MECHANISM OF α-LATROTOXIN ACTION AT THE
FROG NEUROMUSCULAR JUNCTION

by

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A thesis in conformity with the requirements
for the degree of Master of Science
Graduate Department of Physiology
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University of Toronto, Department of Physiology.

ABSTRACT

α-Latrotoxin (α-LTX) is a neurotoxin purified from black widow spider venom that accelerates spontaneous transmitter release independently of extracellular Ca\(^{2+}\). Since transmitter release is Ca\(^{2+}\)-dependent, I hypothesized that α-LTX mobilized intracellular Ca\(^{2+}\). To address this question, standard electrophysiology and confocal imaging techniques were used to measure the rate of transmitter release and ion concentration dynamics respectively in frog motor nerve terminals bathed in CFS. Here I report that transmitter release by α-LTX follows an increase in intracellular Ca\(^{2+}\). The Ca\(^{2+}\) pool targeted by α-LTX is likely to be mitochondrial in origin and not endoplasmic reticulum. An increase in intracellular Na\(^{+}\) precedes the release of Ca\(^{2+}\) suggesting that the mitochondrial Na\(^{+}/Ca\(^{2+}\) exchanger may be activated. There is also evidence to suggest that the mitochondrial permeability transition pore may be involved. Although the release of Ca\(^{2+}\) may accompany the increase in transmitter release produced by α-LTX, it is not necessary.
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LIST OF ABBREVIATIONS

α-Bungarotoxin (α-BuTx)
Ca$^{2+}$-Independent Receptor for α-Latrotoxin (CI RL)
Carbonyl Cyanide m-Chlorophenylhydrazone (CCCP)
CI RL/Latrophilin (CL1)
μ-Conotoxin GlIIIA (μ-CgTx)
Cyclic AMP (cAMP)
Cyclosporin-A (CsA)
Diacyl Glycerol (DAG)
Endoplasmic Reticulum (ER)
End-Plate Potential (EPP)
Inositol trisphosphate (IP$_3$)
α-Latrocrustatoxin (α-LCT)
x-Latroinsectotoxin (x-LIT, where x is α, β, γ, ε, or δ)
α-Latrotoxin (α-LTX)
Miniature End-Plate Potential (MEPP)
Mitochondrial Permeability Transition Pore (MPT pore)
Neuromuscular Junction (NMJ)
Perisynaptic Schwann Cell (PSC)
Phospholipase C (PLC)
Phosphatidylinositol 4,5-bisphosphate (PIP$_2$)
Protein Kinase A (PKA)
Protein Kinase C (PKC)
Sarcoplasmic/Endoplasmic Reticulum Ca$^{2+}$ ATPase (SERCA)
Tetrodotoxin (TTX)
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1 INTRODUCTION

Toxins are substances that are naturally occurring (plant animal, bacteria etc.), exogenous to the victim, and adverse or deleterious when inoculated (Vogt, 1970). The use of toxins in biological research has allowed major advances in various scientific investigations. Neurotoxins are toxins that specifically target nervous tissue. The fascination with neurotoxins among neuroscientists arises from the diverse mechanisms of interaction with neuronal systems. Some neurotoxins affect (primarily or exclusively) the structure and/or function of nerve terminals making the toxins valuable tools for studying neurophysiological mechanisms.

1.1 THE BLACK WIDOW SPIDERS

Out of the hundreds of known neurotoxins, many are produced by arachnids such as scorpions and spiders. For instance the black widow spider (Latrodectus mactans) has been particularly important (Figure 1.1). The most commonly recognized feature of female black widow spiders is a reddish hourglass pattern on the abdomen. Mature female widow spiders generally have a round shape and reach lengths of about 1/4 to 1/3 inch making them the largest of the Cobweb Weavers (Family Therididae). The non-venomous males are brown and half the size of the females.

1.1.1 Habitat

Black widow spiders are located throughout temperate and tropical areas of the world. They are commonly found in ground cover, woodpiles, crevices, barns, and outhouses where they spin irregular, untidy, three-dimensional silk webs. The females hang belly upward, rarely leaving the web except for cold weather and drought. The web is typically used to trap prey, which include a variety of insects (beetles and cockroaches) and other arthropods.

1.1.2 Envenomation

The black widow spider kills its prey by envenomation with highly potent neurotoxins. The oily yellowish venom containing the toxin is usually injected when the spider’s two sharp fangs or chelicerae, penetrate the body of the victim. In humans, black widow spider bites are associated with significant discomfort however they are seldom fatal, posing threats only to the very young and elderly. One to two hours after
Figure 1.1. The black widow spider (*Latrodectus mactans tredecimguttatus*).

http://www.baxter508.k12.ks.us/students/2006/wfry/black_widow_spider.htm
envenomation, bite victims commonly experience symptoms such as muscle spasms, abdominal cramping, weakness of the legs, tightness of the chest, and signs of autonomic hyperactivity (e.g. nausea, vomiting, diarrhea, tachycardia, and hypertension). Although the effects induced by black widow spider venom are broad and include changes in various organs and systems, they can be explained to some extent by stimulation of neurotransmitter release from synaptic terminals.

1.2 α-LATROTOXIN

The venom of the black widow spider contains multiple toxin proteins that have similar physiological effects (e.g. to increase transmitter release) but different phylogenetic targets. So far seven different neurotoxins belonging to the same family have been isolated from crude venom glands (Grishin, 1998). Latrocrustatoxin (α-LCT) is the crustacean-specific neurotoxin, Latroinsectotoxins (α,β,γ,ε, and δ-LIT) are insect-specific neurotoxins, and α-Latrotoxin (α-LTX) is the vertebrate-specific neurotoxin. The latter is the most studied neurotoxin from the venom of Latrodectus mactans tredecimguttatus (the common black widow in the northern Mediterranean) and is the main focus of this thesis.

1.2.1 Purification

Acquiring sufficient toxin to do any meaningful research has proved to be a difficult task because most spiders produce only a very minute quantity of venom. The advantage of using black widow spiders is that they have large venom glands and therefore produce relatively substantial amounts of toxin. When aqueous extracts of crude venom glands are separated by chromatography on a Sephadex gel filtration column, α-LTX and δ-LIT co-elute because they have similar molecular mass. α-LTX can be then be separated from δ-LIT using ion exchange and hydrophobic chromatography (Krasnoperov et al., 1992). The effects of injecting the α-LTX fraction into mice largely coincide with those of the black widow spider bite (LD<sub>50</sub> ~20 μg/kg body weight), whereas α-LCT or any LIT show no toxic activity in mice at doses more than 5 mg/kg of body weight.

Regardless of the isolation methods, a low molecular weight component (M<sub>r</sub> ~8 kDa) composed of 70 amino acid residues always co-purifies with α-LTX (Kiyatkin et al., 1992). This protein is known as latroductin (Pescatori et al., 1995) and is
structurally related to crustacean hyperglycemic hormones (Gasparini et al., 1994). It is unlikely that latrotoxin is the active ingredient in \( \alpha \)-LTX preparations because it does not confer any symptoms of intoxication when injected into mice (Gasparini et al., 1994; Volkova et al., 1995).

1.2.2 Structure

\( \alpha \)-LTX is a slightly acidic protein with an isoelectric point between pH 5.2 - 5.5 and appears to have no glycosylation or association with any proteolytic or lipolytic activities (Frontali et al., 1976). Cloning and sequencing reveals that cDNA encoding \( \alpha \)-LTX contains a 4203 base-pair open reading frame corresponding to a protein composed of 1401 amino acids with a predicted molecular mass of 156 855 Da (Kiyatkin et al., 1990). When \( \alpha \)-LTX is run on an SDS gel, an apparent molecular mass of 131 kDa is revealed for this single polypeptide protein. The difference between the predicted and measured molecular mass is likely due to a post-translational modification of the polypeptide chain, which is initially coded as a precursor. This is supported from observations that expression of full-length \( \alpha \)-LTX cDNA results in an insoluble, functionally inert protein (Kiyatkin et al., 1995). Furthermore, both the N- and C-terminus of \( \alpha \)-LTX are flanked by amino acids that constitute potential recognition sites (R-X-R/K-R) of furin, a subtilisin-like proteolytic enzyme involved in the processing of many protein precursors (Stieneke-Grober et al., 1992). Proteolysis at both sites would produce a protein of exactly 131.5 kDa (encompassing base pairs 142 - 3679, which encode amino acids 1 - 1179). Furthermore, when \( \alpha \)-LTX is digested by trypsin, only peptide fragments in-between these two putative furin cleavage sites are found (Volkova et al., 1991). Therefore, it is possible that during maturation a furin-like protease hydrolyses \( \alpha \)-LTX, producing an active toxin.

All the LTXs contain four readily identifiable domains in their sequence indicating that they have evolved from the same ancestral gene (Figure 1.2). The first is presumed to be a signal sequence; the second, the N-terminal region; the third is composed of ankyrin-like repeats and the fourth, the C-terminal region. The N-terminal region, containing two hydrophobic segments that are positionally well conserved, shows the greatest similarity between all LTXs. In contrast, the sequence preceding the N-terminal domain, which is removed during maturation, shows the least similarity between all LTXs.
Latrotoxins have an N-terminal domain, a central domain consisting of tandem ankyrin repeats, and a C-terminal domain. There is striking similarity in all the Latrotoxins. Only a fragment of α-LCT (which is not shown) has been sequenced to date (Volynskii et al., 1999). However, it is known that α-LCT has similar domain organization to the rest of the Latrotoxins. Figure was taken from (Grishin, 1998)
Identification of ankyrin-like repeats within the toxin molecule is surprising because α-LTX is an extracellular toxin whereas in all other proteins, ankyrin-like repeats are cytoplasmic. Ankyrins constitute a family of proteins with 22 copies of an internal repeat that co-ordinate interaction between various integral membrane proteins and cytoskeletal elements. Ankyrin-like repeats are present in a large number of functionally different proteins (e.g. cell cycle proteins, enzymes, transcription factors), which are widespread from prokaryotes to humans (Bork, 1993) suggesting that they do not have a unique function but rather serve as a general structural domain. For example, it may be used by α-LTX for high affinity binding to integral membrane proteins on the plasma membrane.

1.3 ACTION OF α-LATROTOXIN

α-LTX has been used as tool to study the molecular mechanisms of secretion in a variety of cell systems, such as the neuromuscular junction (NMJ; Longenecker et al., 1970), sensory nerve terminals (Queiroz and Duchen, 1982), synaptosomes (Grasso, 1976), hippocampal cells (Capogna et al., 1996), chromaffin cells (Liu and Misler, 1998), PC12 cells (Grasso et al., 1980), and pancreatic cells (Lang et al., 1998). Many studies have been done on the frog NMJ because it can be investigated with relative ease and convenience, and it has properties in common with many other synapses. The frog NMJ was also used as a model synapse in my research. Therefore most of the subsequent background information will focus on the NMJ, and the effects of α-LTX in other cell systems will only be brought up for comparison and contrast.

1.3.1 Transmitter release

At the frog NMJ, acetylcholine is released both in a steady ‘leak’ (Katz and Miledi, 1977), and in multi-molecular packets, or quanta (Castillo and Katz, 1954). The ‘leak’, or non-quantal component of release, is difficult to measure directly since its major known effect is to produce a small, steady postsynaptic depolarization at an endplate when acetylcholinesterase has been thoroughly inhibited. Quantal release can easily be measured by electrophysiological means because it generates the endplate and miniature endplate potentials (EPPs, MEPPs) in muscle fibres (del Castillo and Katz, 1954). Each MEPP represents the release of one vesicle of acetylcholine molecules (Hurlbut et al., 1990) and is regarded as the smallest unit of the EPP. Unlike
EPPs, which are generated in response to nerve stimulation, MEPPs can occur spontaneously (Fatt and Katz, 1952).

\( \alpha \)-LTX accelerates the occurrence of spontaneous MEPPs from resting levels of about 1-2 per second to levels as high as several hundred per second in muscle fibres of the frog (Longenecker et al., 1970). After reaching a peak, the frequency of spontaneous transmitter release subsides to levels less than 1 per second. What makes \( \alpha \)-LTX interesting is that it can stimulate exocytosis in the complete absence of extracellular Ca\(^{2+} \) so long as another divalent cation is present (Misler and Falke, 1987; Misler and Hurlbut, 1979). It is well accepted that nerve-evoked transmitter release is dependent on Ca\(^{2+} \) entry, therefore the effect of \( \alpha \)-LTX has raised interest in the possibility of a Ca\(^{2+} \)-independent mechanism for exocytosis. There are no known pharmacological inhibitors of \( \alpha \)-LTX action; however, concanavalin A, a lectin from Jack bean, inhibits the action of the toxin non-specifically by greatly reducing its ability to access the nerve terminal (Grasso et al., 1978).

