α -Latrotoxin Releases Calcium in Frog Motor Nerve Terminals

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α -Latrotoxin (α -LTX) is a neurotoxin that accelerates spontaneous exocytosis independently of extracellular Ca^{2+} . Although α -LTX increases spontaneous transmitter release at synapses, the mechanism is unknown. We tested the hypothesis that α -LTX causes transmitter release by mobilizing intracellular Ca^{2+} in frog motor nerve terminals. Transmitter release was measured electrophysiologically and with the vesicle marker FM1-43; presynaptic ion concentration dynamics were measured with fluorescent ion-imaging techniques. We report that α -LTX increases transmitter release after release of a physiologically relevant concentration of intracellular Ca^{2+} . Neither the blockade of Ca^{2+} release nor the depletion of Ca^{2+} from endoplasmic reticulum affected Ca^{2+} signals produced by α -LTX. The Ca^{2+} source is likely to be mitochondria, because the effects on Ca^{2+} mobilization of CCCP (which depletes mitochondrial Ca^{2+}) and

of α -LTX are mutually occlusive. The release of mitochondrial Ca^{2+} is partially attributable to an increase in intracellular Na^+ , suggesting that the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger is activated. Effects of α -LTX were not blocked when Ca^{2+} increases were reduced greatly in saline lacking both Na^+ and Ca^{2+} and by application of intracellular Ca^{2+} chelators. Therefore, although increases in intracellular Ca^{2+} may facilitate the effects of α -LTX on transmitter release, these increases do not appear to be necessary. The results show that investigations of Ca^{2+} -independent α -LTX mechanisms or uses of α -LTX to probe exocytosis mechanisms would be complicated by the release of intracellular Ca^{2+} , which itself can trigger exocytosis.

Key words: α -latrotoxin; presynaptic toxin; mitochondria; calcium; sodium; exocytosis; frog neuromuscular junction/motor nerve terminal

Neurotoxins are important tools for studying synaptic physiology. α -Latrotoxin (α -LTX) is a neurotoxin isolated from the venom of the black widow spider, *Latrodectus mactans tredecimguttatus*. At the frog neuromuscular junction (NMJ) α -LTX increases the frequency of spontaneous transmitter release independently of extracellular Ca^{2+} (Longenecker et al., 1970) despite the fact that Ca^{2+} influx is required for nerve-evoked transmitter release (Bennett, 1999). This implies that there might be a mechanism of transmitter release that could bypass the requirement for Ca^{2+} .

Two theories have been proposed to explain how α -LTX could increase transmitter release independently of extracellular Ca^{2+} . The first suggests that α -LTX forms pores in nerve terminals and that changes in ion conductance could mediate its effect on transmitter release. This idea is supported by the fact that α -LTX can form nonselective cation pores in lipid bilayer membranes (Finkelstein et al., 1976) by oligomerizing into homotetrameric structures (Orlova et al., 2000). However, this mechanism alone cannot explain the specificity of α -LTX for presynaptic nerve terminals (Valtorta et al., 1984). A second theory suggests that α -LTX interacts with a membrane receptor and that activation of a signal transduction mechanism triggers vesicle release. This theory was strengthened when two distinct receptors with nanomolar affinity for α -LTX were isolated and cloned. One is the single-transmembrane-domain cell surface receptor neurexin-1 α (Ushkaryov et al., 1992), and the other is a seven-transmembrane-domain G-protein-coupled receptor, latrophilin/CIRL (Krasnoperov et al., 1997; Leliana et al., 1997), expressed here as CL1. The latter is thought to mediate the actions of α -LTX in the absence of extracellular Ca^{2+} because α -LTX binding to neurexin is Ca^{2+} -dependent (Davletov et al., 1995), but binding to CL1 does not require Ca^{2+} (Davletov et al., 1996). Studies with truncated CL1 mutants transfected into chromaffin cells, however, have demonstrated that receptor activation is

not required for transmitter release by α -LTX (Sugita et al., 1998). Therefore, it seems likely that α -LTX is targeted to presynaptic nerve terminals by the receptor, where it then proceeds to act independently of the receptor; this function could include pore formation.

The mechanism of α -LTX action has been difficult to resolve, and inconsistent results are seen in different cell types. For example, transmitter release by α -LTX is dependent on Ca^{2+} mobilization in rat brain synaptosomes (Davletov et al., 1998; Rahman et al., 1999), whereas in secretory cell lines such as PC12 cells or β -pancreatic cells no changes in intracellular Ca^{2+} are observed in the presence of α -LTX (Meldolesi et al., 1984).

α -LTX has been used widely under the assumption that it is a Ca^{2+} -independent secretagogue at the frog NMJ. However, this assumption has not been tested with Ca^{2+} detection methods. Release of intracellular Ca^{2+} easily could explain the actions of α -LTX in the absence of extracellular Ca^{2+} . Therefore, we decided to test the hypothesis that at frog motor nerve terminals α -LTX causes Ca^{2+} mobilization from intracellular stores and that this triggers transmitter release.

MATERIALS AND METHODS

Animals and experimental treatment. *Rana pipiens* (leopard) frogs (4–5 cm body length; Wards Scientific, St. Catherine's, Ontario) were housed at 15°C in cages with a flow-through water system. Frogs were double-pithed, and the cutaneous pectoris muscles with the innervating pectoralis propius nerve were dissected out (Dreyer and Peper, 1974). Excised muscles were pinned down in a Sylgard-coated (Dow Corning, Midland, MI) preparation dish and maintained at room temperature (20–22°C) in normal physiological saline (NPS) containing (in mM) 120 NaCl, 2 KCl, 1 NaHCO₃, 1.8 CaCl₂, and 5 HEPES, pH-adjusted to 7.2 with NaOH.

