of 1 μM TTX and 30 μM bicuculline. Focal application of 1 nM LTX^{N4C} reversibly increased the frequency of mEPSCs (0.4 Hz for control, 18.6 Hz for LTX^{N4C}, and 4.4 Hz for washout) but did not significantly affect their mean amplitude (18.5 pA for control, 17 pA for LTX^{N4C}, and 18.4 pA for washout). *B*, Pooled data from all recorded cells: mEPSC frequency before (control), 0.5–20 min after the application of 1 nM LTX^{N4C} or LTX^{WT}, after a 27 $\pm$ 3 min wash, and after a second addition of 1 nM mutant. On average, mEPSC frequency was 0.5 $\pm$ 0.07 Hz in control, 8.1 $\pm$ 1.3 Hz after LTX^{N4C} ($n = 18$; $p < 0.001$), and 0.7 $\pm$ 0.3 Hz before and 17.3 $\pm$ 3.7 Hz after LTX^{WT} ($n = 3$; $p < 0.01$). After superfusion, it was 2.5 $\pm$ 0.4 Hz for LTX^{N4C} ($n = 12$) and 18.6 $\pm$ 3.7 Hz for LTX^{WT} ($n = 3$). *C*, Changes in the mean mEPSC frequency and amplitude induced by the recombinant toxins, normalized to the control value for each cell. Note that the effect of LTX^{N4C}, but not LTX^{WT}, decreases after extensive bath perfusion.

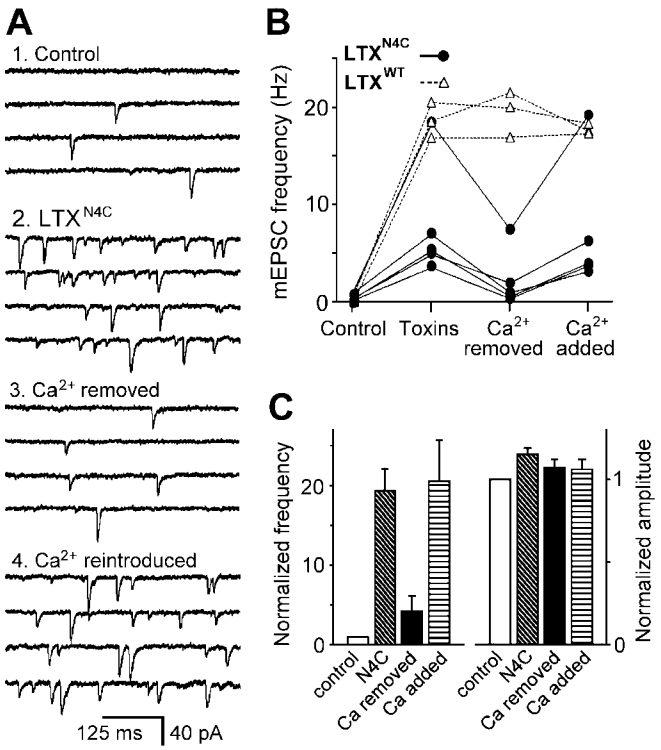

Figure 4. LTX^{N4C} increases mEPSC frequency in a Ca^{2+}_e-dependent manner. *A*, Continuous recordings of mEPSCs before (*1*) and after the application of 1 nM LTX^{N4C} (*2*) in 3 mM Ca^{2+}-containing control saline; this was then replaced with Ca^{2+}-free saline containing 1 mM EGTA (*3*) and later reintroduced again (*4*). *B*, Pooled data from all recorded cells: mEPSC frequency in control, 0.5–20 min after the application of 1 nM LTX^{N4C} or LTX^{WT} (as indicated) in the presence of Ca^{2+}, and after the removal and reintroduction of Ca^{2+}. Note that LTX^{WT}, but not LTX^{N4C}, increases mEPSC frequency, even in the absence of Ca^{2+}. *C*, Mean mEPSC frequency and amplitude normalized to control value for each cell. LTX^{N4C} increases mEPSC frequency in the presence ($n = 5$), but not the absence ($n = 5$), of Ca^{2+} and has no effect on mean mEPSC amplitude under any condition. The frequency of mEPSCs was 0.5 $\pm$ 0.2 Hz in control, 7.7 $\pm$ 2.7 Hz after LTX^{N4C} addition (19.2 $\pm$ 2.5-fold increase; $n = 5$; $p < 0.05$), 2.2 $\pm$ 1.3 Hz in EGTA-containing saline (4.2 $\pm$ 1.3-fold above control; $n = 5$; $p < 0.02$), and 7.2 $\pm$ 3 Hz after switching back to normal saline (20.2 $\pm$ 5.5-fold above control; $n = 5$).

LTX^{N4C} enhanced the rate of occurrence of both types of spontaneous events (Fig. 2). The mean frequencies of these spontaneous EPSCs (sEPSCs) and spontaneous IPSCs (sIPSCs) increased by 26.6 $\pm$ 11.9-fold ($n = 8$) and 16.9 $\pm$ 5.5-fold ($n = 6$), respectively (Fig. 2*C*). However, the mean amplitude of the events was not significantly affected (Fig. 2*C*). Thus, the mutant toxin, like the native one (Capogna et al., 1996b), presynaptically stimulates transmitter release at both glutamatergic and GABAergic hippocampal synapses.