### 1.3.2 Fine Structure

At the ultrastructural level \( \alpha \)-LTX causes synaptic vesicles to become depleted from nerve terminals (Clark et al., 1972; Clark et al., 1970; Rose et al., 1978). Depletion of synaptic vesicles only occurs in the absence of extracellular Ca\(^{2+} \) because vesicle recycling, which normally operates to maintain a constant vesicle supply during physiological stimulation, is blocked under this condition (Ceccarelli and Hurlbut, 1980; Henkel and Betz, 1995; Torri-Tarelli et al., 1990; Valtorta et al., 1988). The fact that the number of vesicles lost correlates with the number of MEPPs counted further suggests that release induced by \( \alpha \)-LTX is quantal in nature (Hurlbut et al., 1990).

\( \alpha \)-LTX appears to leave the architecture of the NMJ unchanged with the exception of motor nerve terminals (Clark et al., 1972; Clark et al., 1970). In toxin-treated preparations bathed in Ca\(^{2+} \)-free saline, nerve terminals become swollen and increase in surface area due to the addition of vesicle membrane. Inside the nerve terminal, mitochondria, endoplasmic reticulum, neurofilaments, and neurotubules appear normal. Other structures around the nerve terminal such as muscle fibres, Schwann cells, fibroblasts, capillaries, and axons also appear to be morphologically unaffected by the toxin.
1.4 MECHANISM OF α-LATROTOXIN ACTION

While α-LTX demonstrates similar physiological effects in many different cells, the complexity and cellular heterogeneity of the preparations used have hampered studies of the mechanism at the biochemical level. For instance, the Ca²⁺ dependency of α-LTX action differs in different cell systems. Unlike the frog NMJ, chromaffin cells only respond to α-LTX when extracellular Ca²⁺ is present (Barnett et al., 1996; Liu and Misler, 1998). Furthermore, the nature of α-LTX-stimulated transmitter release differs in different cell systems. In rat brain synaptosomes, addition of botulinum neurotoxins, which potently blocks vesicular quantal transmitter release, had no effect on the actions of α-LTX in the absence of extracellular Ca²⁺ (Davletov et al., 1998; Rahman et al., 1999). Therefore it was suggested that the Ca²⁺-independent mechanism of α-LTX action was due to non-vesicular leakage of neurotransmitters through pores opened on the plasma membrane. This is in marked contrast to the quantal nature of transmitter release stimulated by α-LTX in Ca²⁺-free saline from motor nerve terminals. The mechanism of α-LTX action remains unknown. To date, three mechanisms have been proposed to explain how α-LTX may increase spontaneous quantal transmitter release at the frog NMJ.

1.4.1 Pore-Forming Hypothesis

α-LTX is known to increase the conductance in black lipid bilayer membranes (Finkelstein et al., 1976). The magnitude of each conductance step is several hundred pS suggesting that the toxin molecule may act as a channel former (carriers exhibit much smaller conductances). These pores are selective for cations with no significant discrimination between Na⁺ and K⁺. They also display substantial permeability to Ca²⁺. α-LTX inserts itself into membranes with a specific polarity (Chanturiya et al., 1996). The pore forming ability of α-LTX is not due to the small molecular weight protein, latrotoxin, since this protein cannot produce channels in black lipid bilayer membranes (Gasparini et al., 1994). However, latrotoxin has been shown to modulate Ca²⁺ influx by α-LTX in synaptosomes (Grishin et al., 1993).

At the frog NMJ, nerve terminals become permeable to molecules of a few hundred Daltons after α-LTX treatment (Davletov et al., 1998). Evidence suggests that α-LTX increases the conductance of the plasma membrane to cations, which short
circuit the nerve terminal spike resulting in blockade of neuromuscular transmission (Longenecker et al., 1970). This suggestion is also consistent with observations that nerve terminals become swollen after treatment with α-LTX (i.e. the toxin causes ions to accumulate within the nerve resulting in an increased osmotic pressure). There are several pieces of evidence to suggest that Na⁺ is the dominant cation mediating the effects of α-LTX in Ca²⁺-free saline. 1) α-LTX depolarizes nerve terminals, which cannot be due to Cl⁻ efflux since toxin-induced channels are selective for cations, 2) replacement of Na⁺ with glucosamine, which is impermeable to the toxin-induced channels in lipid bilayers (A. Finkelstein, unpublished data), greatly reduced the amount of nerve terminal swelling (Gorio et al., 1978), and 3) Mg²⁺ influx is unlikely because of the small electrochemical driving force (see discussion section 4.2.3).

There are some problems associated with the pore-forming hypothesis of α-LTX action. Many molecules are known to increase conductance in bilayers by causing general disruption of the membrane in a non-specific, detergent-like manner (Finkelstein, 1974). According to the hydropathy profile and sequence analysis, α-LTX contains only two hydrophobic regions that are of a sufficient length to constitute a conventional membrane-spanning α-helix and its primary structure does not reveal direct sequence homology with any channel-forming proteins (Kiyatkin et al., 1990). Therefore, it is unlikely that α-LTX would form an ion channel by itself. Even if α-LTX did make the membrane more permeable to cations, it has been shown in frog motor nerve terminals that neither Ca²⁺ nor Na⁺ is required extracellularly for the toxin to increase transmitter release (Gorio et al., 1978).

Proponents of the general pore-forming theory argue that α-LTX could aggregate in solution to form a channel out of several toxin molecules (Lunev et al., 1991). They also argue that α-LTX can directly cause liposomes, on the opposite side of a planar lipid bilayer, to fuse with the membrane, which would by-pass any requirement for extracellular Ca²⁺ (Lishko et al., 1990; Sokolov et al., 1987). However, there is one property about α-LTX that cannot be explained by this theory, and that is the preferential targeting of the toxin to presynaptic nerve terminals (Valtorta et al., 1984).
1.4.2 Receptor Hypothesis

The specific localization of α-LTX at the NMJ suggested that an endogenous receptor might be involved. Furthermore, α-LTX displays high affinity binding in the nanomolar range in PC12 cells, which infers specific sites of interaction on the plasma membrane (Meldolesi et al., 1983). The involvement of receptors in α-LTX action is an attractive proposal because it provides a ready explanation for the phylum specificity of α-LTX. To date, two different families of high-affinity α-LTX receptors have been isolated and cloned (Ushkaryov et al., 1992; Lelianova et al., 1997; Krasnoperov et al., 1997).

1.4.2.1 Neurexins

Neurexins are a family of neuronal cell surface proteins with more than a thousand isoforms. These isoforms are generated in mammals by alternative splicing of transcripts from three genes each containing two promoters. Neurexin Iα is one isoform that was discovered by affinity chromatography using an immobilized α-LTX column (Petrenko et al., 1990). High affinity binding of recombinant neurexin Iα to α-LTX supported the hypothesis that neurexin Iα was a receptor for α-LTX (Davletov et al., 1995). Sequencing reveals that this receptor is composed of an N-terminal signal peptide followed by three overall repeats, an O-linked sugar attachment sequence, a single transmembrane region, and a short cytoplasmic tail (Ushkaryov et al., 1992; Figure 1.3). Each repeat consists of an epidermal growth factor-like domain flanked by two domains that are distantly related to each other and to repeats found in laminin A and sex hormone binding globulin suggesting that they probably serve as cell adhesion molecules. The cytoplasmic carboxyl-terminal end of neurexin Iα may be responsible for transducing extracellular signals to intracellular messages because it binds to CASK, a membrane-associated guanylate kinase homolog that contains multiple domains, several of which perform intracellular signalling roles in other proteins (e.g. CaM kinase domain, SH3 domain; Hata et al., 1996).

No one yet has determined if neurexins are present on motor nerve terminals. In Torpedo electric organ, neurexins are localized only to nerves and not nerve terminals (Russell and Carlson, 1997). Immunoprecipitation studies however, revealed that the cytoplasmic tail of neurexin Iα binds strongly to synaptotagmin, a synaptic vesicle
Figure 1.3. Domain structure of neurexin 1α.

Structure and alternative splicing of neurexin 1α. α-neurexin is shown schematically in the plasma membrane, with extracellular domains on the left and cytoplasmic sequences on the right. Domains are identified as follows: SP, signal peptide; LNS (A) and LNS (B), LNS domains; EGF, EGF-like sequences; CH, O-linked carbohydrate attachment sequences; and TMR, transmembrane regions. Positions of alternative splicing are numbered and are indicated by arrows above the bar diagrams. α-Neurexin contains three cassettes (marked I, II, and III) composed of LNS (A) - EGF - LNS (B) repeats. Figure taken from (Ushkaryov et al., 1992).
protein involved in secretion (Petrenko et al., 1991). Therefore, the action of α-LTX in Ca\(^{2+}\)-free saline could be explained if α-LTX binding to neurexin I\(\alpha\) triggered vesicle exocytosis directly. However, neurexin I\(\alpha\) only binds to α-LTX in the presence of Ca\(^{2+}\) (EC\(_{50}\) ∼30 μM; Davletov et al., 1995; Krasnoperov et al., 1996), so it cannot be responsible for the actions of the toxin in Ca\(^{2+}\)-free saline. This is supported by the fact that α-LTX could still increase transmitter release from nerve terminals that lack neurexin I\(\alpha\) (Geppert et al., 1998). Since my research specifically focuses on the Ca\(^{2+}\)-independent mechanism of α-LTX action, neurexin I\(\alpha\) will not be discussed in further detail.

1.4.2.2 Latrophilin / CIRL

When the search for the Ca\(^{2+}\)-independent receptor for α-LTX continued, another protein, which was able to bind to α-LTX (K\(_D\) ∼0.5 nM) in the presence or absence of any divalent cation was isolated (Davletov et al., 1996; Krasnoperov et al., 1996). This protein was present in receptor preparations purified in the presence of Ca\(^{2+}\) but had been previously perceived as α-LTX bleeding from the affinity column (Petrenko et al., 1991; O’Connor et al., 1993). This protein was cloned and sequenced independently by two labs (Krasnoperov et al., 1997; Lelianova et al., 1997) and was given the name CIRL (Calcium Independent Receptor for α-Latrotoxin) / Latrophilin. For convenience, this receptor will be referred to as CL1 in the rest of the thesis.

1.4.2.2.1 Sequence

Figure 1.4 shows that the large extracellular domain of CL1 begins with a hydrophobic signal peptide, followed by a cysteine-rich stretch homologous to galactose-binding lectin, an extended region of homology to olfactomedin, a mucin-like domain, and a glycosylated spacer fragment. The presence of these structural modules is typical for cell adhesion and extracellular matrix proteins. The cytoplasmic C-terminal portion of CL1 contains 5 cysteine residues that may be palmitoylated. There are also several possible phosphorylation sites in the molecule that may modulate the response to endogenous ligands under physiological conditions. Hydrophobicity plots indicate the presence of seven transmembrane segments, which is the hallmark of serpentine G-protein-coupled-receptors. The putative transmembrane regions of CL1 show the
Figure 1.4. Domain structure of CL1.

A picture showing the proposed transmembrane topology of CL1. The closed arrow in the amino terminus indicates the site of cleavage of the signal peptide sequence. The open arrow denotes the region of endogenous proteolytic processing of CL1. All cysteine residues are marked as SH. The domains with homology to galactose-binding lectin, olfactomedin, mucin, and a cytoplasmic proline-rich cluster are labelled as such. Figure taken from (Krasnoperov et al., 1997).
closest homology to the transmembrane regions of the secretin receptor family, which is part of the G-protein-coupled receptor superfamily (Gilman, 1987; Strader et al., 1994). Most of the receptors bind peptide hormones and are coupled through heterotrimeric G-proteins to the stimulation of various release processes.

1.4.2.2.2 Biochemical Properties

Cells require the integral membrane protein, CL1, for α-LTX binding and action (Lang et al., 1998; Lelianova et al., 1997). CL1 undergoes proteolytic cleavage close to the transmembrane region, producing an unusual two-subunit structure that consists of an 85 kDa fragment and a 120 kDa fragment (Krasnoperov et al., 1997). The latter is glycosylated with a low degree of sialylation (2-4 kDa) and about 25 kDa of N-linked carbohydrates (Davletov et al., 1996). CL1 and α-LTX form specific equimolar complexes with the high-affinity binding site located within the first transmembrane segment of the 85 kDa fragment and the C-terminal half of the 120 kDa fragment (Krasnoperov et al., 1999). Binding of α-LTX to CL1 does not require the lectin-like, olfactomedin-like, and mucin-like domains of the extracellular N-terminus.