Experimental solutions. Ca^{2+} -free saline (CFS) containing (in mM) 120 NaCl, 2 KCl, 1 NaHCO₃, 5 MgCl₂, 10 HEPES, and 5 EGTA, pH-adjusted to 7.2 with NaOH, was used to study the actions of α -LTX in the absence of extracellular Ca^{2+} . When Na^+ and Ca^{2+} were not required, a Na^+ - and Ca^{2+} -free saline (NCFS) was made containing (in mM) 120 choline-Cl[−], 5 MgCl₂, 10 HEPES, and 5 BAPTA tetrapotassium salt (Molecular Probes, Eugene, OR), pH-adjusted to 7.2 with KOH. Before the start of any experiment that required the removal of an ion, preparations were washed (in CFS or NCFS) with four to five bath changes every 10 min for 1 hr. All saline salts and buffers were purchased from Sigma (St. Louis, MO).

For BAPTA-AM experiments a 5 mM stock concentration of BAPTA-AM was made up in dimethylsulfoxide (DMSO; Sigma) and diluted 1:50 to get a working concentration of 100 μM . A 1 M stock concentration of probenecid (Sigma) was made up in ethanol and diluted

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1:1000 to get a working concentration of 1 mM. Pluronic acid (Molecular Probes) was added to assist in the solubilization of probenecid and BAPTA-AM. A working concentration of 2 μM pluronic acid was achieved by diluting a 1 mM stock in DMSO to 1:1000. The final solution was mixed by sonication for several seconds.

Dye loading. Nerve terminals were loaded with the fluorescent dyes Oregon green 488 BAPTA-1-dextran or sodium green-dextran (Molecular Probes; 10,000 molecular weight) for measuring changes in presynaptic Ca^{2+} or Na^+ , respectively, by forward-filling the dye through the cut end of the innervating motor nerve. The muscles were washed first in a Petri dish with CFS for 10 min to remove excess Ca^{2+} . With a pair of sharp scissors the motor nerve was cut ~ 1 cm proximal to the muscle in a CFS bath. Then the preparation was transferred to a 1.5 ml rectangular well (containing CFS) that was cut out of a Sylgard-coated Petri dish. An adjacent small well contained 1 μl of the dye indicator at a concentration of 5 mM (in distilled water). The freshly cut end of the nerve was drawn into the dye-filled well, and a Vaseline border was made to isolate the contents of the two wells. Once the CFS was replaced with NPS, the dish was sealed with Parafilm (American National Can, Greenwich, CT) and stored at 15°C for 12–20 hr. During the incubation period the indicators were taken up by the axons and carried to the nerve terminals.

For FM1-43 (Molecular Probes) imaging the muscles were incubated with the dye (2 μM) in NPS for 15 min. During this incubation period the nerve was stimulated with 100 pulses (5 sec at 20 Hz) every 30 sec to induce vesicle recycling and uptake of the dye. Once the vesicles were loaded with FM1-43, the preparation was washed thoroughly with NPS to remove any extracellular and nonspecifically bound dye.

Blockade of spontaneous action potentials and postjunctional receptors. In CFS, spontaneous firing of action potentials can cause muscle fibers to twitch and also can load nerve terminals with Na^+ . The latter is known to have a physiological effect on transmitter release (Zengel et al., 1994). Therefore, the firing of spontaneous action potentials was blocked by the addition of 4 μM tetrodotoxin (TTX; Sigma) to block Na^+ channels.

When recording miniature endplate potentials (MEPPs) and intracellular Ca^{2+} fluorescence in NPS, we used μ -conotoxin GIIIA (10 $\mu\text{g}/\text{ml}$; Bachem, Torrance, CA) to block muscle Na^+ channels (Sosa and Zengel, 1993). This allowed us to block muscle contractions but preserved nerve action potentials and MEPPs. When only fluorescence imaging was required, α -bungarotoxin (5 $\mu\text{g}/\text{ml}$), which blocks nicotinic receptors, was used instead to block muscle contractions. TTX was added to the bath when, subsequently, the muscles were transferred to CFS.

Electrophysiology. Transmitter release was monitored by intracellular recordings in a muscle fiber via 5–15 M Ω glass microelectrodes (World Precision Instruments, Everett, WA) filled with 3 M KCl. Transmitter release was evoked by stimulating the motor nerve (0.2 Hz) at twice the threshold voltage that was required for muscle contraction in NPS. Responses were amplified (Neuroprobe amplifier, AM Systems, Carlsborg, WA), digitized (10 kHz, 12 bit; Labmaster interface, Scientific Solutions, Solon, OH), and averaged in groups of three to five by TOMAHACQ (T. A. Goldthorpe, University of Toronto), a program for PC data acquisition systems. Concurrently, a digital recording of the experiment (VR-10 digital data recorder, Instrutech, Great Neck, NY) was made for later analysis of MEPP frequency.

MEPP records stored on tapes were digitized by a Digidata 1200 Interface A/D Converter (Axon Instruments, Foster City, CA) that used Axoscope (Axon Instruments) data acquisition software and were analyzed with Mini Analysis software v4.0.1 (Synaptosoft, Leonia, NJ). MEPPs were counted by hand, and the frequency was calculated from the time that was required to record 100 MEPPs. During the height of α -LTX action the frequency of quantal release is so high that it is difficult to count MEPPs accurately. Therefore, MEPPs were counted when there was a positive inflection of the membrane potential that was greater than the level of noise. Although this method underestimated the frequency of spontaneous transmitter release during toxin action, the absolute frequency was not essential for any of the hypotheses that were tested here. Any treatment that attenuated the action of α -LTX by $>50\%$ was considered to have a significant effect.