We then showed that the recombinant toxins affect glutamatergic mEPSCs in a manner similar to that of the previously characterized native LTX (Capogna et al., 1996a,b). Spontaneous mEPSCs, mediated by AMPA–kainate glutamate receptors, were recorded in whole-cell voltage-clamped CA3 pyramidal cells at -70 mV (in the presence of TTX and bicuculline, to block action potential-evoked release and $GABA_A$ receptors, respectively). Focal applications of either LTX^{N4C} or LTX^{WT} increased the frequency of mEPSCs but did not affect their amplitudes (Fig. 3), consistent with a purely presynaptic site of action. The effect occurred with a variable latency (0.5–20 min) probably attributable to restricted diffusion of toxins within tissue and was not accompanied by any detectable change in the holding current. On average, LTX^{N4C} increased the mEPSC frequency by 23.5 $\pm$ 5-fold ($n = 18$) (Fig. 3*B*,*C*). Only in one cell did the mEPSC frequency remain unchanged for longer than 20 min after LTX^{N4C} application. LTX^{WT} was somewhat more potent and enhanced the mEPSC frequency by 34.4 $\pm$ 11.8 times ($n = 3$) (Fig. 3*B*,*C*).

One important difference between LTX^{N4C} and LTX^{WT} that we discovered here was the reversibility of the action of the mutant on washout. Indeed, the mEPSC frequency evoked by LTX^{WT} never decreased, even after a very extensive wash (Fig. 3*B*). In contrast, ~25 min superfusion dramatically reduced the increase in mEPSC frequency initially triggered by LTX^{N4C}. Was this inhibition caused by LTX^{N4C} washout, desensitization of toxin receptors, or exhaustion of the LTX-sensitive pool of vesicles? To answer this question, a new aliquot of LTX^{N4C} was added to the recording chamber after the wash, and this clearly restored the high mEPSC frequency ($n = 3$) (Fig. 3*B*). Thus, the mutant toxin can dissociate from its receptors and be removed by washing, providing additional evidence for its inability to insert itself into the membrane and form pores.

Thus, LTX^{N4C} is active at synapses and promotes spontaneous release of neurotransmitters but does not permanently insert into the presynaptic membrane, indicating that it acts via a reversible interaction with its receptors.

LTX^{N4C}-induced spontaneous release requires extracellular Ca^{2+}

We found previously that, to stimulate the receptor-dependent action, LTX requires Ca^{2+}_e (Davletov et al., 1998; Ashton et al.,

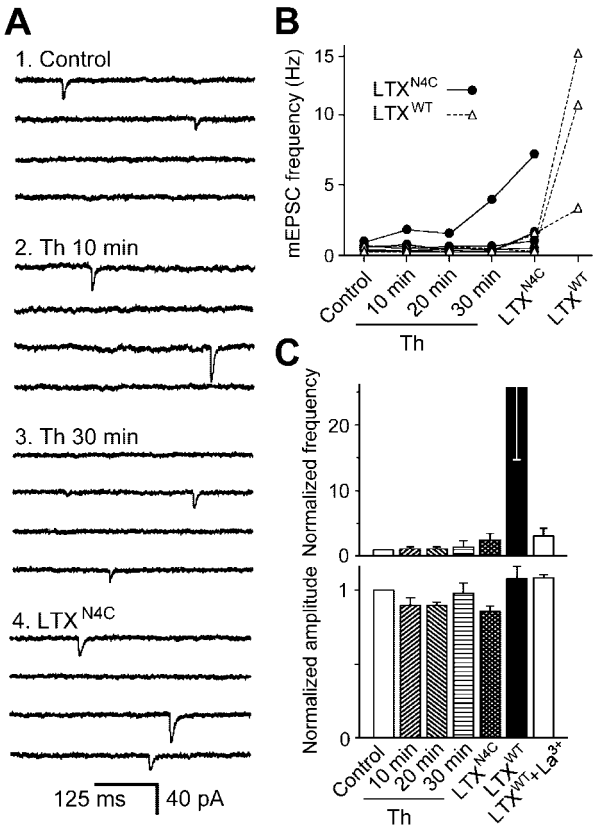

Figure 5. The depletion of intracellular Ca^{2+} stores by Th blocks the increase of mEPSC frequency induced by LTX^{N4C}. *A*, Continuous recordings of mEPSCs before (*1*) and after 10 min (*2*) or 30 min (*3*) application of Th, and 15 min after subsequent addition of LTX^{N4C} (*4*). *B*, Pooled data from all recorded cells: mEPSC frequency in control, 10, 20, or 30 min after the superfusion with Th, and after subsequent sequential applications of LTX^{N4C} and LTX^{WT}. *C*, Mean mEPSC frequency (top) and amplitude (bottom) normalized to control value for each cell. Superfusion with Th for 10, 20, or 30 min did not significantly change mEPSC frequency ($n = 9$; $p > 0.4$) and inhibited the effect of LTX^{N4C} (only 2.1 ± 0.5-fold increase above Th for 30 min; $n = 9$; $p > 0.08$) but did not attenuate the action of LTX^{WT} (15.1 ± 3.2-fold increase above Th for 30 min; $n = 3$; $p < 0.05$). The latter was, however, substantially blocked after the subsequent addition of 100 μM La^{3+} (3.1 ± 1.1-fold increase above Th for 30 min; $n = 4$; $p > 0.1$). The mean mEPSC amplitude was not altered by any of these conditions.