CL1 interacts with intracellular proteins involved in signalling and exocytosis. In the presence of low micromolar magnesium and GDP, the heterotrimeric G proteins Gαq/11 and Gαo co-purify with CL1 (Rahman et al., 1999). Gαq/11 activates phospholipase C (PLC), which breaks down phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol trisphosphate (IP3), which releases Ca2+ from intracellular stores, and diacyl glycerol (DAG), which activates protein kinase C (PKC). The other G-protein, Gαo, inhibits adenylate cyclase, which indirectly reduces protein kinase A (PKA) activation because of decreased cyclic AMP (cAMP) production. In addition, Gαo can modulate the function of ion channels. Syntaxin, a component of the machinery thought to be critical for inducing the fusion of vesicles with their target membranes and a central component of the SNARE hypothesis (Rothman, 1994), also co-purifies with CL1 (Krasnoperov et al., 1997). Currently the significance of this interaction is unknown, however it is tempting to speculate that protein-protein interactions could mediate the Ca2+-independent actions of α-LTX on exocytosis.
1.4.2.2.3 Homologues and Distribution

CL1 is just one member of a family of three closely related G-protein-coupled receptors (Ichtchenko et al., 1999; Matsushita et al., 1999; Sugita et al., 1998). CLs are multiply spliced showing strong similarity within their lectin, olfactomedin, and transmembrane domains but strong differences in the C-terminal regions. CL2 and CL3 have far less affinity for α-LTX than CL1. CL1 is predominantly expressed in nervous tissue and is most abundant in the cerebral cortex. CL3 is also specific to nervous tissue, while CL2 is ubiquitously expressed. The CLs differential tissue distribution and binding affinities to α-LTX suggest that these receptors may have different functions upon binding to their endogenous ligand.

1.5 PURPOSE OF THIS STUDY

α-LTX is interesting to study not only in itself but because it can be employed as a tool to investigate specific aspects of presynaptic physiology. A better knowledge about α-LTX action will give us more insight in terms of molecular and functional definition of nerve terminals. For my study, α-LTX action was studied at the frog NMJ because it is the most thoroughly characterized model for the study of quantal release via small synaptic vesicles.

1.5.1 Hypothesis

Since α-LTX can increase spontaneous transmitter release in the absence of external Ca\(^{2+}\) it is widely believed that all its actions are independent of Ca\(^{2+}\). This is a logical conclusion because frog nerve terminals bathed in Ca\(^{2+}\)-free saline for extended periods of time still respond to α-LTX. However, it is possible that soaking in Ca\(^{2+}\)-free saline did not deplete Ca\(^{2+}\) stores and that α-LTX works by releasing intracellular Ca\(^{2+}\).

I tested the hypothesis that α-LTX mobilizes Ca\(^{2+}\) from intracellular stores. I next asked which organelles could supply this Ca\(^{2+}\) and how α-LTX triggered the release of Ca\(^{2+}\). Finally I tested the hypothesis that Ca\(^{2+}\) release is necessary and sufficient to produce the effects of α-LTX.
2 MATERIAls AND METHODS

2.1 ANIMALS AND EXPERIMENTAL TREATMENT

*Rana pipiens* (Leopard) frogs (4-5 cm body length; Wards Scientific, St. Catherines, Ontario, Canada) 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; Figure 2.1A). 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 120 mM NaCl, 2 mM KCl, 1 mM NaHCO₃, 1.8 mM CaCl₂, and 5 mM HEPES (pH adjusted to 7.2 with NaOH).

2.2 EXPERIMENTAL SOLUTIONS

To test for altered evoked transmitter release, synaptic transmission was monitored in low Ca²⁺ saline (LCS), which is similar to NPS except that the CaCl₂ concentration was lowered to 0.5 mM and 3.6 mM MgCl₂ was added. Ca²⁺-free saline (CFS) containing 120 mM NaCl, 2 mM KCl, 1 mM NaHCO₃, 5 mM MgCl₂, 10 mM HEPES, and 5 mM EGTA (pH adjusted to 7.2 with NaOH) was used to study the actions of α-LTX in the absence of extracellular Ca²⁺. When Na⁺ and Ca²⁺ were not required, a Na⁺ and Ca²⁺-free saline (NCFS) was made containing 120 mM choline chloride, 5 mM MgCl₂, 10 mM HEPES, and 5 mM EGTA (pH adjusted to 7.2 with KOH). Before the start of any experiment, preparations were washed in LCS, CFS, or NCFS every 10 minutes (4-5 bath changes) for an hour. All saline salts and buffers were purchased from Sigma (St. Louis, MO).

2.3 IMMUNOCYTOCHEMISTRY

Preparations processed for CL1 staining were fixed in NPS containing 4% paraformaldehyde (5°C) for 10 minutes and rinsed in NPS for 60 minutes. Preparations were incubated in a NPS staining solution containing 10% bovine serum albumin (BSA, Sigma) and 0.3% Triton X-100 (Boehringer Mannheim) for 30 minutes to minimize non-specific staining and to permeabilize the membrane respectively. This was followed by a 60 minute incubation with the rabbit anti-CL1 monoclonal antibody (provided by Dr. Yuri A. Ushkaryov, Imperial College, London, UK) and then a 60 minute
Figure 2.1. Legend on page 18.
(A) Location of the cutaneous pectoris muscles in Rana pipiens. This frog genus has two cutaneous pectoris muscles, which are thin skin muscles located on the ventral side of the animal in the mid-thorax region just off the midline. These muscles are only attached to the body along its perimeter. Therefore, the muscle with the innervating nerve can easily be excised by cutting the anchoring connective tissue around the preparation. (B) Illustration of the forward-filling method for loading nerve terminals with fluorescent ion indicators conjugated to dextrans. Muscles were bathed in a large well containing NPS while the cut end of the nerve was drawn into a small well containing 5 mM dye. The Vaseline border not only served to prevent the two wells from mixing but also served to keep the nerve from drying out. (C) Illustration of the experimental set-up for confocal imaging and electrophysiology. Muscles were pinned down in a custom-made chamber. The nerve was drawn into a suction electrode, which was used for stimulation. Nerve-evoked transmitter release and Ca^{2+} signals were detected with standard intracellular recording and confocal imaging techniques. The dashed circle represents the field of view through the microscope lens. Adapted from illustrations by John Georgiou.
rinse in NPS. Preparations were re-incubated in 10 µg/mL goat anti-rabbit IgG secondary antibody conjugated to FITC (Sigma) for 45 minutes and then rinsed with NPS staining solution for 30 minutes. Preparations were mounted onto glass slides using a drop of Slowfade anti-fade reagent (Molecular Probes, Eugene, Or) to reduce photo-bleaching.

2.4 DYE LOADING

Nerve terminals were loaded with the fluorescent indicator Oregon Green 488 BAPTA-1 Dextran or Sodium Green Dextran (Molecular Probes, Eugene, OR; 10,000MW) for measuring changes in presynaptic Ca^{2+} or Na^{+} respectively. When conjugated to dextrans (large, biologically inert, hydrophilic polysaccharides), these indicators can move by axoplasmic transport but do not get compartmentalized, pumped out, bound to cellular proteins, or loaded into other cells (e.g. Schwann cells and muscle cells). These properties give more accurate descriptions about the changes in the intracellular Ca^{2+} concentration, especially during experiments performed over long periods of time.

Dextran indicators were loaded into nerve terminals by forward-filling the dye through the cut-end of the motor nerve (Figure 2.1B). Muscles were first washed in a Petri dish with CFS for 10 minutes in order to get rid of any excess Ca^{2+}. The absence of Ca^{2+} helps to keep the cut end of the nerve open since Ca^{2+} is believed to be responsible for membrane sealing. With a pair of sharp scissors, the motor nerve was cut about 1 cm proximal to the muscle in a CFS bath. The preparation was then transferred to a 1.5 ml rectangular well (containing CFS) that was cut out of a Sylgard-coated Petri dish. Adjacent to this chamber was a small well, which contained 2 μl of the dextran indicator at a concentration of 5 mM (in distilled water). With a pair of forceps, the freshly cut end of the nerve was drawn into the dye-filled well and a Vaseline border was made in order to keep the contents of the two wells from mixing. 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 hours. During the incubation period, indicators were taken up through the nerve by the axon anterograde transport system and carried out to the nerve terminals.
2.5 BLOCKADE OF SPONTANEOUS ACTION POTENTIALS

In Ca²⁺-free saline containing EGTA, membranes become easily excitable. This is due to buffering of Ca²⁺ ions that normally screen negative surface charges on the outer plasma membrane surface. In the absence of these screening charges, the transmembrane potential becomes smaller thereby making it easier to reach threshold for action potential firing. Spontaneous firing of muscle action potentials can make electrophysiology and fluorescence imaging technically difficult because they cause muscle fibres to twitch. Spontaneous firing of nerve action potentials is also unwanted because the nerve terminal becomes loaded with Na⁺, which can have a physiological effect on transmitter release (Zengel et al., 1994). Therefore spontaneous action potential firing was blocked by addition of tetrodotoxin (TTX, Sigma, St. Louis, MO) to the CFS bath. This neurotoxin, isolated from the puffer fish, specifically blocks voltage-gated Na⁺ channels in neuronal membranes. However, if it is added in high enough concentrations (4 μM), it will also block voltage-gated Na⁺ channels in muscle membranes.

2.6 BLOCKADE OF POSTJUNCTIONAL RECEPTORS

At the frog NMJ, postsynaptic nicotinic acetylcholine receptors are located opposite presynaptic active zones. When these receptors bind acetylcholine, they pass a net inward current that causes the muscle membrane to depolarize. If this depolarization is sufficient to reach threshold, a muscle action potential is generated which leads to muscle contraction. If the muscle were allowed to contract, it would make nerve-evoked Ca²⁺ signals difficult to image. Therefore α-Bungarotoxin (α-BuTx, 10 μg/ml, Sigma, St. Louis, MO) was used to block muscle contractions during nerve stimulation. α-BuTx irreversibly blocks nicotinic acetylcholine receptors without interfering with acetylcholine release from motor nerve endings (Chang and Lee, 1957). Application of α-BuTx makes it impossible to record any end-plate potentials (EPPs) or miniature end-plate potentials (MEPPs), so this toxin was only used for experiments with fluorescence imaging that did not require measurement of transmitter release.

Sometimes it was necessary, after measuring nerve-evoked Ca²⁺ signals, to record transmitter release. As a result, μ-Conotoxin GIIIA (μ-CgTx, 10 μg/mL, Bachem, Torrance, CA) was used instead of α-BuTx because it preferentially blocks Na⁺ channels in muscles (Sosa and Zengel, 1993). This allowed us to block muscle
contractions during nerve stimulation, which were required for Ca\textsuperscript{2+} imaging controls, without compromising the recording of MEPPs.

2.7 RECORDING TRANSMITTER RELEASE

Transmitter release was monitored by intracellular recordings in the muscle fibre using 5-15 M\textohm 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 required for muscle contraction in NPS. Responses were amplified (Neuroprobe amplifier, Transidyne General corporation), digitized (Labmaster interface, Scientific Solutions, Solon, Ohio) and averaged in groups of 3-5 by TOMAHACQ (T.A. Goldthorpe, University of Toronto), a program for PC data acquisition systems. Changes in nerve-evoked transmitter release were assayed on-line by measuring the amplitude of EPPs. Concurrently, a digital recording of the experiment (VR-10 digital data recorder) was stored onto tape (Zenith VHS recorder) for later analysis of MEPP frequency.

2.7.1 Analyzing Spontaneous Transmitter Release

MEPP data stored on tapes were digitized by a Digidata 1200 Interface A/D Converter (Axon Instruments) using Axoscope Data Acquisition software and stored in an “abf” file. MEPP records were displayed using Jaejin Mini Analysis software v4.0.1 (Synaptosoft Inc; Figure 2.2), which reads ‘*.abf files’. MEPPs were counted by hand in 127.5 msec time bins and the frequency was calculated every minute from the time it took to see 100 MEPPs. During the height of \(\alpha\)-LTX action, quantal release is so high that it is difficult to count the number of MEPPs accurately. Therefore, MEPPs were counted when the trace made a positive inflection that was greater than the level of noise. This method underestimated the frequency of spontaneous transmitter release during toxin action, however knowing the absolute frequency was not necessary for any of my hypotheses. What was important was the relative change in toxin-induced transmitter release produced by each experimental treatment. Therefore, any treatment that attenuated the action of \(\alpha\)-LTX by over 50% was considered to have a large effect.
Figure 2.2. Screenshot of Jaejin Mini-analysis software program version 4.01 for PCs.
2.8 FLUORESCENCE MICROSCOPY

Dye-loaded nerve terminals located on surface fibres were chosen for all experiments. Fluorescence ($F$) was measured with a Bio-Rad 600 (Hercules, CA) confocal laser scanning microscope using 1% of the maximum laser intensity. Oregon Green and Sodium Green dyes were excited using the 488 nm line of the argon ion laser, and the emitted fluorescence was detected through a low pass filter with a 515 nm cut-off. Confocal images were acquired using a 40x water immersion objective (0.55 NA; Nikon) and averaged in groups of 3.