Fluorescence imaging. Dye-loaded nerve terminals located on surface fibers were chosen for all experiments. Fluorescence (F) was measured with a Bio-Rad 600 (Hercules, CA) confocal laser-scanning microscope that used 1% of the maximum laser intensity for the ion indicators. Oregon green and sodium green dyes were excited by using the 488 nm line of the argon ion laser, and the emitted fluorescence was detected via a low-pass filter with a 515 nm cutoff. Confocal images were acquired by using a 40 $\times$ water-dipping objective (0.55 numerical aperture; Nikon) and were averaged in groups of three.

Confocal images were acquired digitally with data acquisition software provided by Bio-Rad. Image files were analyzed later with BFOCAL, a program for PC analysis of Bio-Rad images written by T. A. Goldthorpe (University of Toronto). Changes in fluorescence were measured from a region of interest on the nerve terminal, which displayed the greatest dynamic range after nerve stimulation, and were expressed as:

$$\% \Delta F/F = [(F_{\text{response}} - F_{\text{resting}})/F_{\text{resting}}] \times 100\%.$$

Fluorescence images also were captured by using a Nikon Optiphot microscope equipped with a 40 $\times$ (0.55 numerical aperture; Nikon) water-dipping objective, xenon lamp, and CCD camera (Cohu 4915) for FM1-43

experiments. Changes in fluorescence were processed with Axon Imaging Workbench software (Axon Instruments) and expressed as $\% \Delta F/F$ (see above). Measurements were made from several clusters of vesicles at 2 min intervals and adjusted by background subtraction.

Chemicals. CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and thapsigargin were purchased from Calbiochem (San Diego, CA). BAPTA-AM was purchased from Molecular Probes, and α -LTX was bought from Latoxan (Valence, France).

Statistical analysis and figures. All values are reported as the mean $\pm$ SEM. An independent Student's *t* test was used to determine statistical significance at a 95.0% confidence level. *N*, *n* refers to the number of muscles (i.e., preparations) and the number of endplates, respectively. SigmaPlot 4 graphing software (Jandel Scientific, San Rafael, CA) and Corel Draw 8 (Corel, Ottawa, Canada) were used to graph and display the data.

RESULTS

α -LTX increases intracellular Ca^{2+} and transmitter release

In normal physiological saline, stimulation of the motor nerve at 10 Hz for 5 sec produced a rapid rise in Ca^{2+} fluorescence of $40 \pm 2\%$ above baseline ($N, n = 7, 7$; Fig. 1A). Increasing the stimulation frequency to 20 and 40 Hz produced larger Ca^{2+} signals (72 ± 1 and $107 \pm 1\%$, respectively; $N, n = 7, 7$ for both) because of the more frequent opening of voltage-gated Ca^{2+} channels (Robitaille and Charlton, 1992). This control was performed for every nerve terminal that we examined to demonstrate the dynamic range of the indicator and detection system.

Ca^{2+} was removed by washing the preparations with CFS (see Materials and Methods). When the nerve was restimulated with 10, 20, and 40 Hz stimulation in CFS (Fig. 1A), no Ca^{2+} signals were produced ($N, n = 7, 7$ for each stimulation frequency). This suggests that the bath was nominally free of unchelated Ca^{2+} and that any changes in intracellular Ca^{2+} by α -LTX could not be attributable to Ca^{2+} entry.

When α -LTX (0.5 nM) was applied to nerve terminals bathed in CFS, the average change in Ca^{2+} fluorescence was 55% (Table 1). Figure 1B shows a typical result in which there was $\sim 62\%$ increase in fluorescence. The increase in Ca^{2+} fluorescence with the application of 0.5 nM α -LTX was similar to that produced by 10 Hz nerve stimulation in NPS (Fig. 1A). Ca^{2+} signals were not significantly different when a 10-fold higher concentration of α -LTX (5 nM) was applied ($50 \pm 6\%$; $N, n = 6, 6$). Unlike nerve stimulation, which increased intracellular Ca^{2+} by Ca^{2+} entry, α -LTX increased intracellular Ca^{2+} by Ca^{2+} mobilization from intracellular stores. The time course of Ca^{2+} elevation by α -LTX is much slower than that obtained with nerve stimulation (several minutes compared with a few seconds).

Slightly after elevating the intracellular Ca^{2+} concentration, α -LTX also caused a gross increase in spontaneous transmitter release as MEPP frequency increased from 1–2 to ~ 3 –400 MEPPs/sec (Fig. 1B). Then, over the course of 10 min, the frequency of spontaneous transmitter release declined to low levels (<1 MEPP/sec). The run-down in MEPP frequency is attributable to synaptic vesicle depletion (Clark et al., 1970, 1972), because vesicle recycling does not occur in CFS after treatment with α -LTX (Ceccarelli and Hurlbut, 1980; Henkel and Betz, 1995).