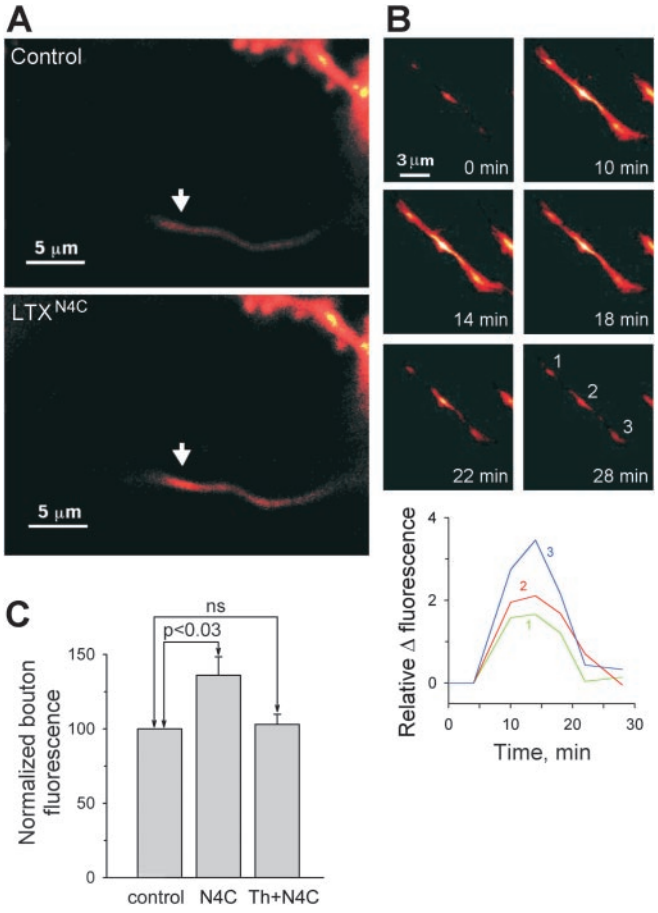

Figure 6. LTX^{N4C} produces an increase in the basal Ca^{2+} fluorescence of CA3 pyramidal cell nerve terminals. *A*, Top, A length of axon from a CA3 pyramidal neuron can be visualized after injection of the calcium indicator dye Oregon Green 488 BAPTA-1. A presynaptic bouton is marked by an arrow. Note the piece of dendrite, studded with dendritic spines, at the top right corner of the image. Bottom, Fifteen minutes after the application of 1 nM LTX^{N4C}, the basal fluorescence of the axon has increased. *B*, Top, Ca^{2+} fluorescence in presynaptic boutons before and after the application of LTX^{N4C}. Sequential frames of an axon with three boutons (numbered 1–3) were taken at indicated times; 1 nM toxin was added at 0 min. Bottom, Relative increase in fluorescence determined for boutons 1–3 above. Note that the fluorescence reaches maximal values between 10 and 15 min from toxin application but then gradually decreases to the control level. *C*, A summary histogram revealing that LTX^{N4C} produces a significant increase in basal fluorescence of the axon ($n = 6$; $p < 0.03$). Cells treated with 4 μM Th show no increase in basal fluorescence after exposure to LTX^{N4C} ($n = 3$). ns, Nonsignificant.

2000, 2001) (Volynski et al., unpublished observations). Because LTX^{N4C} acts by interacting with receptors but without forming pores, we tested whether this action also needed Ca^{2+}_e. For this purpose, an increase in the mESPC frequency was first induced by LTX^{N4C} in normal saline, and then the medium was replaced with Ca^{2+}-free saline supplemented with 1 mM EGTA. Although this medium replacement was done briefly to avoid LTX^{N4C} washout, it significantly reduced the mEPSC frequency, which, however, increased again during the reintroduction of normal saline containing 3 mM Ca^{2+} (Fig. 4). None of these manipulations affected mEPSC amplitude (Fig. 4*C*). In contrast, after the administration of LTX^{WT}, the removal of Ca^{2+}_e had no effect on the frequency of mEPSCs (Fig. 4*B*,*C*). Thus, the absence of Ca^{2+}_e greatly inhibits the action on spontaneous release of the mutant but not wild-type LTX.

Receptor-mediated LTX^{N4C} action involves mobilization of stored Ca^{2+}

Biochemical data (Davletov et al., 1998; Ashton et al., 2001) suggest that the receptor-mediated LTX action requires Ca^{2+} mobilization from intracellular stores. The involvement of Ca^{2+} stores was addressed here by studying the action of LTX^{N4C} in the presence of 10 μM Th that depletes such stores (Thastrup et al., 1990). Th itself only slightly affected the rate of occurrence of mEPSCs, in agreement with previous evidence at hippocampal synapses (Savic and Sciancalepore, 1998). In particular, the frequency of mEPSCs increased by only 1.5 ± 0.7-fold after 30 min Th treatment (Fig. 5*A*,*B*). Subsequent addition of LTX^{N4C} for up to 20 min did not significantly increase the frequency of mEPSCs (Fig. 5*C*). In striking contrast, LTX^{WT} (added to the Th-treated slices after the mutant) was still able to increase mEPSC frequency (Fig. 5*C*), demonstrating that Th did not act by simply impairing the secretory apparatus. Thus, Th occludes the action of the mutant toxin, indicating that LTX^{N4C}-induced release may require mobilization of Ca^{2+} from presynaptic Th-sensitive stores.