2.8.1 Perisynaptic Schwann Cell $\text{Ca}^{2+}$

Perisynaptic Schwann cells (PSCs) are peripheral glial cells that are found in close proximity to nerve terminals at the NMJ. Changes in PSC $\text{Ca}^{2+}$ were imaged by loading excised muscles with the cell permeant indicator fluo-3 AM (10 µM, Molecular Probes) containing 0.02% pluronic acid (Molecular Probes) in NPS for 90 minutes at room temperature. Once its acetomethoxyester (AM) groups are cleaved by esterases inside PSCs, Fluo-3 responds to $\text{Ca}^{2+}$ by increasing its fluorescence.

2.8.2 Image analysis

Confocal images were digitally acquired using 'mpl' data acquisition software provided by Bio-Rad and stored as '*.pict' files. File images were later analyzed using BFOCAL, a program for PC analysis of Bio-Rad images written by T.A. Goldthorpe (University of Toronto). Changes in fluorescence ($F$) were measured from a region of interest on the nerve terminal and were expressed as $\% \Delta F / F = (F_{\text{response}} - F_{\text{resting}}) / F_{\text{resting}} \times 100\%$.

2.8.3 Nerve Terminal Diameter Analysis

Changes in nerve terminal diameter (i.e. the amount of swelling) after $\alpha$-LTX treatment were determined from fluorescent-labelled nerve terminals using BFOCAL. Measurements of nerve terminal width made from regions of interest were expressed as $\% \Delta D / D = (D_{\text{response}} - D_{\text{resting}}) / D_{\text{resting}} \times 100\%$. 


2.9 **Chemicals**

CCCP (carbonyl cyanide m-chlorophenylhydrazone) and thapsigargin were both purchased from Calbiochem (San Diego, CA). BAPTA-AM was purchased from Molecular Probes (Eugene, OR). Cyclosporin A was bought from Sigma Aldrich (Oakville, Can) and α-LTX was from Latoxan (France).

2.10 **STATISTICAL ANALYSIS**

All values are reported as the mean ± the standard error of the mean (SEM). An independent Student t-test was used to determine statistical significance at a 95.0% confidence level (i.e. α = 0.05). N,n refers to the number of muscles (i.e. preparations) and the number of endplates respectively.

2.11 **FIGURES**

Sigma Plot 4 graphing software (Jandel Scientific, San Rafael, CA) and Corel Draw 8 (Corel, Ottawa, Can) were used to graph and display the data.
3 RESULTS

3.1 IS CL1 LOCATED AT THE FROG NMJ?

At the frog NMJ, studies using antibodies to α-LTX show that α-LTX binds specifically to motor nerve terminals and does not bind to Schwann cells, muscle fibres, capillaries, or preterminal axons (Valtorta et al., 1984). The specificity of α-LTX binding is likely due to the presence of a toxin receptor, however no discrimination was made between neurexin Iα and CL1 because the experiments were done in the presence of Ca$^{2+}$, which allows α-LTX to bind to both receptors. In order to verify that CL1 was present at frog motor nerve terminals, immunocytochemistry was performed on a preparation using anti-CL1 antibodies (Figure 3.1, N,n = 1,3). CL1 immunoreactivity was predominantly found at the NMJ with very little staining found elsewhere. The receptor appears to be distributed along the length of the nerve terminal in a banded pattern, which is characteristic of active zone staining at this synapse. There also appears to be staining around the periphery of the nerve terminal. This suggests that CL1 may target α-LTX specifically to presynaptic nerve terminals in the absence of Ca$^{2+}$. 
Figure 3.1. Anti-CL1 immunofluorescence in frog cutaneous pectoris muscle.

Confocal grey scale images from NMJs labelled with rabbit anti-CL antibodies and revealed with goat anti-rabbit-FITC. Pictures were taken from the same muscle at 3 different endplates. Bar, 20 μm.
3.2 DOES α-LTX INCREASE INTRACELLULAR Ca^{2+} WITH TRANSMITTER RELEASE IN CFS?

3.2.1 Is the Tissue Bath Nominally Free of Ca^{2+}?

In order to investigate the Ca^{2+}-independent effects of α-LTX, it was important to set up conditions that were nominally free of extracellular Ca^{2+}. Before each experiment, dye-loaded surface nerve terminals were first stimulated in NPS and nerve terminal intracellular Ca^{2+} measured. This control was performed because it gave information about the dynamic range of the dye and some indication that the Ca^{2+} handling mechanisms in the nerve terminal were functioning properly. In NPS, stimulating the nerve at 10 Hz for 5 seconds produced a rapid rise in Ca^{2+} fluorescence 40 ± 2% above baseline (N = 7; Figure 3.2 A). Increasing the stimulation frequency up to 20 and 40 Hz produced larger Ca^{2+} signals (72 ± 1% and 107 ± 1% respectively; N = 7 for both) due to the accumulation of intracellular Ca^{2+}.

Ca^{2+} was then removed from the bath by washing preparations (4 - 5 bath changes) every 15 minutes for an hour with CFS. When the nerve was re-stimulated with 10,20, and 40 Hz stimulation (Figure 3.2 B), no Ca^{2+} signals were produced (0.3 ± 0.2%, 0.4 ± 0.2%, and -0.2 ± 0.4% respectively; N = 7 for all) suggesting that the bath was nominally free of extracellular Ca^{2+}. Therefore any changes in the level of cytoplasmic Ca^{2+} in response to α-LTX could not be due to Ca^{2+} entry.

3.2.2 α-LTX and Presynaptic Ca^{2+}

When α-LTX (0.5 nM) was applied after nerve terminals were bathed in CFS for 60 minutes, a 55 ± 9% (N,n = 9,10) increase in Ca^{2+} fluorescence was observed (Figure 3.3). This increase in Ca^{2+} fluorescence was similar to that produced by 10 Hz nerve stimulation from the same nerve terminal in NPS (Figure 3.2). Unlike nerve stimulation, which increased the intracellular Ca^{2+} fluorescence by increasing Ca^{2+} entry, α-LTX must have increased the intracellular Ca^{2+} fluorescence by mobilizing Ca^{2+} from intracellular stores because the experiment was conducted under conditions in which Ca^{2+} entry does not occur. Furthermore, the observation that α-LTX raised presynaptic Ca^{2+} fluorescence at a slower rate than nerve stimulation (several minutes compared to a few seconds), suggested that the toxin may be operating through a multi-step Ca^{2+} release pathway rather than allowing a rapid influx of Ca^{2+}. Unlike the
Figure 3.2. Ca²⁺ Fluorescence after nerve stimulation.

(A) Presynaptic Ca²⁺ signals in NPS in response to 10, 20, and 40Hz nerve stimulation (N,n = 7,7 for each). (B) Absence of presynaptic intracellular Ca²⁺ signals in CFS in response to the same stimulation frequencies (N,n = 7,7 for each). Nerve stimulation was set to twice the threshold value for muscle contraction for a 5 second duration. Stimulated Ca²⁺ signals, which recovered in tens of seconds (Figure 3.2), the Ca²⁺ signal after α-LTX treatment did not recover in tens of minutes (Figure 3.3).
3.2.3 α-LTX and Transmitter Release

Slightly after elevating the intracellular \( \text{Ca}^{2+} \) concentration, α-LTX (0.5 nM) caused MEPP frequency to greatly accelerate from resting values of about 1-2 MEPPs per second to peak values greater than 300 MEPPs per second (Figure 3.3). Several minutes afterwards, the MEPP frequency eventually declined back to low levels (less than 1 s\(^{-1}\)). This was probably due to vesicle depletion (Clark et al., 1972; Clark et al., 1970) because vesicle recycling does not occur in the absence of extracellular \( \text{Ca}^{2+} \) when nerve terminals are treated with α-LTX (Ceccarelli and Hurlbut, 1980; Henkel and Betz, 1995). Similar results were obtained in 4 other experiments.

3.2.4 Dose-dependence of α-LTX Action

Both the time to onset and rate of α-LTX action on transmitter release and intracellular \( \text{Ca}^{2+} \) were dose-dependent. When α-LTX was applied at 5 nM (ten times the \( K_d \sim 0.5 \text{ nM} \) for the CL1-α-LTX complex (Davletov et al., 1996)), both the time to onset as well as the rate at which transmitter release and presynaptic \( \text{Ca}^{2+} \) increased was more rapid (< 2 minutes compared to ~20 minutes \( N,n = 3,3 \); < 10 minutes compared to ~25 minutes \( N,n = 3,3 \), respectively). However, there did not appear to be any significant difference in the peak MEPP frequency (\( f_{\text{MEPP}} > 300 \text{ s}^{-1} \); \( N,n = 3,3 \)) or the peak \( \text{Ca}^{2+} \) fluorescence (50 ± 6%; \( N,n = 6,6 \); \( P > 0.6 \)) when α-LTX was added at 5 nM compared to 0.5 nM. When the concentration of α-LTX was reduced ten-fold (i.e. 0.05 nM), no significant increase in intracellular \( \text{Ca}^{2+} \) or transmitter release was observed after 1.5 hours of recording. This suggests that the concentration might have been too low to be effective or that the time to onset would be too long to make any experiments practical. Most experiments were performed using an α-LTX concentration of 0.5 nM unless otherwise stated.
The graph represents a simultaneous recording of spontaneous quantal transmitter release frequency (blue) and Ca$^{2+}$ fluorescence (red) from motor nerve terminals after treatment with 0.5 nM α-LTX (arrow). Pictures of nerve terminal fluorescence and traces of spontaneous release activity are given at three time points during the experiment (1, 2, and 3). Bar for fluorescence images, 20 μm. Bar for electrophysiology traces, 0.5 mV by 10 msec. Similar results were obtained in 2 other experiments.

**Figure 3.3.** Effect of α-LTX on transmitter release and presynaptic Ca$^{2+}$. 
3.3 DOES α-LTX RELEASE Ca\(^{2+}\) FROM ENDOPLASMIC RETICULUM?

3.3.1 The Effect of U-73122

Activation of PLC makes IP\(_3\) from PIP\(_2\), which mobilizes Ca\(^{2+}\) from endoplasmic reticulum (ER). Receptors that activate PLC generally belong to the 7 transmembrane class of G-protein coupled receptors. Since CL is a 7 transmembrane receptor, I tested the involvement of PLC in the action of α-LTX. Nerve-muscle preparations were incubated with the PLC inhibitor, U73122 (50 μM) for 1 hour in CFS and then α-LTX (0.5 nM) was added. In the presence of U-73122, α-LTX increased the frequency of spontaneous transmitter release and the intracellular Ca\(^{2+}\) concentration similar to control values (Table 1).

3.3.2 The Effect of Thapsigargin

There are many pathways that lead to the release of Ca\(^{2+}\) from ER. U-73122 is a drug that blocks one of them. Rather than trying inhibitors to block each pathway, I took a more indirect approach by determining the amount of Ca\(^{2+}\) that could be released from ER using thapsigargin, and the extent to which this Ca\(^{2+}\) could increase transmitter release. Thapsigargin increases intracellular Ca\(^{2+}\) indirectly by blocking uptake of Ca\(^{2+}\) into ER. When thapsigargin (20 μM) was applied to nerve terminals bathed in Ca\(^{2+}\)-free saline for 1 hour, there was very little change in intracellular Ca\(^{2+}\) or transmitter release (Table 1). Furthermore, when α-LTX (0.5 nM) was added after thapsigargin, a robust increase in Ca\(^{2+}\) fluorescence (Figure 3.4) and transmitter release was still observed (Table 1). This suggests that ER does not contain enough Ca\(^{2+}\) to support the actions of α-LTX in CFS and is probably not the primary Ca\(^{2+}\) pool targeted.