α -LTX does not mobilize Ca^{2+} from endoplasmic reticulum (ER)

CL1 has been classified as a seven-transmembrane receptor coupled to the G-protein, $\text{G}_{\alpha_{11}}$ (Rahman et al., 1999). This G-protein can activate phospholipase C (PLC) to produce inositol trisphosphate (IP_3), which mobilizes Ca^{2+} from the ER. Therefore, the PLC inhibitor U-73122 was used to determine whether the activation of PLC was responsible for the action of α -LTX on transmitter release and Ca^{2+} mobilization. Nerve–muscle preparations were incubated with U-73122 (50 μM) for 1 hr in CFS, and then α -LTX (0.5 nM) was applied. In the presence of U-73122, α -LTX still increased MEPP frequency and intracellular Ca^{2+} similar to controls (Fig. 2A, Table 1).

We next tried to inhibit the release of Ca^{2+} from ER by first depleting the store with thapsigargin. When thapsigargin

Figure 1. Effect of α -LTX on transmitter release and presynaptic Ca^{2+} in Ca^{2+} -free saline. *A, Left*, Presynaptic intracellular Ca^{2+} signals in NPS in response to 10, 20, and 40 Hz nerve stimulation. *A, Right*, Absence of presynaptic Ca^{2+} signals in CFS in response to the same stimulation frequencies. The motor nerve was stimulated for 5 sec at twice the voltage that was required for muscle contraction. *Inset* pictures show indicator fluorescence in the presynaptic terminal at the peak of the response at each frequency of stimulation. Similar results were obtained in seven preparations. *B*, The *bottom* graph shows measurements from a simultaneous recording of spontaneous quantal transmitter release frequency (MEPP frequency, blue) and Ca^{2+} fluorescence (red) in the same motor nerve terminal as in *A* after treatment with 0.5 nM α -LTX (arrow). Pictures of nerve terminal fluorescence and MEPP recordings are given at three time points during the experiment (1–3, *top* panels). The intracellular Ca^{2+} concentration increased before the increase in transmitter release. All of the data in this figure are from a single endplate. Similar results were obtained in two other experiments that recorded MEPPs and intracellular Ca^{2+} simultaneously.

(20 μM) was applied to nerve terminals bathed in CFS for 1 hr, there was very little change in the intracellular Ca^{2+} concentration (Fig. 2*B*) or transmitter release (Table 1). However, when α -LTX (0.5 nM) was applied after thapsigargin, a significant increase in Ca^{2+} fluorescence (Fig. 2*B*) and an acceleration of spontaneous transmitter release were still observed (Table 1). Although Ca^{2+} in ER stores can have subtle physiological effects at the frog NMJ (Narita et al., 1998), our data suggest that it is not sufficient to support the actions of α -LTX in CFS. Therefore, ER is probably not the primary Ca^{2+} pool affected by α -LTX.

α -LTX mobilizes Ca^{2+} from mitochondria

Another major Ca^{2+} -storing organelle found in nerve terminals is the mitochondrion. Unlike ER, these stores may not be depleted readily in CFS because of the large internally negative membrane potential (~ 150 – 200 mV) opposing the efflux of Ca^{2+} . Several drugs, such as CCCP, are well known to interfere with mitochondrial metabolism and can cause mitochondria to lose their Ca^{2+} . When CCCP (10 μM) was applied to nerve terminals bathed in CFS, a significant rise in intracellular Ca^{2+} ($50 \pm 12\%$; $N, n = 5, 5$) and transmitter release (102 ± 4 MEPPs/sec; $N, n = 3, 3$) was

Table 1. Summary of drug effects on the actions of α -LTX

Treatment	Ca^{2+} Fluorescence ($\%\Delta F/F \pm \text{SEM}$)		MEPP Frequency (MEPPs/sec $\pm \text{SEM}$)	
α -LTX (0.5 nM)	55 $\pm$ 9	(<i>N</i> , <i>n</i> = 9,10)	340 $\pm$ 19	(<i>N</i> , <i>n</i> = 5,5)
α -LTX (0.5 nM) after U-73122 (50 μM)	54 $\pm$ 6	(<i>N</i> , <i>n</i> = 4,7; <i>p</i> = 0.76)	353 $\pm$ 8	(<i>N</i> , <i>n</i> = 3,3; similar to 0.5 nM α -LTX)
Thapsigargin (20 μM)	16 $\pm$ 7	(<i>N</i> , <i>n</i> = 2,3; <i>p</i> = 0.04*)	3.5 $\pm$ 2	(<i>N</i> , <i>n</i> = 3,3)
α -LTX (0.5 nM) after thapsigargin (20 μM)	60 $\pm$ 17	(<i>N</i> , <i>n</i> = 2,3; <i>p</i> = 0.5)	359 $\pm$ 11	(<i>N</i> , <i>n</i> = 2,2; similar to 0.5 nM α -LTX)
α -LTX (0.5 nM) after CCCP (10 μM)	2.4 $\pm$ 1	(<i>N</i> , <i>n</i> = 3,3; <i>p</i> = 0.00005*)	–	
CCCP (10 μM)	50 $\pm$ 12	(<i>N</i> , <i>n</i> = 5,5; <i>p</i> = 0.74)	102 $\pm$ 4	(<i>N</i> , <i>n</i> = 3,3; lower than 0.5 nM α -LTX)
CCCP (10 μM) after α -LTX (0.5 nM)	1.3 $\pm$ 1	(<i>N</i> , <i>n</i> = 4,4; <i>p</i> = 0.01*)	–	

The effect of α -LTX (0.5 nM) on Ca^{2+} fluorescence was measured after muscles were incubated with 50 μM U-73122, 20 μM thapsigargin, or 10 μM CCCP in CFS. The effect of 0.5 nM α -LTX on transmitter release was measured after muscles were incubated with U-73122 or thapsigargin. A *t* test was used to test for significant differences between the peak Ca^{2+} fluorescence found with 0.5 nM α -LTX alone (control) and the peak Ca^{2+} fluorescence found with α -LTX applied after a drug, with a drug applied after α -LTX, or with a drug alone. Similarly, qualitative measurements (see Materials and Methods) were made for MEPP frequency (baseline $\sim$ 1 MEPP/sec). Data marked with an asterisk indicate statistical significance. *N*, *n*, Number of muscles, number of nerve terminals. Transmitter release was not recorded where a dash (–) is indicated. Not all experiments measured fluorescence and MEPP frequency simultaneously.