Because LTX^{WT} was still active in Th-treated slices, it must have acted via the mechanism absent in the mutant toxin, presumably membrane pore formation. LTX pores can be efficiently blocked by La^{3+} (Ashton et al., 2001), and, indeed, this cation

greatly inhibited the action of the wild-type toxin in cultures treated previously for 15–25 min with 10 μM Th (Fig. 5*C*). In the La^{3+} experiments, the mEPSC frequency was 1.7 ± 0.3 Hz before and only 4.6 ± 1.1 Hz after LTX^{WT} ($p > 0.1$; $n = 4$).

Presynaptic release of stored Ca^{2+} was directly demonstrated in experiments in which calcium indicator was iontophoretically injected into CA3 pyramidal cells, permitting unambiguous visualization of the cell axon and boutons (Fig. 6*A*, top) (for detailed methods, see Emptage et al., 2001). Basal fluorescence within an axon remained constant for extended periods (>1 hr) after dye loading. The addition of LTX^{N4C} to the recording chamber produced a significant (36 ± 12.4%; $n = 6$ cells in 6 slices) increase in basal fluorescence within synaptic boutons (Fig. 6*A*, bottom). This increase in fluorescence is temporally linked with an increase in synaptic activity within the slice, because both basal fluorescence and synaptic activity reached their peaks within 15 min after addition of LTX^{N4C} and then subsided (Fig. 6*B*). Note that relative increases and decay rates of fluorescence differed between individual boutons (Fig. 6*B*, bottom), indicating that mutant toxin acted independently on each presynapse. These findings suggest that the rise in $[Ca^{2+}_e]$ (whose behavior is consistent with its origin from intracellular Ca^{2+} stores rather than influx) precedes, and probably causes, the increase in the frequency of miniature postsynaptic events. Unlike the synaptic boutons, cell dendrites showed no significant increase (7.4 ± 3.2%; $n = 6$ cells in 6 slices) in fluorescence over the same time period. Finally, cells that had been treated for 20 min with 4 μM Th showed no increase in basal fluorescence within the boutons after exposure to LTX^{N4C} (Fig. 6*C*).

We then tested the involvement of other components of the Ca^{2+} signaling cascade. First, we found that the phospholipase C (PLC) inhibitor aminosteroid U73122 (Smith et al., 1990; Thompson et al., 1991) potently attenuated the LTX^{N4C} effect (Fig. 7*A*). An inactive analog of this drug, U73343, was used as a control and did not affect the mutant-evoked increase in frequency of mEPSCs. PLC activation produces inositol(1,4,5)-trisphosphate (IP_3) and leads to subsequent release of Ca^{2+} from IP_3-sensitive stores. This action of IP_3 can be inhibited by 2-APB, a cell-permeable antagonist of the IP_3-gated Ca^{2+}-release channels (Ma et al., 2000; Chorna-Ornan et al., 2001). Indeed, 2-APB abolished the effect of LTX^{N4C} (Fig. 7*A*). To further determine the type of Ca^{2+} stores involved, we used ryanodine that inhibits distinct, IP_3-insensitive Ca^{2+}-release channels. As shown in Figure 7*B*, ryanodine did not affect the mEPSC frequency increase brought about by LTX receptor activation, indicating that ryanodine receptors are virtually not involved in LTX^{N4C} action.

Other properties of LTX^{N4C}-induced spontaneous release

The LTX receptor-transduction mechanism requires both extracellular and stored Ca^{2+}. Although LTX^{N4C} did not act by grossly increasing permeability of the plasma membrane to Ca^{2+}, as did LTX^{WT} (Fig. 1*C*), it might potentially stimulate exocytosis by allowing a small influx of Ca^{2+}_e (undetectable by radioactive measurements) at the active zones. Therefore, we tested the effect of mutant toxin in the presence of inhibitors of Ca^{2+} channels and LTX pores.

First, we tested Cd^{2+}, a blocker of voltage-dependent Ca^{2+} channels (VDCCs). However, 100 μM Cd^{2+}, which blocked voltage-dependent Ca^{2+} channels and abolished evoked EPSCs (data not shown), did not affect the LTX^{N4C} action (Fig. 7*C*). We then tested an inhibitor of store-operated plasma membrane Ca^{2+} channels (SOCs), SKF 96365 (Merritt et al., 1990). These channels can function at hippocampal synapses and mediate