As a control, the action of thapsigargin was tested in perisynaptic Schwann cells which had been loaded with a Ca\(^{2+}\) indicator. These glial cells, which wrap around motor nerve terminals, are known to contain thapsigargin-sensitive Ca\(^{2+}\) stores (Robitaille, unpublished results). When thapsigargin was applied to perisynaptic Schwann cells at the same concentration (20 μM) in NPS, there was an increase in Ca\(^{2+}\) (Figure 3.5) suggesting that this compound has a physiological effect at the frog NMJ. In agreement, Narita et al. (1998) have found subtle physiological effects of thapsigargin directly at frog motor nerve terminals.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ca(^{2+}) Fluorescence (%ΔF/F ± SEM)</th>
<th>N, n</th>
<th>MEPP Frequency (s(^{-1}) ± SEM)</th>
<th>N, n</th>
<th>Significance (relative to α-LTX 0.5 nM)</th>
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<tr>
<td>α-LTX (0.5 nM)</td>
<td>55 ± 9</td>
<td>9, 10</td>
<td>340 ± 19</td>
<td>5, 5</td>
<td>-</td>
</tr>
<tr>
<td>α-LTX (5 nM)</td>
<td>50 ± 6</td>
<td>6, 6</td>
<td>332 ± 11</td>
<td>3, 3</td>
<td>p = 0.69</td>
</tr>
<tr>
<td>α-LTX (0.5 nM) after U-73122 (50 μM)</td>
<td>54 ± 6</td>
<td>4, 7</td>
<td>353 ± 8</td>
<td>3, 3</td>
<td>p = 0.76</td>
</tr>
<tr>
<td>Thapsigargin (20 μM)</td>
<td>16 ± 7</td>
<td>2, 3</td>
<td>3.5 ± 2</td>
<td>2, 2</td>
<td>p = 0.04</td>
</tr>
<tr>
<td>α-LTX (0.5 nM) after Thapsigargin (20 μM)</td>
<td>60 ± 17</td>
<td>2, 3</td>
<td>359 ± 11</td>
<td>1, 1</td>
<td>p = 0.5</td>
</tr>
<tr>
<td>α-LTX (0.5 nM) after CCCP (10 μM)</td>
<td>2.4 ± 1</td>
<td>3, 3</td>
<td>NA</td>
<td>-</td>
<td>p = 0.00005 *</td>
</tr>
<tr>
<td>CCCP (10 μM)</td>
<td>50 ± 12</td>
<td>5, 5</td>
<td>102 ± 4</td>
<td>3, 3</td>
<td>p = 0.74</td>
</tr>
<tr>
<td>CCCP (10 μM) after α-LTX (0.5 nM)</td>
<td>1.3 ± 1</td>
<td>4, 4</td>
<td>NA</td>
<td>-</td>
<td>p = 0.01 *</td>
</tr>
</tbody>
</table>

Table 1. Legend on page 33.
Table 1. Summary table of various drugs on the actions of α-LTX and on their own.

The effect of α-LTX (0.5 nM) on transmitter release and Ca\textsuperscript{2+} fluorescence was measured after muscles were incubated with 50 μM U-73122 in CFS. The changes in Ca\textsuperscript{2+} fluorescence and transmitter release by thapsigargin (20 μM) or CCCP (10 μM) in CFS were also documented. A t-test was used to test for significant differences between the experimental Ca\textsuperscript{2+} fluorescence measurements and the control Ca\textsuperscript{2+} fluorescence measurements (0.5 nM α-LTX). Those marked with an asterisk indicate statistical significance.
Figure 3.4. The effect of thapsigargin on the actions of α-LTX.

Nerve terminals were first bathed in CFS for 1 hour. Changes in intracellular Ca\textsuperscript{2+} were measured in the presence of TTX (4 μM). Application of thapsigargin (20 μM, orange bar) produced a small increase in intracellular Ca\textsuperscript{2+}, but when α-LTX (0.5 nM, yellow bar) was subsequently applied there was an increase in intracellular Ca\textsuperscript{2+} similar to that obtained in the absence of thapsigargin. Similar results were observed in 2 other nerve terminals. Refer to Table 1 for the average peak Ca\textsuperscript{2+} fluorescence.
Figure 3.5. The effect of α-LTX on PSC intracellular Ca\textsuperscript{2+}.

Graph showing the change in intracellular Ca\textsuperscript{2+} after application of thapsigargin (20 μM, orange bar) to a PSC bathed in NPS.
3.4 DOES \(\alpha\)-LTX RELEASE \(\text{Ca}^{2+}\) FROM MITOCHONDRIA?

3.4.1 The Effect of CCCP

Mitochondria are another major \(\text{Ca}^{2+}\) storing organelle found in nerve terminals. Unlike ER, these stores may not be readily depleted in CFS because the large internally negative mitochondrial membrane potential (\(-150 - 200 \text{ mV}\)) opposes efflux of \(\text{Ca}^{2+}\). Several drugs, well known to interfere with mitochondrial metabolism, can cause mitochondria to lose their \(\text{Ca}^{2+}\). For instance, CCCP disrupts the mitochondrial respiratory chain by permeabilizing the membrane to \(\text{H}^+\). When CCCP (10 \(\mu\)M) was applied to nerve terminals bathed in CFS, a significant rise in intracellular \(\text{Ca}^{2+}\) (50 ± 12, \(N,n \ 5,5\)) and transmitter release (\(f_{\text{MEPP}} \sim 100 \text{ s}^{-1}\)) was produced (see Table 1 for summary). The amount of \(\text{Ca}^{2+}\) mobilized by CCCP on average was similar in magnitude to that mobilized by \(\alpha\)-LTX (\(P > 0.7\)) suggesting that mitochondria are likely candidates to be the source of \(\text{Ca}^{2+}\).

In order to determine if \(\alpha\)-LTX mobilizes \(\text{Ca}^{2+}\) from mitochondria, CCCP (10 \(\mu\)M) was first used to deplete mitochondrial \(\text{Ca}^{2+}\) stores from nerve terminals bathed in CFS. CCCP was added with oligomycin (10 \(\mu\)g/ml) to prevent the reverse action of the mitochondrial ATPase from consuming ATP. Oligomycin on its own had no effect on \(\text{Ca}^{2+}\) homeostasis (data not shown). Once the \(\text{Ca}^{2+}\) signal had stabilized after addition of CCCP, addition of \(\alpha\)-LTX (0.5 nM) produced no further increase in intracellular \(\text{Ca}^{2+}\) (2.4 ± 0.4%; \(N,n = 3,3\); Figure 3.6). Similarly, when nerve terminals bathed in CFS were pre-treated with \(\alpha\)-LTX (0.5 nM), addition of CCCP (10 \(\mu\)M) also produced no further change in intracellular \(\text{Ca}^{2+}\) (1.3 ± 0.7%, \(N = 4\), Figure 3.7). Thus, the effects of CCCP and \(\alpha\)-LTX on \(\text{Ca}^{2+}\) mobilization were mutually occlusive, suggesting that the toxin likely targets mitochondrial \(\text{Ca}^{2+}\) pools. In both cases, the result was not due to dye saturation because the dynamic range of the dye, determined before the experiment by nerve stimulation in NPS as shown in Figure 3.2, was at least 2 times larger than the \(\text{Ca}^{2+}\) signal produced by either agent.
**Figure 3.6.** Legend on page 38.
Figure 3.6. CCCP prevents Ca$^{2+}$ release by α-LTX.

(A) Change in Ca$^{2+}$ fluorescence was measured after application of CCCP (10 μM, first orange bar) followed by α-LTX (0.5 nM, second orange bar) from a nerve terminal bathed in CFS with TTX (4 μM). There was a 10 minute wash period (W, first yellow bar) with CFS in-between application of CCCP and α-LTX. NPS (i.e. containing 1.8 mM Ca$^{2+}$) was added back to the bath after α-LTX (second yellow bar). (B) Bar graph representing the average peak Ca$^{2+}$ fluorescence achieved as a % of control after treatment with α-LTX with and without pre-treatment with CCCP. Without CCCP pre-treatment (α-LTX control, blue bar), the peak Ca$^{2+}$ fluorescence was calculated as the maximum change in fluorescence (F' - F) by α-LTX relative to rest (N,n = 9,10). With CCCP pre-treatment (α-LTX after CCCP, red bar), the peak Ca$^{2+}$ fluorescence was calculated as the additional change in fluorescence (F'' - F') by α-LTX after that produced by CCCP relative to rest (N,n = 3,3).
Figure 3.7. Legend on page 40.
**Figure 3.7.** α-LTX prevents Ca$^{2+}$ release by CCCP.

Same type of experiment as Figure 3.6 except in the reverse order (i.e. α-LTX added before CCCP). (A) Change in Ca$^{2+}$ fluorescence was measured after application of α-LTX (0.5 nM, first orange bar) followed by CCCP (10 μM, yellow bar) from a nerve terminal bathed in CFS with TTX (4 μM). NPS (i.e. containing 1.8 mM Ca$^{2+}$) was added back to the bath after CCCP (second orange bar). (B) Bar graph representing the average peak Ca$^{2+}$ fluorescence achieved as a % of control after treatment with CCCP with and without pre-treatment with α-LTX. Without α-LTX pre-treatment (CCCP control, blue bar), the peak Ca$^{2+}$ fluorescence was calculated as the maximum change in fluorescence (F' - F) by CCCP relative to rest (N,n = 5,5). With α-LTX pre-treatment (CCCP after α-LTX, red bar), the peak Ca$^{2+}$ fluorescence was calculated as the additional change in fluorescence (F'' - F') by α-LTX after that produced by CCCP relative to rest (N,n = 4,4).
3.5 DOES α-LTX INCREASE INTRACELLULAR Ca\(^{2+}\) BY INCREASING INTRACELLULAR Na\(^{+}\)?

There is currently evidence for three different mechanisms of Ca\(^{2+}\) efflux from mitochondria (Gunter and Pfeiffer, 1990), one of which is Na\(^{+}\)-dependent. Na\(^{+}\)-Ca\(^{2+}\) exchangers on the mitochondrial plasma membrane can extrude Ca\(^{2+}\) from mitochondria using the energy from the Na\(^{+}\) concentration gradient when intracellular Na\(^{+}\) increases. To determine if α-LTX causes intracellular Na\(^{+}\) to increase, I forward-filled Sodium Green dextran into terminals and applied α-LTX in CFS. α-LTX (0.5 nM) caused the Na\(^{+}\) fluorescence to increase by 42 ± 4% (N,n = 5,5; Figure 3.8).

Furthermore, α-LTX (0.5 nM) also caused marked swelling of nerve terminals, which is dependent upon extracellular Na\(^{+}\) (Figure 3.9; Gorio et al., 1978). The increase in nerve terminal diameter preceded the increase in transmitter release suggesting that swelling of the nerve terminal could not be due to the rapid addition of vesicle membrane. If we assume that nerve terminal swelling is a result of Na\(^{+}\) influx, then this influx of Na\(^{+}\) logically precedes the release of Ca\(^{2+}\). Therefore I hypothesized that Na\(^{+}\) influx might activate the Na\(^{+}\)/Ca\(^{2+}\) exchanger and thereby cause the release of Ca\(^{2+}\) from mitochondria.

The increase in intracellular Na\(^{+}\) was not due to entry of Na\(^{+}\) through voltage-gated Na\(^{+}\) channels because addition of TTX (4 μM) did not affect the Na\(^{+}\) signal. At the frog NMJ, the plasma membrane of nerve terminals become permeable to small molecules (< 400 Da) after treatment with α-LTX (Davletov et al., 1998). This channel or pore in the plasma membrane is not only conductive to Na\(^{+}\) but also to Ca\(^{2+}\). When nerve terminals were treated with α-LTX in CFS and then given back extracellular Ca\(^{2+}\), there was a rapid increase in intracellular Ca\(^{2+}\) that would saturate the signal (Figure 3.10). The increase in intracellular Ca\(^{2+}\) was not due to influx through voltage-gated Ca\(^{2+}\) channels because Cd\(^{2+}\) (100 μM) which blocks all voltage-gated Ca\(^{2+}\) channels rapidly, did not block this effect. Cd\(^{2+}\) on its own did not cause any changes in the levels of intracellular Ca\(^{2+}\).

To test whether an increase in intracellular Na\(^{+}\) was necessary to produce the increase in intracellular Ca\(^{2+}\) by α-LTX, extracellular Na\(^{+}\) was removed (Figure 3.11). When α-LTX was applied to nerve terminals bathed in NCFS (i.e. no added Ca\(^{2+}\) or Na\(^{+}\)), the intracellular Na\(^{+}\) actually decreased (-16.7 ± 2%; N,n = 3,3). The decrease in
Figure 3.8. Time-course of the effect of α-LTX on the intracellular Na⁺ concentration.

Intracellular Na⁺ fluorescence was measured after the addition of α-LTX (0.5 nM) in CFS in the presence of TTX (4 μM). Bar, 20 μM.
Figure 3.9. Temporal relationship between the increase in nerve terminal diameter, intracellular Ca$^{2+}$, and transmitter release.