Figure 2. The effect of U73122 and thapsigargin on α -LTX-induced Ca^{2+} mobilization. Nerve terminals were bathed first in CFS containing 4 μM TTX for 1 hr. Then changes in intracellular Ca^{2+} were measured after the addition of 50 μM U-73122 (*A*) or 20 μM thapsigargin (*B*), followed by 0.5 nM α -LTX 1 hr later. Similar results were observed from six other U-73122-treated nerve terminals and two other thapsigargin-treated nerve terminals (see Table 1). Neither drug prevented the release of intracellular Ca^{2+} by α -LTX.

produced (Table 1). The amount of Ca^{2+} mobilized by CCCP on average was not significantly different from that mobilized by α -LTX (Table 1), suggesting that mitochondria are likely to be the Ca^{2+} source.

To determine whether α -LTX mobilizes Ca^{2+} from mitochondria, we first used CCCP (10 μM) to deplete mitochondrial Ca^{2+} stores from nerve terminals bathed in CFS. This was done in the presence of oligomycin (10 $\mu\text{g}/\text{ml}$), which prevents the reverse action of the mitochondrial ATPase from consuming ATP (Budd and Nicholls, 1996). Oligomycin on its own had no effect on Ca^{2+} homeostasis (data not shown). Once the Ca^{2+} signal had stabilized after the addition of CCCP, the addition of α -LTX (0.5 nM) did not produce any further increase in intracellular Ca^{2+} ($2.4 \pm 1\%$; *N*, *n* = 3,3; Fig. 3*A*). Similarly, when nerve terminals bathed in CFS were pretreated with α -LTX (0.5 nM), the addition of CCCP (10 μM) produced no further change in intracellular Ca^{2+} ($1.3 \pm 1\%$; *N*, *n* = 4,4; Fig. 3*B*). Because the effects of CCCP and α -LTX on Ca^{2+} mobilization were mutually occlusive, this suggests that α -LTX targets mitochondrial Ca^{2+} pools. In both cases, further increases in the Ca^{2+} signal were not prevented as a result of dye saturation because the dynamic range of the dye, determined before the experiment by nerve stimulation in NPS (see Fig. 1*A*), was on average at least two times larger than the Ca^{2+} signal produced by α -LTX or CCCP. Furthermore, replacing the bath with NPS at the end of the experiment rapidly produced a much larger Ca^{2+} signal than that produced by any combination of CCCP and α -LTX (Fig. 3*A,B*). Both of these observations indicate that larger Ca^{2+} signals could have been detected in these occlusion experiments. The Ca^{2+} signal produced by adding Ca^{2+} back to the bath was insensitive to the Ca^{2+} channel blocker Cd^{2+} (100 μM CaCl_2 added to saline; data not shown). This suggests that Ca^{2+} must have entered through toxin-induced pores and not through Ca^{2+} channels.

It is possible that stimulus-dependent Ca^{2+} entry during the experiment may have caused mitochondria to accumulate Ca^{2+} . Similarly, it is possible that, during the long incubation to allow dye transport to terminals, mitochondria accumulated Ca^{2+} to the extent that the results are an artifact of the incubation time. To examine these possibilities, we avoided the long incubation time by loading the dye for only 3 hr at room temperature into nerves cut close to the muscle. Furthermore, the nerve was left unstimulated for the duration of the experiment, and 4 μM TTX was added to prevent spontaneous nerve activity. When 10 μM CCCP was applied to nerve terminals bathed in CFS, a large Ca^{2+} signal was produced ($155 \pm 1\%$; *N*, *n* = 1,5). Because this signal is greater than the signal produced by the terminals incubated overnight and by stimulation during the experiment, we conclude that Ca^{2+} release by mitochondria is not an artifact of incubation time or nerve-evoked activity.

Ca^{2+} mobilization by α -LTX is Na^+ -dependent

We next asked how α -LTX signals the mitochondria to release Ca^{2+} . Because α -LTX forms a pore in frog nerve terminals (Davletov et al., 1998), we tested the hypothesis that Na^+ entry through this pore causes mitochondria to lose Ca^{2+} . It has been shown previously that methods that increase intracellular Na^+ at nerve terminals also cause an increase in transmitter release (Baker and Crawford, 1975; Meiri et al., 1981; Atwood et al., 1983). To determine whether α -LTX increases intracellular Na^+ , we detected changes in the intracellular Na^+ concentration with the fluorescent indicator sodium green-dextran loaded in nerve terminals that were bathed in CFS. Application of α -LTX (0.5 nM) caused the Na^+ signal to increase by $42 \pm 4\%$ (*N*, *n* = 5,5). Similar responses were observed with 5 nM α -LTX (Fig. 4*A*). Entry of Na^+ , however, was not attributable to the opening of voltage-gated Na^+ channels because these were blocked by 4 μM TTX.