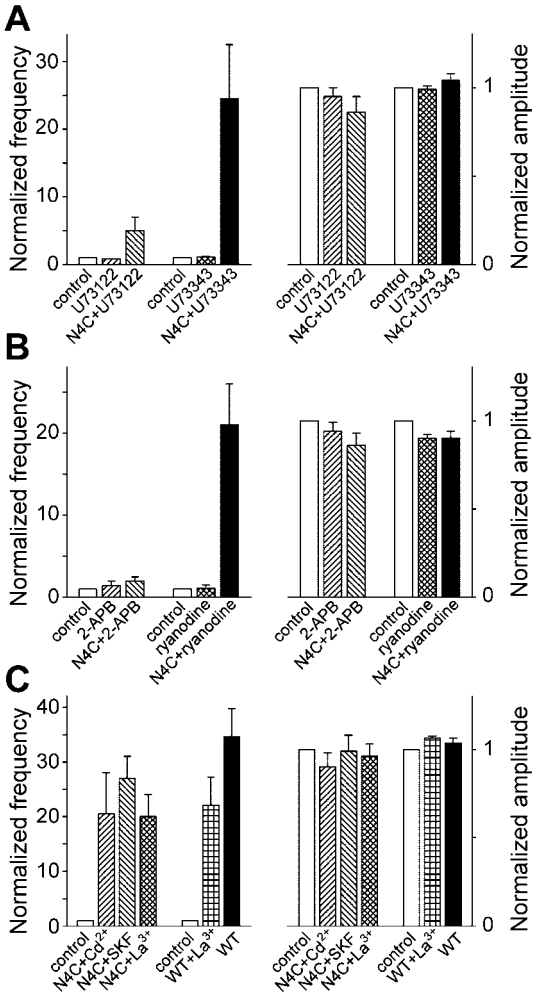

Figure 7. LTX^{N4C} action involves the activation of the PLC–IP_3–Ca^{2+} signaling cascade but not the opening of Ca^{2+} channels or LTX pores. *A–C*, Normalized mean frequency and amplitude of mEPSC evoked by LTX^{N4C} in hippocampal slices treated with the following: 2 μM U73122 or 2 μM U73343 (*A*); 50 μM 2-APB or 20 μM ryanodine (*B*); 50 μM SKF 96365, 100 μM Cd^{2+}, or 100 μM La^{3+} (*C*). All of the data in the figure are normalized to the control value for each cell. On average, the mEPSC frequency was as follows (in Hz): controls, from 0.5 to 1.4; U73122, 0.7 ± 0.2; U73343, 0.9 ± 0.2; 2-APB, 1.7 ± 0.6; ryanodine, 1.2 ± 0.6; Cd^{2+}, 0.7 ± 0.1; SKF 96365, 0.8 ± 0.2; La^{3+}, 1.1 ± 0.1. Subsequently added LTX^{N4C} increased the mEPSC frequency as follows (in Hz): control, 24 ± 3.2 ($n = 8$; $p < 0.001$); U73122, 4 ± 1 ($n = 4$; $p < 0.05$); U73343, 18.1 ± 1.8 ($n = 3$; $p < 0.01$); 2-APB, 2.1 ± 0.4 ($n = 5$; $p > 0.7$; nonsignificant); ryanodine, 21.1 ± 4.4 ($n = 4$; $p < 0.04$); Cd^{2+}, 11 ± 0.7 ($n = 5$; $p < 0.0001$; $p > 0.26$ compared with mEPSC frequency increase by LTX^{N4C} in control cultures; Fig. 3*C*); SKF 96365, 15.3 ± 3.7 ($n = 4$; $p < 0.03$; $p > 0.06$ compared with data in Fig. 3*C*); La^{3+}, 21.8 ± 2.9 ($n = 5$; $p < 0.002$; $p < 0.001$ compared with data in Fig. 3*C*). LTX^{WT} increased the frequency to 14.1 ± 3.2 Hz in the presence of La^{3+} and 22.1 ± 1.6 Hz after the removal of La^{3+} ($n = 4$; $p < 0.02$ and $p < 0.0009$, respectively). The toxins had no effect on the mean mEPSC amplitude under any condition. Note that 2-APB significantly reduces and U73122 inhibits the effect of LTX^{N4C}, whereas La^{3+} attenuates the activity of LTX^{WT} but does not affect LTX^{N4C}.

Ca^{2+}_e influx during activation by cytosolic Ca^{2+} released from stores (Emptage et al., 2001). Again, a 20 min application of this drug did not prevent the LTX^{N4C}-induced increase in mEPSC frequency (Fig. 7*C*).

Finally, La^{3+}, which potently blocks VDCCs, SOCs, and the LTX-induced pores, did not affect the mEPSCs frequency increase induced by LTX^{N4C} but strongly attenuated the effect of LTX^{WT} (Fig. 7*C*). In contrast, as described above, in cultures treated with Th, La^{3+} almost completely blocked the effect of LTX^{WT} (Fig. 5*C*). Together, these results clearly illustrate the