Figure showing the increase in nerve terminal diameter (green), in relation to the change in Ca$^{2+}$ fluorescence and transmitter release (blue dashed line), after application of $\alpha$-LTX (0.5 nM) from the same nerve terminal in Figure 3.3. The preparation was bathed in CFS with addition of 4 $\mu$M TTX. This temporal correlation was observed in 2 other experiments.
<table>
<thead>
<tr>
<th>Bath Conditions</th>
<th>Ca\textsuperscript{2+} Fluorescence Images</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Ca\textsuperscript{2+} (rest)</td>
<td>![Image of fluorescence images]</td>
</tr>
<tr>
<td>0 Ca\textsuperscript{2+} (+2 hours)</td>
<td>![Image of fluorescence images]</td>
</tr>
<tr>
<td>+ 1.8 mM Ca\textsuperscript{2+}</td>
<td>![Image of fluorescence images]</td>
</tr>
</tbody>
</table>

**Figure 3.10.** Ca\textsuperscript{2+} permeability of the nerve terminal membrane by α-LTX.

Resting Ca\textsuperscript{2+} fluorescence of 2 nerve terminals from 2 different muscles imaged at \( t = 0 \) min (top, right and left panels). Nerve terminal Ca\textsuperscript{2+} fluorescence 2 hours after treatment with α-LTX (0.5 nM, middle, right panel) and without (left panel). During the 2 hour application of α-LTX (middle, right panel), the intracellular Ca\textsuperscript{2+} concentration increased then decreased. Nerve terminal Ca\textsuperscript{2+} fluorescence 2 minutes after adding Ca\textsuperscript{2+} (1.8 mM) back to the bath (bottom, right and left panels). For the terminal treated with α-LTX, 100 μM Cd\textsuperscript{2+} was added prior to the addition of Ca\textsuperscript{2+}. The rapid increase in intracellular Ca\textsuperscript{2+} was seen in every nerve terminal treated with α-LTX.
Figure 3.1. The role of Na⁺ on the mechanism of α-LTX-induced Ca²⁺ release.

Graph showing the effect of α-LTX (0.5 nM) in CFS (blue) and NCFS (red). Values were normalized to the effects of the toxin in CFS and displayed as a per cent of control. The left pair and right pair of bar graphs show the change in intracellular Na⁺ and Ca²⁺ respectively after application of α-LTX in CFS and NCFS.
intracellular Na\(^+\) was not due to the loss of dye because adding back the Na\(^+\) caused the intracellular Na\(^+\) to increase above resting levels. There are two possibilities that could explain the loss of Na\(^+\): 1) removal of extracellular Na\(^+\) caused the Na\(^+\)-Ca\(^{2+}\) exchanger on the plasma membrane to operate in reverse, or 2) intracellular Na\(^+\) passed out through the same \(\alpha\)-LTX-dependent pores which allowed it to enter in CFS or Na\(^+\) leaked out via Na\(^+\) channels. In either case, Na\(^+\) entry by \(\alpha\)-LTX does not occur when nerve terminals are bathed in NCFS. When changes in intracellular Ca\(^{2+}\) were measured in NCFS, the effect of \(\alpha\)-LTX on Ca\(^{2+}\) mobilization was attenuated by over 70% \((N,n = 4,13)\). This suggests that Ca\(^{2+}\) efflux from mitochondria by \(\alpha\)-LTX is partially Na\(^+\)-dependent.

### 3.6 CYCLOSPORIN-A

The fact that removing extracellular Na\(^+\) did not totally block the Ca\(^{2+}\) signal produced by \(\alpha\)-LTX, suggests that another mechanism may be operating. While Ca\(^{2+}\) movements across the inner mitochondrial membrane normally occur via specific transporters, there is also a latent transport system, known as the mitochondrial permeability transition (MPT) pore, which can render the inner membrane permeable to many structurally unrelated molecules and ions including Ca\(^{2+}\). Cyclosporin-A (CsA) is the strongest known inhibitor of the permeability transition pore (Broekemeier et al., 1989). To determine if CsA blocked the effect of \(\alpha\)-LTX on Ca\(^{2+}\) mobilization, preparations were incubated for 1 hour in CFS containing 500 \(\mu\)M CsA. Although CsA had no effect on \(\alpha\)-LTX-induced transmitter release \((> 90\%\) of control, \(N,n = 4,4)\), \(\alpha\)-LTX-induced Ca\(^{2+}\) mobilization was attenuated by \(> 78\%\) \((N,n = 3,3;\) Figure 3.12). This result suggests that \(\alpha\)-LTX could be activating multiple mechanisms to trigger the release of Ca\(^{2+}\) from mitochondria or that in frogs, CsA does not block Ca\(^{2+}\) efflux from mitochondria completely. It is not likely that residual Na\(^+\) in the NCFS bath could have caused the release of Ca\(^{2+}\) because in NCFS, it was shown that the nerve terminal loses Na\(^+\).
Figure 3.12. The effect of CsA on the actions of α-LTX.

(A) Time-course (red line graph) of the change in the intracellular Ca\(^{2+}\) concentration after the application of α-LTX (0.5 nM) from a nerve terminal treated with 500 μM CsA for an 1 hour in CFS with TTX (4 μM). The control in blue (0.5 nM α-LTX) is also plotted on the same graph. The arrows on both curves represents the time of α-LTX application. (B) Bar graph showing the average effect of α-LTX on peak intracellular Ca\(^{2+}\) fluorescence (N,n = 3,3) and peak transmitter release (N,n = 4,4) with (red) and without (blue) treatment with CsA.
3.7 IS Ca\(^{2+}\) NECESSARY FOR \(\alpha\)-LTX-INDUCED EXOCYTOSIS?

The correlation between the rise in intracellular Ca\(^{2+}\) and the rise in transmitter release makes it easy to presume that the former caused the latter because it is well known that Ca\(^{2+}\) is required for synchronous transmitter release. Therefore, to determine if the rise in intracellular Ca\(^{2+}\) was necessary for the rise in transmitter release induced by \(\alpha\)-LTX, the cell permeant Ca\(^{2+}\) chelator BAPTA-AM (\(K_D \sim 100\) nM) was used. BAPTA attenuates nerve-evoked exocytosis because it chelates Ca\(^{2+}\) entering through Ca\(^{2+}\) channels near active zones (Adler et al., 1991).

3.7.1 The Effect of BAPTA-AM

Transmitter release and intracellular Ca\(^{2+}\) were assayed simultaneously from the same nerve terminal after 30 minutes incubation with 100 \(\mu\)M BAPTA-AM in CFS. Application of 0.5 nM \(\alpha\)-LTX caused Ca\(^{2+}\) fluorescence to increase by only 9.4% (\(N,n = 1,1\)) but still caused transmitter release to increase well over 300 MEPPs/sec (Figure 3.13). Furthermore, BAPTA chelated enough free Ca\(^{2+}\) such that the rise in transmitter release preceded the rise in intracellular Ca\(^{2+}\). If transmitter release by \(\alpha\)-LTX were Ca\(^{2+}\)-dependent, then one would expect the rise in intracellular Ca\(^{2+}\) to come first.

The effect of BAPTA-AM on neurotransmission is not long lasting (Figure 3.14) and this probably explains why the Ca\(^{2+}\) signal was not completely eliminated in Figure 3.13. The decrease in effectiveness of BAPTA-AM is probably due to its extrusion by anion pumps (Ouanounou et al., 1996). Therefore, to get a strong effect of BAPTA at the time of \(\alpha\)-LTX action: 1) an anion pump inhibitor, probenecid (1 mM), was added to minimize loss of BAPTA from the cytosol, 2) a second treatment of BAPTA-AM was given 15 minutes after the first (final concentration 200 \(\mu\)M) to get a longer-lasting effect of the chelator, and 3) \(\alpha\)-LTX was added at 10 times the normal concentration to hasten the action of the toxin (time to onset < 2 min). Following these criteria ensured that the effects of the toxin were observed when Ca\(^{2+}\) buffering was at its strongest. BAPTA significantly attenuated the toxin-induced rise in Ca\(^{2+}\) fluorescence by ~94% (\(N,n = 3,3\)) but had no effect on the acceleration of transmitter release (\(f_{\text{MEPP}} > 300/\text{sec}, N,n = 2,2\); Figure 3.15). The data suggests that Ca\(^{2+}\) released by \(\alpha\)-LTX from intracellular stores does not play a major role in toxin-induced exocytosis.
Figure 3.13. Time course of the effect of BAPTA-AM on the actions of α-LTX.

Nerve terminals bathed in CFS were pre-treated with 100 μM BAPTA-AM for 30 minutes. Changes in transmitter release (blue) and intracellular Ca^{2+} (red) were simultaneously measured after application of 0.5 nM α-LTX (arrow). The average peak MEPP frequency (blue dashed line) and peak Ca^{2+} fluorescence (red dashed line) without BAPTA found in other experiments (i.e. controls) is also indicated on the graph.
Changes in EPP amplitudes were measured after bath application of BAPTA-AM (300 μM) and plotted as a percent of control. The experiment was carried out in LCS because muscle contractions are blocked in this solution (Crawford, 1973). The nerve was stimulated by a twin-pulse protocol every 15 seconds to minimize depression of nerve-evoked responses (Redman and Silinsky, 1994). Each point represents the average of 3 amplitudes measured from the first EPP. The EPP amplitude initially increases because BAPTA prevents the opening of Ca\(^{2+}\)-gated K\(^+\) channels, which normally act to shorten the AP (Robitaille et al., 1993). The traces above the graph are representative of the muscle membrane response to nerve stimulation. Notice when the action of BAPTA was strongest, there was no paired-pulse facilitation. Even with continued presence of BAPTA-AM, the effect of the chelator eventually reversed. This effect was observed in 1 other experiment.
Figure 3.15. Action of α-LTX after modified application of BAPTA-AM.

Peak Ca\(^{2+}\) fluorescence and peak MEPP frequency achieved by α-LTX (5 nM) from terminals treated with (red) or without (blue) BAPTA-AM (200 μM) in the presence of 1 mM probenecid. Preparations were bathed in CFS supplemented with TTX (4 μM). Values were normalized and expressed as a percent of control (5 nM α-LTX in CFS).
4 DISCUSSION

4.1 SUMMARY

α-LTX increases transmitter release at the frog NMJ in a Ca\(^{2+}\)-free environment after binding to a G-protein-coupled receptor. This neurotoxin increased the intracellular Ca\(^{2+}\) concentration by mobilizing Ca\(^{2+}\) predominantly from mitochondria. α-LTX also raised the intracellular Na\(^{+}\) concentration, which was partially responsible for the release of Ca\(^{2+}\) from mitochondria. The rise in the intracellular Ca\(^{2+}\) concentration was also blocked partially by an inhibitor of the mitochondrial permeability transition pore. Thus, α-LTX may have multiple actions on the release of Ca\(^{2+}\) from mitochondria. Although the increase in intracellular Ca\(^{2+}\) was correlated with the rise in transmitter release, the former probably does not cause the latter.

4.2 α-LTX-STIMULATED TRANSMITTER RELEASE

The frog NMJ is a classical preparation for studying quantal transmitter release and has accounted for most of the pioneering research on α-LTX. In the absence of extracellular Ca\(^{2+}\), α-LTX accelerates spontaneous transmitter release several hundred fold above the resting levels at the frog NMJ. The stimulation of secretion in CFS occurs in a variety of other cell systems including mammalian NMJs (Okamoto et al., 1971), synaptosomes (Nicholls et al., 1982), PC12 cells (Grasso et al., 1980), insulin-secreting cell lines (Lang et al., 1998), and hippocampal slices (Capogna et al., 1996). This implies that α-LTX may be stimulating transmitter release through a universal mechanism, which is not just specific to amphibians.

At the frog NMJ, there is a relatively long latency period between the time of toxin addition and the time of toxin action. This latency period may be due to steric hindrance because α-LTX is a very large protein (MW 130 kDa) that has to diffuse through a mesh of connective tissue and extracellular matrix proteins in order to get access to nerve terminals. Adding a lectin such as concanavalin A, which attaches to cell surface sugar residues, can block the actions of α-LTX completely (Grasso et al., 1978; Rubin et al., 1978) by preventing the toxin from binding to its receptor [Meldolesi, 1982 #14].
4.2.1 Vesicle Depletion

$\alpha$-LTX stimulates transmitter release in CFS until nerve terminals are completely depleted of vesicles (Clark et al., 1972; Clark et al., 1970; Gorio et al., 1978; Watanabe and Meldolesi, 1983; Watanabe et al., 1983). In the presence of $Ca^{2+}$, nerve terminals retain their population of synaptic vesicles after treatment with $\alpha$-LTX suggesting that vesicle recycling may be dependent on extracellular $Ca^{2+}$ (Ceccarelli and Hurlbut, 1980; Torri-Tarelli et al., 1990; Valtorta et al., 1988). Vesicle recycling is not blocked by $\alpha$-LTX because endocytosis can be triggered by re-introducing $Ca^{2+}$ after vesicles have been depleted by $\alpha$-LTX in CFS (Henkel and Betz, 1995). Since $\alpha$-LTX still depletes vesicles in CFS even though $Ca^{2+}$ is released from intracellular stores and remains elevated after vesicle depletion it seems that vesicle recycling either does not require $Ca^{2+}$ or requires a lot more $Ca^{2+}$ (in small localized areas) than released in these experiments.