Figure 3. CCCP and α -LTX release Ca^{2+} from the same store. *A, Left*, Change in Ca^{2+} fluorescence when α -LTX was applied after 10 μM CCCP, followed by 0.5 nM α -LTX to a nerve terminal bathed in CFS with 4 μM TTX and 10 $\mu\text{g/ml}$ oligomycin. There was a 10 min wash period (*Wash*) with CFS between the application of CCCP and α -LTX. When NPS (i.e., containing 1.8 mM Ca^{2+}) was applied (Ca^{2+}) after α -LTX, there was a large increase in Ca^{2+} fluorescence. *A, Right*, Bar graph compares the additional average peak Ca^{2+} fluorescence achieved when α -LTX was applied after CCCP [i.e., $(\Delta F/F)_{\alpha\text{-LTX} + \text{CCCP}} = ([F_{\alpha\text{-LTX} + \text{CCCP}} - F_{\text{rest}}] - [F_{\text{CCCP}} - F_{\text{rest}}]) / F_{\text{rest}}$] with the average peak Ca^{2+} fluorescence achieved when α -LTX was applied alone in other experiments [i.e., $\Delta F/F_{\alpha\text{-LTX}} = ([F_{\alpha\text{-LTX}} - F_{\text{rest}}] / F_{\text{rest}})$]. The value for $\Delta F/F_{\alpha\text{-LTX}}$ was normalized to 100%. An asterisk indicates a significant difference in Ca^{2+} fluorescence relative to control. *B*, Change in Ca^{2+} fluorescence when CCCP was applied after α -LTX. The bar graph comparisons are the same as in *A*.

To determine whether the increase in intracellular Na^{+} caused the increase in intracellular Ca^{2+} , we removed extracellular Na^{+} by choline substitution (Fig. 4*B*). When α -LTX was applied to nerve terminals bathed in NCFS (i.e., no Ca^{2+} or Na^{+}), the Na^{+} fluorescence decreased ($-16.7 \pm 2\%$; $N, n = 3, 3$). The decrease in intracellular Na^{+} was not attributable to dye loss because the Na^{+} signal increased when extracellular Na^{+} was reintroduced. Thus, unlike results in CFS, increases in the intracellular Na^{+} concentration do not occur when nerve terminals are treated with α -LTX in NCFS. When changes in intracellular Ca^{2+} were measured in NCFS, the effect of α -LTX on Ca^{2+} mobilization was reduced by 70% ($N, n = 4, 13$; Fig. 4*B*). This suggests that Na^{+} influx is necessary for Ca^{2+} mobilization by α -LTX.

Is Na^{+} entry required for α -LTX-dependent exocytosis?

To determine whether transmitter release by α -LTX still occurred in the absence of extracellular Na^{+} and Ca^{2+} , we could not use electrophysiological techniques because there would have been no Na^{+} -dependent postsynaptic current. Therefore, we measured changes in fluorescence from terminals for which the vesicles had been loaded with FM1-43 by nerve stimulation (see Materials and Methods; Cochilla et al., 1999). In this manner, FM1-43 was taken up into vesicles during endocytosis and was released during exocytosis. When α -LTX was applied in NCFS, the nerve terminals, which had accumulated FM1-43 previously, lost most of their fluorescence in 40 min (Fig. 4*C*). Similar results were obtained in two other experiments. This suggests that Na^{+} entry is not required by α -LTX to stimulate the fusion of synaptic vesicles.

Relationship between Ca^{2+} and α -LTX-induced transmitter release

To examine the role of Ca^{2+} in mediating the effects of α -LTX on spontaneous transmitter release, we used the cell-permeant Ca^{2+} chelator BAPTA-AM to quell changes in intracellular free Ca^{2+} . To maximize the effect of BAPTA-AM at the time of α -LTX action, (1) we added an anion pump inhibitor, probenecid (1 mM), to minimize the loss of BAPTA from the cytosol (Ouanounou et al., 1996); (2) we gave a second treatment of BAPTA-AM 15 min after the first (final concentration 200 μM) to get a longer-lasting effect of the chelator; and (3) we added α -LTX at 10 times the normal concentration to hasten the action of the toxin (time to onset, <2 min) 15 min after the second BAPTA-AM addition. Following these criteria ensured that the effects of α -LTX were observed when Ca^{2+} buffering was at its strongest. In CFS, BAPTA-AM significantly reduced the toxin-induced increase in Ca^{2+} fluorescence by $\sim 94\%$ ($N, n = 3, 3$) but had no effect on the acceleration of transmitter release (>300 MEPPs/sec; $N, n = 2, 2$; Fig. 5). The data suggest that Ca^{2+} released by α -LTX from intracellular stores does not play a major role in toxin-induced exocytosis.