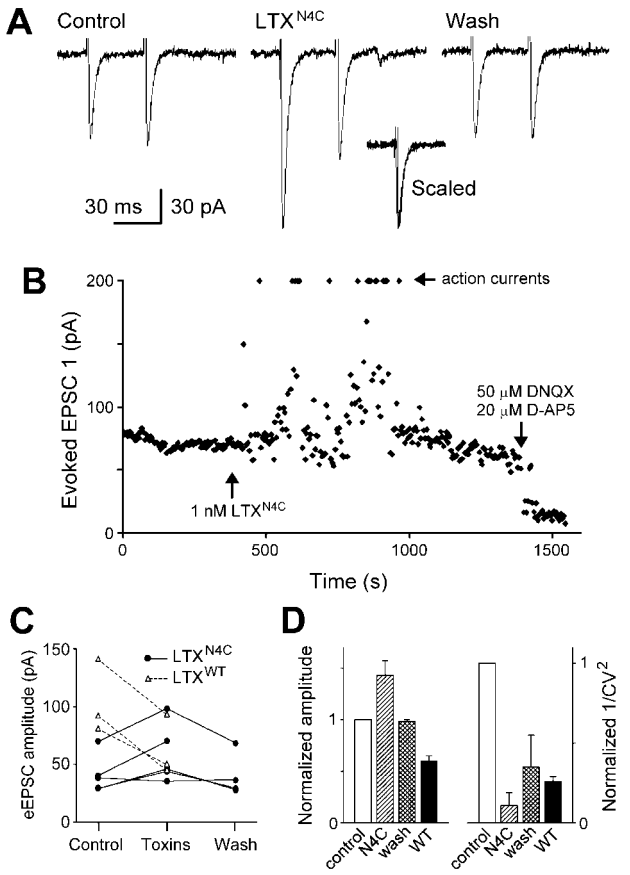

Figure 8. Effects of LTX^{N4C} on evoked EPSCs. *A*, Single traces of EPSCs evoked in a CA3 neuron by pairs of stimuli in the dentate gyrus (interval between stimuli, 50 msec) before and after LTX^{N4C} application. The mutant toxin reversibly enhanced the amplitude of evoked EPSCs. The mean values for this cell were as follows: control, EPSC1, 69 ± 2 pA; control, EPSC2, 72 ± 3 pA; LTX^{N4C}, EPSC1, 98 ± 28 pA; LTX^{N4C}, EPSC2, 86 ± 32 pA; wash, EPSC1, 69 ± 6 pA; wash, EPSC2, 68 ± 7 pA. *B*, The time course of the LTX^{N4C} effect on the amplitude of EPSCs evoked by the first stimuli in the same cell. After LTX^{N4C} addition (arrow), some inward currents elicited peak-saturated voltage-clamp-recorded action potentials (more than −200 pA; labeled as action currents). *C*, Pooled data, Peak amplitudes of evoked EPSCs (eEPSCs) in control, after the addition and washout of LTX^{N4C}, and after the addition of LTX^{WT}. On average, the amplitude of the EPSCs was 41 ± 7 pA before and 58 ± 11 pA after LTX^{N4C} and 41 ± 9 pA after washing out ($n = 5$; but $n = 4$ washed); it was 105 ± 14 pA before and 63 ± 12 pA after LTX^{WT} ($n = 3$). *D*, EPSC peak amplitudes and 1/CV^2 values normalized to the control value for each cell. LTX^{N4C} enhanced EPSC amplitudes and depressed 1/CV^2 ($n = 5$). EPSC amplitudes fully recovered on LTX^{N4C} washout, whereas 1/CV^2 recovered only partially ($n = 5$). In contrast, LTX^{WT} decreased EPSC peak amplitudes but also caused a higher variability of neuronal responses.

dual mode of action of LTX^{WT}: (1) pore formation (absent in LTX^{N4C}) and (2) receptor-mediated signaling (present in both toxins). La^{3+} and Th selectively inhibit these two major mechanisms in an additive manner: when the ionophoretic activity is blocked by La^{3+}, LTX^{WT} only acts by stimulating the receptor; when the receptor transduction pathway is perturbed by, for example, Th, pores formed by wild-type LTX still cause exocytosis.

These results also suggest that Ca^{2+}_e supports LTX^{N4C} action by either entering terminals through some unusual Ca^{2+} channels insensitive to Cd^{2+}, SKF 96365, and La^{3+} or acting as an extracellular cofactor for the LTX receptor signaling cascade.

LTX^{N4C} increases evoked release

One of the characteristic effects of native LTX is its ability to reduce the amplitude of evoked synaptic responses (Ceccarelli and Hurlbut, 1980; Capogna et al., 1996b). We confirmed that LTX^{WT} acts in the same manner and decreases the amplitude of EPSCs elicited by stimulation delivered within the dentate gyrus (Fig. 8). In contrast, application of LTX^{N4C} enhanced the responses in four of five cells (Fig. 8*A–D*) and simultaneously increased the frequency of spontaneous events in all five cells. The time course of the action of the mutant on evoked currents was similar to that on mEPSCs, and this effect was fully reversed by a wash. On average, within 20 min of LTX^{N4C} application, peak amplitude of EPSCs became 1.4 ± 0.1-fold higher than in control ($n = 5$; $p < 0.04$) but recovered to 98% of control value on washout (Fig. 8*B–D*). Such an increase in the peak amplitude of the evoked synaptic currents was not attributable to spontaneous synaptic events coinciding in time with the evoked currents, because the evoked currents before and after LTX^{N4C} perfectly superimposed after rescaling (Fig. 8*A*). The 1/CV^2 of the EPSCs was reduced by LTX^{N4C} from 259 ± 149 to 13 ± 4 ($n = 5$) (Fig. 8*D*), indicating much higher variability of evoked responses than in control. We also tested the effect of mutant toxin on paired-pulse facilitation elicited by two stimuli delivered with a 50 msec interval. As expected, because of the enhanced transmitter release in response to the first pulse, the paired-pulse ratio was slightly decreased by the mutant from 1.1 ± 0.1 to 0.88 ± 0.02 ($n = 4$) (Fig. 8*A*).

Thus, our findings indicate that LTX^{N4C} stimulates not only spontaneous but also action potential-evoked neurotransmitter release by a mechanism compatible with receptor-mediated changes in the intraterminal Ca^{2+} concentration.