It has been suggested that $\alpha$-LTX causes the preferential secretion of small synaptic vesicles in frog motor nerve terminals because large dense core vesicles (LDCV) containing CGRP (calcitonin gene-related peptide) and calcitonin were not depleted by toxin treatment (Matteoli et al., 1988). This raised the possibility that there may be two different mechanisms for vesicle secretion. In this study however, pictures were taken from different nerve terminals before and after toxin action, which is not a good representation of single cells. In $\beta$ cells and nerve endings innervating oesophageal smooth muscle, $\alpha$-LTX stimulates the release of LDCV in a $Ca^{2+}$-independent manner (Lang et al., 1998; Ny et al., 1997). Thus, differential mechanisms of secretion revealed by $\alpha$-LTX still remain controversial.

4.2.2 Independence of Extracellular $Ca^{2+}$

In tissue preparations such as the frog NMJ, removal of extracellular $Ca^{2+}$ can be difficult because $Ca^{2+}$ can get trapped in constricted places like the synaptic cleft. Even when the preparation is washed several times with a saline containing no added $Ca^{2+}$, there is probably leak of $Ca^{2+}$ from muscle fibres. Thus, proving that the bath is nominally free of $Ca^{2+}$ can be difficult given the problems associated with tissue preparations. In many previous studies on the frog NMJ, it was assumed that the actions of $\alpha$-LTX were independent of $Ca^{2+}$ because 1 mM EGTA was added to the bath. My results were the first to show using $Ca^{2+}$ indicator dyes that in CFS.
supplemented with 5 mM EGTA, no Ca^{2+} entry can be detected after high frequency nerve stimulation. This was an important control because it proved that transmitter release by α-LTX could not be due to an influx of contaminant Ca^{2+}.

In chromaffin cells and rat brain synaptosomes, α-LTX does not require extracellular Ca^{2+} for binding but it does need it for action (Davletov et al., 1998; Michelena et al., 1997). Interestingly, stimulation of transmitter release in both of these systems by α-LTX is not mediated by the influx of external Ca^{2+}. At the frog NMJ, it appears that α-LTX does not require extracellular Ca^{2+} for binding or action, because even when EGTA was replaced with the same concentration of BAPTA salt, which has a faster forward rate constant for Ca^{2+} binding (Adler et al., 1991), the actions of α-LTX were not affected (data not shown). Other preparations, such as hippocampal slices and PC12 cells, also respond to α-LTX by increasing release in CFS. It should be noted that synaptosomes can respond to α-LTX without external Ca^{2+}, but it is argued that this release is non-quantal because addition of botulinum neurotoxins, which normally block quantal transmitter release (Jahn and Niemann, 1994), had no effect. The requirement for external Ca^{2+} is just one of many issues that has been difficult to resolve as a result of studying α-LTX in different preparations.

4.2.3 Dependence on Extracellular Divalent Cations

α-LTX can only stimulate exocytosis independently of extracellular Ca^{2+} provided that another divalent cation is present such as Mg^{2+} (Misler and Falke, 1987; Misler and Hurlbut, 1979). This observation was not due to a lack of binding because α-LTX can still associate with CL in the absence of divalent cations (Davletov et al., 1996). It was hypothesized that α-LTX increased transmitter release at the frog NMJ in the absence of Ca^{2+} by increasing the permeability of the nerve terminal to Mg^{2+}, because removal of Mg^{2+} prevented the toxin effect. Assuming a free intracellular Mg^{2+} concentration of about 1-4 mM (Baker and Crawford, 1972), one would expect Mg^{2+} to enter terminals given an extracellular concentration of 5 mM and a resting membrane potential of −70 mV. However, it does not seem likely that an increase in intracellular Mg^{2+} could fully account for the actions of α-LTX on transmitter release because the mechanism of exocytosis is not sensitive to changes in the intracellular Mg^{2+} concentration (Miledi, 1973). Alternatively, Mg^{2+} may be modulating the actions of α-LTX. For instance, in rat
brain synaptosomes, Ca\(^{2+}\) influx by \(\alpha\)-LTX is enhanced in the presence of extracellular Mg\(^{2+}\) (Davletov et al., 1998). Finally it is possible that frog CL is different from mammalian CL and requires divalent cations to allow binding of \(\alpha\)-LTX.

4.3 \(\alpha\)-LTX STIMULATED Ca\(^{2+}\) MOBILIZATION

The role of Ca\(^{2+}\) in triggering exocytosis has been well established for many years (for a review, see Bennett, 1999). The ability of \(\alpha\)-LTX to increase spontaneous transmitter release in a manner that is independent of extracellular Ca\(^{2+}\) has led many people to believe that secretion can occur by a route that circumvents the requirement for Ca\(^{2+}\). However, increasing the intracellular Ca\(^{2+}\) concentration without Ca\(^{2+}\) entry through Ca\(^{2+}\) channels is sufficient to trigger exocytosis (Miledi, 1973; Charlton et al., 1982; Bennett, 1999). My study was the first to demonstrate that \(\alpha\)-LTX causes Ca\(^{2+}\) release from intracellular stores without requiring extracellular Ca\(^{2+}\).

It is unlikely that the increase in Ca\(^{2+}\) fluorescence that I saw was due to an influx of Mg\(^{2+}\). In order to get half the signal that Ca\(^{2+}\) produces by binding to Oregon Green BAPTA, the Mg\(^{2+}\) concentration would have to increase by a 100-fold because the dye is 200 times less sensitive to Mg\(^{2+}\) than Ca\(^{2+}\). However, owing to the increased cation conductance, \(\alpha\)-LTX probably depolarizes the nerve terminal and this would drastically reduce the inwardly directed electrochemical gradient for Mg\(^{2+}\) entry. Furthermore, after CCCP, \(\alpha\)-LTX gave no increase in Ca\(^{2+}\) signal even though Mg\(^{2+}\) should have entered. The same argument can be made for both the CsA and the Na\(^{+}\) replacement experiments as well. So even though there could still be an increase in intracellular Mg\(^{2+}\), the intracellular Mg\(^{2+}\) concentration does not increase enough with \(\alpha\)-LTX to account for the majority of the Ca\(^{2+}\) signal.

Synaptic vesicles contain a high concentration of Ca\(^{2+}\) in the frog nerve terminal (Grohovaz et al., 1996; Pezziati and Grohovaz, 1999). Therefore it might be possible that vesicles release Ca\(^{2+}\) into the synaptic cleft, which then re-enters the nerve terminal. This seems unlikely for two reasons: (1) the increase in intracellular Ca\(^{2+}\) preceded the increase in transmitter release by minutes and (2) any Ca\(^{2+}\) escaping into the extracellular milieu would have been quickly buffered by the high concentration of EGTA or BAPTA. In addition, vesicles could release Ca\(^{2+}\) into the cytoplasm and docked vesicles would be exquisitely placed to cause their own exocytosis by releasing Ca\(^{2+}\). However, BAPTA should have taken up Ca\(^{2+}\) released from distal vesicles. It is
also possible that the intimate relationship of vesicles with active zones precludes timely action of BAPTA to buffer Ca\(^{2+}\) released there. The intracellular release of Ca\(^{2+}\) by synaptic vesicles cannot be ruled out by my experiments.

4.3.1 Ca\(^{2+}\) Signals in Other Cell Systems

Rat brain synaptosomes are the only other preparation where \(\alpha\)-LTX has been shown to release stored Ca\(^{2+}\). The mechanism of Ca\(^{2+}\) release differs from frog NMJ because it depends upon extracellular Ca\(^{2+}\). In the absence of extracellular Mg\(^{2+}\), it has been shown that radioactively labelled Ca\(^{2+}\) uptake caused by \(\alpha\)-LTX is blocked (i.e. no Ca\(^{2+}\) entry; Davletov et al., 1998). Therefore, they argued that there must be Ca\(^{2+}\) mobilization because transmitter release was significantly reduced by high concentrations of BAPTA-AM. In the absence of extracellular Ca\(^{2+}\), BAPTA-AM had no effect on release by \(\alpha\)-LTX so it was concluded that Ca\(^{2+}\) was not mobilized and that transmitter release was due to leakage. Changes in intracellular Ca\(^{2+}\) have been measured more directly in PC12 cells and pancreatic \(\beta\) cells using Ca\(^{2+}\)-fluorescent indicator dyes (Lang et al., 1998; Meldolesi et al., 1984). In both of these systems, \(\alpha\)-LTX increased transmitter release but not intracellular Ca\(^{2+}\) in CFS, which supports the observation made in synaptosomes. The fact that \(\alpha\)-LTX mobilizes Ca\(^{2+}\) in frog motor nerve terminals in synaptosomes makes things a little more complicated because it suggests that the toxin probably has different actions in different cell systems.

4.4 NEURONAL Ca\(^{2+}\) STORES

Ca\(^{2+}\) stores within nerve terminals can function simultaneously both as a sink and source of Ca\(^{2+}\) ions. At nerve terminals, Ca\(^{2+}\) is found in ER, mitochondria, and synaptic vesicles (Meldolesi et al., 1988). Using high-resolution ultrastructural mapping, Ca\(^{2+}\) in frog motor nerve terminals is predominantly found in synaptic vesicles and the lumen of smooth ER cisternae (Grohovaz et al., 1996; Pezzati and Grohovaz, 1999). While parts of mitochondria also appear to contain Ca\(^{2+}\) at a lower concentration than vesicles, the distribution of these parts positive are irregular and often in clear apposition to cisternae of ER.
4.4.1 The Endoplasmic Reticulum

Ca\textsuperscript{2+} mobilization from ER normally occurs by activation of Ca\textsuperscript{2+} release channels incorporated in the ER membrane. These Ca\textsuperscript{2+} channels fall into two major classes: those gated by IP\textsubscript{3} (IP\textsubscript{3} receptor) and those gated by Ca\textsuperscript{2+} (ryanodine receptor). IP\textsubscript{3} is an intracellular second messenger, which is generated from the breakdown of PIP\textsubscript{2} by PLC. When IP\textsubscript{3} binds to its receptor, it triggers the release of Ca\textsuperscript{2+}. The release of Ca\textsuperscript{2+} by IP\textsubscript{3} receptor activation is enhanced when Ca\textsuperscript{2+} and IP\textsubscript{3} are present together. IP\textsubscript{3} receptors are blocked by heparin (Smith and Gallacher, 1994) and xestospongin (Gafni et al., 1997). Ryanodine receptors are activated by increases in intracellular Ca\textsuperscript{2+} and the source of Ca\textsuperscript{2+} can be either extracellular or from intracellular stores. This property of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release can set a chain reaction of ryanodine receptor activation, which results in a Ca\textsuperscript{2+} wave propagating along the ER membrane. Ryanodine receptor activation is blocked by ryanodine (Jenden and Fairhurst, 1969) and dantrolene (Ohta et al., 1990).

The Ca\textsuperscript{2+}-sensitivity of the IP\textsubscript{3} and ryanodine receptor is enhanced by the uptake of Ca\textsuperscript{2+} into the ER lumen. Ca\textsuperscript{2+} uptake is handled by high-affinity Ca\textsuperscript{2+} ATPases (SERCA pumps, i.e. sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+} ATPases), which require energy to load Ca\textsuperscript{2+} into the ER against its concentration gradient. The ER membrane is generally 'leaky' to Ca\textsuperscript{2+} so in order to maintain a high concentration of Ca\textsuperscript{2+} in the ER, the SERCA pumps are continuously active. Inhibition of SERCA pumps by thapsigargin (Thastrup et al., 1990) leads to an increase in intracellular Ca\textsuperscript{2+} because of the disruption in Ca\textsuperscript{2+} homeostasis across the ER membrane, i.e. ER cannot take up Ca\textsuperscript{2+} but still loses it.

In rat brain synaptosomes, α-LTX has been shown to mobilize Ca\textsuperscript{2+} from ER (Davletov et al., 1998; Rahman et al., 1999). It is suggested that α-LTX binding to CL1 stimulates PLC activity and the production of IP\textsubscript{3} because (i) CL1 co-elutes with G\textsubscript{a/q11}, a G-protein coupled to PLC activation, (ii) the PLC inhibitor, U-73122, blocks α-LTX-induced transmitter release, and (iii) dumping of ER Ca\textsuperscript{2+} by thapsigargin also blocks transmitter release by α-LTX. In other cell systems, α-LTX has been shown to stimulate IP\textsubscript{3} production in PC12 cells (Rosenthal et al., 1990; Vicentini and Meldolesi, 1984) and COS cells transfected with CL1 (Lelianova et al., 1997), supporting the hypothesis that CL1 activation mobilizes Ca\textsuperscript{2+} from IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} stores. However, this mechanism
cannot explain the actions of α-LTX at the frog NMJ because it requires the presence of extracellular Ca\textsuperscript{2+}. This implies that the source of Ca\textsuperscript{2+}, or the mechanism by which Ca\textsuperscript{2+} is released by α-LTX, must be different in frog motor nerve terminals.