DISCUSSION

α -Latrotoxin releases intracellular Ca^{2+}

The first finding here is that α -LTX causes an increase in the intracellular Ca^{2+} concentration to physiologically significant levels that are sufficient to trigger exocytosis. Because these experi-

Figure 4. The role of Na^+ in the action of α -LTX. *A*, α -LTX increases intracellular Na^+ . The graph shows measurements from a simultaneous recording of spontaneous transmitter release (black dots) and Na^+ fluorescence (white dots) in a motor nerve terminal after treatment with 5 nM α -LTX (bar). Similar results were obtained in two other experiments. *B*, α -LTX-induced Na^+ and Ca^{2+} signals (0.5 nM) in CFS (black) and NCFS (white) with 4 μM TTX. Values were normalized to the effects of the α -LTX in CFS and were displayed as a percentage of control. The left and right pairs of bar graphs show the change in intracellular Na^+ and Ca^{2+} , respectively, after the application of α -LTX in CFS and NCFS. Both results in NCFS were significantly different from results in CFS (*). *C*, α -LTX causes exocytosis in the absence of extracellular Na^+ and Ca^{2+} . The graph shows the changes in vesicular FM1-43 fluorescence after the application of 5 nM α -LTX to nerve terminals bathed in NCFS containing 4 μM TTX. Similar results were obtained in two other experiments. The insets show pictures of the terminal when α -LTX first was applied (5 min) and then 15 and 30 min later. Note the disappearance of fluorescent spots that correspond to clusters of labeled vesicles. In these images the contrast has been reversed so that bright areas appear dark.

ments were performed in CFS, there must have been a release of intracellular Ca^{2+} in the motor nerve terminals. It is clear from these results that presynaptic terminals do not lose all of their organelle Ca^{2+} during a 1 hr incubation in CFS.

The source of released Ca^{2+}

The next set of experiments revealed some details about the source of this released Ca^{2+} . In nerve terminals, Ca^{2+} is found in ER, mitochondria, and synaptic vesicles (Meldolesi et al., 1988). High-resolution electron spectroscopic imaging showed that Ca^{2+} in frog motor nerve terminals was found predominantly in synaptic vesicles and the lumen of smooth ER cisternae (Grohovaz et al., 1996; Pezzati and Grohovaz, 1999). Parts of mitochondria also appear to contain Ca^{2+} but at a lower concentration than vesicles. In rat brain synaptosomes, α -LTX binding to CL1 stimulates PLC that mobilizes Ca^{2+} from intracellular stores (Davletov et al., 1998). Although α -LTX stimulates the breakdown of phosphoinositides (Vicentini and Meldolesi, 1984), this is not critical to the toxin mechanism because an α -LTX mutant, which on binding still triggers the breakdown of phosphoinositides, cannot stimulate exocytosis (Ichtchenko et al., 1998). Furthermore, activation of PLC by α -LTX in synaptosomes is dependent on the presence of extracellular Ca^{2+} (Davletov et al., 1998). At the NMJ the source of released Ca^{2+} by α -LTX is unlikely to be the ER, because a PLC inhibitor and a blocker of ER Ca^{2+} uptake both failed to affect α -LTX-induced Ca^{2+} signals.

Our occlusion experiments showed that CCCP, which is known to release mitochondrial Ca^{2+} , released the same pool of Ca^{2+} as that released by α -LTX. The amount of Ca^{2+} released by CCCP is

similar to that released by α -LTX and is sufficient to cause a sustained increase in spontaneous transmitter release from a resting value of ~ 1 to 102 MEPP/sec (Table 1; Alnaes and Rahamimoff, 1975; Zengel et al., 1994). Therefore, the release of Ca^{2+} by α -LTX is likely to reach physiologically significant concentrations.

Mitochondria in lizard motor nerve terminals acquire and release Ca^{2+} during physiological stimulation (David et al., 1998; David, 1999). In contrast to our results, David (1999) did not report that CCCP could release mitochondrial Ca^{2+} although the uptake of Ca^{2+} was blocked. However, his study used the Ca^{2+} indicator Oregon green BAPTA-5N, which has much lower affinity than the Oregon green BAPTA-1-dextran (60 μM vs 170 nM) used in our experiments. In addition to species differences, another difference between our study and that of David (1999) is that we used 10-fold more CCCP and applied it for a longer period of time; this enhances the possibility of detecting Ca^{2+} release.

We cannot rule out the possibility that synaptic vesicles, which occupy most of the terminal volume, could release Ca^{2+} also (for review, see Gonçalves et al., 2000). For instance, Gonçalves et al. (1998) showed that synaptic vesicles can acquire Ca^{2+} and that uptake is blocked by CCCP. Release of Ca^{2+} by vesicles was not demonstrated.

Mechanism of α -LTX signaling

α -LTX elevated the intracellular Na^+ concentration as expected from the action of a nonspecific cation channel (Finkelstein et al., 1976) inserted in the presynaptic membrane. In the absence of extracellular Na^+ , α -LTX caused the loss of Na^+ , and the release of stored Ca^{2+} was reduced greatly. Therefore, it appears that

Figure 5. α -LTX causes transmitter release with minimal change in the intracellular Ca^{2+} concentration. Shown are peak Ca^{2+} fluorescence and peak MEPP frequency achieved by 5 nM α -LTX from terminals treated with (white) or without (black) 200 μM BAPTA-AM in CFS supplemented with 4 μM TTX and 1 mM probenecid. Values for Ca^{2+} fluorescence and MEPP frequency were normalized and expressed as a percentage of control (5 nM α -LTX in CFS). α -LTX-induced Ca^{2+} fluorescence after BAPTA-AM was reduced significantly as compared with α -LTX control (*).

Na^+ influx caused by α -LTX is primarily responsible for the mobilization of Ca^{2+} , possibly by activating the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Because removing extracellular Na^+ did not block completely all of the Ca^{2+} that was released, there may have been an additional mechanism operating.

Black widow spider venom causes nerve terminals to swell in a Na^+ -dependent manner (Gorio et al., 1978). We confirmed, with observations of terminals filled with fluorescent indicators, that swelling with α -LTX occurs in CFS but does not occur in NCFS (data not shown). It is unlikely that swelling is responsible for the effects of α -LTX on transmitter release, because it has been shown that the frequency of exocytotic fusion events is reduced considerably as terminals swell (Solsona et al., 1998). Moreover, our experiments in NCFS showed that swelling was not required for α -LTX effects.