Discussion

For several decades, LTX has been used as a tool to study synaptic transmission, but its mechanism of action is still unclear (Rosenthal and Meldolesi, 1989; Südhof, 2001; Ushkaryov, 2002). On the one hand, LTX binds two different presynaptic receptors, NRX and LPH, and there is evidence that activation of a receptor sends an exocytotic signal (Bittner et al., 1998; Davletov et al., 1998; Ashton et al., 2001). On the other hand, the toxin forms cation-selective pores in the presynaptic plasma membrane, allowing influx of Ca^{2+} and influx–efflux monovalent cations with subsequent stimulation of exocytosis (Scheer et al., 1986; Filippov et al., 1994; Hurlbut et al., 1994). The receptors do not directly participate in pore formation but facilitate LTX insertion into the membrane and channel formation (Hlubek et al., 2000; Volynski et al., 2000). In addition, an alternative mechanism has been proposed, consisting of partial translocation of LTX through the plasma membrane, followed by intracellular interaction with the exocytotic machinery (Khvotchev and Südhof, 2000). At the physiological level, interpretation of LTX effects has been also problematic as a result of the toxin acting simultaneously as an ionophore and as a receptor ligand. In this paper, for the first time, we characterize the physiological properties of the LTX receptor-mediated transmitter release by using a non-pore-forming LTX^{N4C}.

LTX^{N4C} (Ichtchenko et al., 1998) differs from the wild-type toxin only by a four amino acid insert between the N-terminal and the ankyrin-containing domains. As a result, LTX^{N4C} and LTX^{WT} are very similar in how they bind the receptors (Fig. 1*B*) (Ichtchenko et al., 1998). However, in total contrast with the conclusions of these authors (Ichtchenko et al., 1998), the mutation has not made LTX^{N4C} inactive. Our experiments (Figs. 2–5) show that, in the presence of Ca^{2+}, the mutant toxin is almost as efficient as the wild-type or native LTX in stimulating spontaneous release (Capogna et al., 1996a,b).

At the same time, LTX^{WT} and LTX^{N4C} display several inter-

esting and particularly important differences, which reveal the receptor-mediated transduction mechanism of the toxin. First, on the basis of our electron microscopic studies (Orlova et al., 2000), the mutation has been introduced inside the compact "body" domain that determines LTX oligomerization. Distortions in this domain are likely to hinder the formation of ring-like toxin tetramers. Indeed, LTX^{N4C} forms only dimers but not tetramers (Volynski et al., unpublished observations). Because the tetramers represent LTX pores (Orlova et al., 2000), the mutant toxin must be incapable of pore formation, and we clearly showed this both biochemically and electrophysiologically (Fig. 1*C*) (Volynsky et al., unpublished observations). Consistently, whereas the binding of LTX^{WT} to neurons is irreversible because of its membrane incorporation (Fig. 3) (Volynski et al., 2000) (Volynski et al., unpublished observations), LTX^{N4C} can be washed away (Figs. 3, 8), indicating a reversible interaction with a receptor and ruling out the mechanism on the basis of toxin internalization. During LTX^{N4C} dissociation, its effect ceases and, thus, can only be mediated by a receptor.

Second, unlike LTX^{WT}, LTX^{N4C} requires Ca^{2+}_e to stimulate spontaneous release (Fig. 4). One explanation could be that Ca^{2+} is required for the binding of LTX^{N4C} to NRX and that only this interaction triggers secretion. This does not appear to be the case, because the mutant toxin also acts in the presence of Sr^{2+}, which does not support LTX interaction with NRX (Volynski et al., unpublished observations) (Davletov et al., 1998). These findings are in agreement with our previous results indicating that Ca^{2+} is essential for the LPH-mediated signaling rather than LTX binding (Davletov et al., 1998; Rahman et al., 1999; Ashton et al., 2001).

Another striking difference is that LTX^{N4C} enhances EPSCs evoked by electrical stimulation of presynaptic fibers, whereas LTX^{WT} inhibits such EPSCs (Fig. 8). This effect is accompanied by a trend to paired-pulse inhibition and a marked increase in the trial-to-trial variability of responses, both indicating a presynaptic site of action. As our experiments illustrate (Fig. 6), the mutant toxin increases the intraterminal Ca^{2+} concentration, and this could explain an increased efficacy of incoming action potentials in stimulating exocytosis. This observation also implies that the inhibition of evoked currents by native LTX (Ceccarelli and Hurlbut, 1980; Capogna et al., 1996b) results from the influx–efflux of cations through LTX pores, leading to depolarization of the presynaptic plasma membrane and subsequent inactivation of presynaptic action potentials. The lack of depression of evoked responses is yet more evidence that LTX^{N4C} does not form cation-permeable pores, which would alter the presynaptic membrane potential.

The use of La^{3+} provides another important insight into the differences between the mechanisms of action of the wild-type and mutant toxins: this trivalent cation blocks the ionophoretic mode of LTX (Hurlbut et al., 1994; Van Renterghem et al., 2000; Ashton et al., 2001) and consistently attenuates, but does not abolish, the effect of LTX^{WT} (Fig. 7*C*), thus limiting its action only to receptor stimulation. Indeed, in the presence of La^{3+}, LTX^{WT} and LTX^{N4C} behave very similarly. Furthermore, the full activity of LTX^{N4C} in the presence of La^{3+} unequivocally proves that the mutant does not act via pore formation (Fig. 7*C*). These observations strongly support the idea that LTX^{WT} has a dual mechanism of action, whereas LTX^{N4C} is only able to act via the receptor. Consistently, the wild-type toxin is fully inhibited only by Th and La^{3+} together, whereas the action of the mutant is blocked by Th alone (Fig. 5). Our study agrees with previous evidence that the Ca^{2+}-independent effect of native LTX is mainly attributable to its pore formation (Ashton et al., 2000, 2001).

Contrary to our results, some previous findings (Ichtchenko et al., 1998; Sugita et al., 1998) have suggested that LTX causes transmitter release without sending receptor-mediated exocytotic signals. First, these authors found that LTX^{WT}-evoked hormone secretion in PC12 cells was potentiated by overexpression of signaling-deficient LPH variants. However, wild-type LTX can form pores with any receptor (Volynski et al., 2000) and cause secretion, bypassing any intracellular signaling (Fig. 5). The second work was based on the use of LTX^{N4C} in the absence of Ca^{2+} on synaptosomes prestimulated with high K^+. However, LTX^{N4C} requires Ca^{2+}_e (Fig. 4), whereas high K^+ inhibits the subsequent receptor-mediated LTX action (Ashton et al., 2001). Also, in their experiments, La^{3+} completely blocked the effect of LTX^{WT} (Ichtchenko et al., 1998), indicating that these authors studied only the pore-mediated release.

Combined, our results may suggest the following mechanism of the receptor-mediated action of LTX. The toxin stimulates a presynaptic receptor, possibly LPH, which is linked to $G_{\alpha q/11}$ (Rahman et al., 1999) known to be involved in Ca^{2+} homeostasis. The downstream effector of $G_{\alpha q/11}$ is PLC. Indeed, LTX^{N4C} was demonstrated to activate PLC and stimulate Ca^{2+}_e-dependent hydrolysis of phosphoinositides (Ichtchenko et al., 1998), whereas the inhibition of PLC by U73122 greatly attenuated the receptor-dependent LTX action (Fig. 7*A*). Activated PLC increases the intraterminal concentration of IP_3, which in turn induces release of Ca^{2+} from intracellular stores. This rise of cytosolic Ca^{2+} (similar to presynaptic residual Ca^{2+}) will increase the probability of release and, consequently, the rate of spontaneous exocytosis and the amplitude of evoked release. In fact, mobilization of Ca^{2+} appears to play a pivotal role in LTX receptor signaling because the effect of LTX^{N4C} is abolished by both an inhibitor of IP_3-induced calcium release (2-APB) and by depletion of stores with Th (Figs. 5, 7). Chelation of cytosolic Ca^{2+} by membrane permeable BAPTA AM also blocks the LTX^{N4C} action (Ashton et al., 2001). Moreover, we directly show that LTX^{N4C} induces a rise in the presynaptic $[Ca^{2+}]$ (Fig. 6). Th blocks the LTX^{N4C} effect on both secretion and the rise of calcium, indicating that the latter must be the underlying mechanism of the receptor-mediated action.

The rise of cytosolic Ca^{2+} resulting from LTX^{N4C}-induced activation of intracellular stores can be sufficient to increase the frequency of spontaneous exocytosis and enhance evoked release, as demonstrated for other systems (Hashimoto et al., 1996; Tse et al., 1997; Emptage et al., 2001). An interesting feature of the LTX receptor signaling, however, is that it requires Ca^{2+}_e, although the latter can be replaced with Sr^{2+}_e without any attenuation of the effect (Volynski et al., unpublished observations). Notably, cyclopiazonic acid (CPA), which depletes intracellular Ca^{2+} stores by selectively blocking sarcoplasmic–endoplasmic reticulum Ca^{2+}/ATPases (SERCA pumps), formally behaves similar to LTX^{N4C}, increasing the basal presynaptic Ca^{2+} fluorescence and the frequency of mEPSCs in a manner dependent on extracellular Ca^{2+} (Emptage et al., 2001). It is possible, therefore, that LTX^{N4C} and CPA share a similar mechanism of action. Another possibility is that the LTX receptor-induced Ca^{2+} release activates influx of extracellular Ca^{2+} or Sr^{2+} via some plasma membrane channels. Indeed, the inhibitory effect of 2-APB in our experiments could be attributable to this drug not only inhibiting IP_3 receptors but also directly blocking some SOCs (Kukkonen et al., 2001). However, La^{3+} that potently blocks SOCs (Diver et al., 2001) had no effect on the high rate of spontaneous exocytotic

events evoked by LTX^{N4C} (Fig. 7*C*). Our experiments also rule out the involvement of various other plasma membrane Ca^{2+}-permeant channels because SKF 96365 and Cd^{2+} did not inhibit the LTX^{N4C} action (Fig. 7*C*). Thus, if Ca^{2+} influx is indeed required for the effect of the LTX receptor, it must occur through some rather unusual Ca^{2+} channels. Finally, it is possible that Ca^{2+} and Sr^{2+} serve as extracellular cofactors for the LTX receptor signaling. Future work using other model systems will be required to distinguish between these two possibilities.

The properties of LTX^{N4C} are also very similar to those of α-latrocrustatoxin, a structural homolog of LTX. At crayfish neuromuscular junctions, the crustatoxin increased the rate of spontaneous (and the amplitude of evoked) synaptic responses in a $Ca^{2+}{}_e$- or $Sr^{2+}{}_e$-dependent, but lanthanide-insensitive, manner and raised the cytosolic $[Ca^{2+}]$ (Elrick and Charlton, 1999), suggesting that different LTX-related toxins can stimulate exocytosis through similar mechanisms.

In conclusion, our findings demonstrate that, by using LTX^{N4C}, it is possible to uncouple the receptor- and pore-mediated actions of LTX. The future use of this unique tool will allow determining the individual roles of LTX receptors in signal transduction and the pore formation by native LTX.

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