α -LTX increases spontaneous exocytosis in the absence of extracellular Ca^{2+} provided that Mg^{2+} or another divalent cation is present (Misler and Hurlbut, 1979; Misler and Falke, 1987). Although our results cannot rule out the possibility that α -LTX allows the entry of extracellular Mg^{2+} , it is unlikely that an increase in intracellular Mg^{2+} is responsible for the Ca^{2+} signal, because the Ca^{2+} indicator dye is ~ 200 times less sensitive to Mg^{2+} than Ca^{2+} (Molecular Probes). Because α -LTX probably depolarizes the nerve terminal by increasing membrane conductance to Na^+ , the extent to which the intracellular Mg^{2+} concentration could increase would be small. Furthermore, even if α -LTX caused the intracellular Mg^{2+} concentration to increase, Mg^{2+} is not a good substitute for Ca^{2+} in triggering transmitter release (Miledi, 1973). The most likely explanation for the requirement of extracellular Mg^{2+} is that it is required for α -LTX to form functional pores in the membrane (Orlova et al., 2000).

Release of Ca^{2+} is not necessary for α -LTX action

α -LTX does not require extracellular Na^+ to stimulate exocytosis. We showed that α -LTX triggers exocytosis of FM1-43-labeled vesicles in the absence of extracellular Na^+ and Ca^{2+} (see Fig. 4C). Under these conditions the intracellular Ca^{2+} signal is reduced greatly. This supports previous ultrastructural data demonstrating that nerve terminals lose their vesicles after treatment with black widow spider venom in Na^+ - and Ca^{2+} -free saline (Gorio et al., 1978). Similarly, Na^+ is not required by α -LTX to stimulate the secretion of radiolabeled neurotransmitters from rat brain synaptosomes (Deri et al., 1993; Storchak et al., 1994).

The Ca^{2+} dependence of α -LTX action on exocytosis has been controversial. For instance, in rat brain synaptosomes and adrenal

chromaffin cells some studies show that transmitter release by α -LTX depends on the presence of extracellular Ca^{2+} and a rise in intracellular Ca^{2+} (Davletov et al., 1998; Liu and Misler, 1998; Rahman et al., 1999). However, other studies in these same systems have shown that α -LTX does not require any increase in intracellular Ca^{2+} to stimulate exocytosis (Meldolesi et al., 1984; Michelena et al., 1997). Studies on PC12 cells and β -pancreatic cells have reached the latter conclusion (Meldolesi et al., 1984; Lang et al., 1998).

At the frog NMJ, α -LTX appears to stimulate vesicular exocytosis independently of extracellular Ca^{2+} and any increase in intracellular Ca^{2+} . When the amplitude of Ca^{2+} signals was reduced vastly in NCFS (see Fig. 4C) or after the application of an intracellular Ca^{2+} chelator (see Fig. 5), the effect of α -LTX appeared undiminished. Although we cannot prove that the chelator controlled Ca^{2+} signals in microdomains at vesicle fusion sites in these experiments, as little as 25 μM BAPTA-AM can reduce stimulus-evoked transmitter release in this preparation drastically (Robitaille and Charlton, 1992; Robitaille et al., 1993). It therefore appears that acceleration of exocytosis by α -LTX can occur by a mechanism different from that used in normal Ca^{2+} -regulated secretion. This is a plausible conclusion because, in systems in which synaptotagmin function is impaired by peptide injection or genetic mutation, Ca^{2+} -regulated secretion by ionophores and depolarizing agents is impaired, yet acceleration of transmitter release by α -LTX remains unaffected (Geppert et al., 1994; Thomas and Elferink, 1998). Similarly, munc13-1, a phorbol ester receptor essential for Ca^{2+} -dependent exocytosis in glutamatergic neurons, is not required for exocytosis by α -LTX (Augustin et al., 1999). In contrast, it is possible that α -LTX increases the sensitivity of transmitter release to Ca^{2+} . For instance, in permeabilized cells α -LTX causes more transmitter release than Ca^{2+} -ionophores or high K^+ solutions, given the same extracellular Ca^{2+} concentration (Davletov et al., 1998). We also have seen that the frequency of spontaneous transmitter release with α -LTX far exceeds that obtained with CCCP, although the Ca^{2+} signals produced by both agents are similar.

The action of α -LTX contrasts with that of α -latrocrustatoxin (α -LCTX), a similar toxin in black widow spider venom. α -LCTX also causes increased spontaneous transmitter release in crustacean synapses, but this action requires only the elevation of intracellular Ca^{2+} concentration subsequent to Ca^{2+} entry via a pore (Elrick and Charlton, 1999).

In conclusion, our results provide a more complete picture of the actions of α -LTX at the frog NMJ, the classic preparation in which α -LTX action first was described. Our data show that assumptions about Ca^{2+} independence of drug and toxin effects in the absence of extracellular Ca^{2+} must be tested. Experiments that are designed to test hypotheses of Ca^{2+} -independent mechanisms of α -LTX action would be confused by the exocytosis triggered by intracellular Ca^{2+} release. Furthermore, α -LTX is used frequently as a tool to obtain Ca^{2+} -independent exocytosis, and the interpretation of these experiments too may be complicated by the release of intracellular Ca^{2+} . The data also show that the main effect of α -LTX in the NMJ is not via a Ca^{2+} -dependent mechanism.

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