In CFS, α-LTX does not release Ca\textsuperscript{2+} from ER by IP\textsubscript{3} receptor activation in frog motor nerve terminals. Unlike synaptosomes, the PLC inhibitor U-73122 did not block the increase in transmitter release or Ca\textsuperscript{2+} mobilization by α-LTX. This was not surprising since U-73122 only affects the Ca\textsuperscript{2+}-dependent effects of α-LTX (Rahman et al., 1999). Even though U-73122 only blocks one mechanism of Ca\textsuperscript{2+} release from the ER (inhibition of PLC\textbeta), it appears that the ER does not contain enough Ca\textsuperscript{2+} to fully account for the amount of release induced by α-LTX. When cytoplasmic Ca\textsuperscript{2+} levels fall in CFS, the ER loses Ca\textsuperscript{2+}. Furthermore, the fact that α-LTX could still mobilize a significant amount of Ca\textsuperscript{2+} after depleting Ca\textsuperscript{2+} from ER with thapsigargin, suggests that ER is not the major Ca\textsuperscript{2+} pool. In the presence of extracellular Ca\textsuperscript{2+}, this story could be different, however the goal of the thesis was to parse out the more interesting mechanism of α-LTX action in CFS.

4.4.2 The Mitochondrion

Ca\textsuperscript{2+} homeostasis within mitochondria is regulated by several transport mechanisms (for a review see (Gunter and Pfeiffer, 1990)). Mitochondria in presynaptic nerve terminals have been shown to buffer Ca\textsuperscript{2+} loads during repetitive nerve stimulation (David et al., 1998). Therefore, it is thought that mitochondria help buffer large increases in intracellular Ca\textsuperscript{2+} by acting as a temporary sink before slowly releasing it. In this way, mitochondria protect neurons against high-sustained intracellular Ca\textsuperscript{2+} concentrations, which are believed to be the trigger for the initiation of cell death (Berridge et al., 1998). The importance of mitochondria is shown by experiments in which inhibition of mitochondria alters the temporal pattern of transmitter release and Ca\textsuperscript{2+} signalling (Brodin et al., 1999; Ichas et al., 1997; Tang and Zucker, 1997).
4.4.2.1 Ca\textsuperscript{2+} Influx

4.4.2.1.1 Ca\textsuperscript{2+} Uniporter

Entry of Ca\textsuperscript{2+} into the mitochondrial matrix occurs via a Ca\textsuperscript{2+} uniporter. Ca\textsuperscript{2+} influx into mitochondria is not coupled to the movement of any other ions. Instead, Ca\textsuperscript{2+} uses the steep electrochemical gradient (~-150 mV to -200 mV inside the mitochondria) created by H\textsuperscript{+} efflux from the mitochondrial matrix during respiration. Since the Ca\textsuperscript{2+} affinity of the mitochondrial uniporter is relatively low compared to the ER SERCA pump, the uniporter is probably only significant when the cytosolic concentrations of Ca\textsuperscript{2+} exceed ~500 nM (Pozzan et al., 1994). Inside the mitochondrial matrix are several Ca\textsuperscript{2+}-sensitive dehydrogenases that are members of the TCA cycle (Denton and McCormack, 1990). It is believed that these enzymes may be involved in linking ATP production to energetic demands of the cell during physiological activity (Hajnoczky et al., 1995). In other words, when Ca\textsuperscript{2+} accumulates in mitochondria as an indirect result of increased cellular activity, it triggers the mitochondrial respiratory chain to produce more ATP.

4.4.2.2 Ca\textsuperscript{2+} Efflux

\textit{\alpha}-LTX was shown to mobilize Ca\textsuperscript{2+} from mitochondrial stores because depletion of these stores with CCCP blocked \textit{\alpha}-LTX-induced Ca\textsuperscript{2+} mobilization and vice versa. CCCP is a protonophore that uncouples oxidative phosphorylation and depolarizes the mitochondrial membrane by disrupting the H\textsuperscript{+} concentration gradient, which causes the release of Ca\textsuperscript{2+}. My results show that CCCP releases a comparable amount of Ca\textsuperscript{2+} to \textit{\alpha}-LTX in frog motor nerve terminals bathed in CFS. In contrast, it is believed the Ca\textsuperscript{2+} concentration in the mitochondrial matrix is not much higher than the cytosol concentration of Ca\textsuperscript{2+} so the release of mitochondrial Ca\textsuperscript{2+} could not possibly generate much of a signal. However, motor nerve terminals innervating the cutaneous pectoris muscle contain many mitochondria (Herrera et al., 1985). Therefore it is not unreasonable to think that the collective release of Ca\textsuperscript{2+} from all the mitochondria could produce a substantial increase in intracellular Ca\textsuperscript{2+}. It is also possible that stimulus-dependent Ca\textsuperscript{2+} entry during the experiment caused mitochondria to load Ca\textsuperscript{2+}.

The peak frequency of spontaneous transmitter release by CCCP in the absence of extracellular Ca\textsuperscript{2+} was about a 100 MEPPs/second, which is similar to values...
reported previously (Molgo and Pecot-Dechavassine, 1988). This value is much lower than the peak MEPP frequencies attained with α-LTX (3 - 400 MEPPs/second), despite the fact both α-LTX and CCCP produce similar Ca\(^{2+}\) signals. This suggests that α-LTX sensitizes transmitter release to Ca\(^{2+}\). Alternatively, the release of Ca\(^{2+}\) could just be an epiphenomenon and exocytosis is being triggered by a mechanism that bypasses the requirement for Ca\(^{2+}\).

4.4.2.2.1 \(\text{Na}^+$/Ca\(^{2+}\)$ Exchanger

Under physiological conditions, Ca\(^{2+}\) is given back to the cytosol from the mitochondrial matrix using energy derived from the H\(^+\) or Na\(^+\) concentration gradient. In the nervous system, the Na\(^+$/Ca\(^{2+}\)$ exchanger, which transports 2 Na\(^+\) ions for each Ca\(^{2+}\) ion, has been explored for many years and is thought to be the dominant mechanism by which Ca\(^{2+}\) is extruded from mitochondria. After stimulation-induced increases in cytosolic Ca\(^{2+}\), the mitochondrial Na\(^+$/Ca\(^{2+}\)$ exchanger appears to prolong the recovery of the resting cytosolic Ca\(^{2+}\) concentration because it releases Ca\(^{2+}\) that had accumulated within mitochondria (Babcock et al., 1997). Activation of the Na\(^+$/Ca\(^{2+}\)$ exchanger is also sensitive to the intracellular Na\(^+\) concentration (Hoyt et al., 1998). Compared to Ca\(^{2+}\) influx, the rate at which Ca\(^{2+}\) is removed from the mitochondrial matrix by Na\(^+$/Ca\(^{2+}\)$ exchange is relatively slow and easily saturated.

α-LTX mobilizes Ca\(^{2+}\) from mitochondria partly by increasing intracellular Na\(^+\). α-LTX has also been shown to increase intracellular Na\(^+\) in synaptosomes and PC12 cells (Deri and Adam-Vizi, 1993; Grasso et al., 1982). The ability of α-LTX to increase intracellular Na\(^+\) is consistent with previous findings at the frog NMJ. For instance, it has been shown using extracellular recordings that α-LTX causes the action potential to disappear (Longenecker et al., 1970). This is not due to inhibition of voltage-gated Na\(^+\) channels because α-LTX does not have any affinity for anything else in CFS but CL. Furthermore, α-LTX causes nerve terminals to swell by osmosis, which is not observed when extracellular Na\(^+\) is removed (Gorio et al., 1978). I have shown that the increase in nerve terminal diameter is not due to exocytosis of non-recycling vesicles because the latter follows the former. The fact that Ca\(^{2+}\) release by α-LTX was not completely inhibited by the removal of Na\(^+\) suggests that choline used as a Na\(^+\) replacement may be acting as a weak surrogate for Na\(^+\) since choline can probably also pass through the
large conductance cation pores. However, I have found no evidence in the literature to support choline activation of the Na⁺/Ca²⁺ exchanger.

4.4.2.2 The Mitochondrial Permeability Transition

More recently, the MPT pore has been explored as another possible route of Ca²⁺ release (Bernardi, 1996). The MPT pore is a cation-permeable channel that is gated by voltage and the opening is favoured by several factors such as depolarization, intramatrix Ca²⁺, and oxidizing agents. It is believed that under physiological conditions, Ca²⁺ accumulation in mitochondria stimulates respiration and proton efflux thus increasing matrix pH. A decrease in the matrix H⁺ concentration facilitates MPT pore opening, which leads to a collapse of the mitochondrial H⁺ gradient and membrane potential, the outward movement of Ca²⁺ through the channel and an influx of H⁺ into the matrix. The decrease in mitochondrial pH leads to closing of the channel. Thus, the MPT pore behaves like a Ca²⁺-induced Ca²⁺ release channel similar to the ryanodine receptors found in the ER membrane. In addition to this low conductance mode, the MPT pore can also switch to a high conductance mode (Ichas and Mazat, 1998). Opening of the MPT pore in the high conductance mode appears to be irreversible and has profound consequences for cell function. In the high conductance mode, this channel is capable of passing large molecules with a molecular weight up to 1.5 kDa into the cytosol. Apoptosis-inducing factor (AIF) and cytochrome c are just a couple examples of destructive molecules that could leak into the cytoplasm because they are believed to be the triggers for cell death (for a review see (Kroemer et al., 1998)).

Experiments with CsA provided evidence that a mechanism of Ca²⁺ release in addition to Na⁺/Ca²⁺ exchange may be operating since removing extracellular Na⁺ did not completely block the release of Ca²⁺ by α-LTX. Inhibition of the MPT pore by CsA does not appear to require inhibition of calcineurin activity because CsA analogs, which do not inhibit calcineurin activity, can still block the MPT pore (Bernardi et al., 1994). However, the effect of CsA was also not completely effective at blocking the actions of α-LTX. It could be that CsA is not a potent inhibitor of the MPT pore in frog or that the block of the MPT pore by CsA is not long lasting (Gorio et al., 1978).

Normally, MPT pore opening as a result of mitochondrial Ca²⁺ accumulation results in osmotic swelling and rupturing of the outer mitochondrial membrane. Mitochondrial swelling occurs in frog motor nerve terminals that are treated with α-LTX.
in Ca\textsuperscript{2+}-containing saline (Gorio et al., 1978). However, when \( \alpha \)-LTX is applied in CFS, mitochondria appear morphologically normal, which questions the involvement of the MPT pore. All my CsA experiments were done in CFS, which means that Na\textsuperscript{+} was still present in the bath. Therefore, CsA could just be blocking the Na\textsuperscript{+}-dependent effects of \( \alpha \)-LTX by occluding the non-selective cation pore on the plasma membrane and that the MPT pore is not involved. In the future, experiments with CsA will have to be done in Na\textsuperscript{+} and CFS to eliminate this possibility.

4.5 Ca\textsuperscript{2+} HANDLING MECHANISMS

Very little Ca\textsuperscript{2+} entering the cytosol actually remains as free Ca\textsuperscript{2+} because it is rapidly buffered or extruded from the cell (for a review see (Miller, 1991)). Much of the Ca\textsuperscript{2+} entering the cytoplasm is immediately buffered by high affinity Ca\textsuperscript{2+} binding proteins such as calmodulin, parvalbumin, calcineurin, calbindin-D\textsubscript{28K}, and S100 protein. The cytosol also contains a variety of low affinity Ca\textsuperscript{2+} binding molecules such as citrate, nucleotides, and inositol phosphates, which may participate in Ca\textsuperscript{2+} buffering when the intracellular Ca\textsuperscript{2+} concentration gets high. Ca\textsuperscript{2+} is removed from the cytosol by several mechanisms. High affinity, ATP driven Ca\textsuperscript{2+} pumps found on the plasma membrane (Ca\textsuperscript{2+} ATPases) are responsible for pumping Ca\textsuperscript{2+} out of the cell. Ca\textsuperscript{2+} is also expelled from cells through low-affinity, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers. These exchangers remove Ca\textsuperscript{2+} ions using the energy derived from the Na\textsuperscript{+} electrochemical gradient. Finally, Ca\textsuperscript{2+} ions can be removed from the cytosol by sequestration into various organelles such as the Golgi apparatus, nucleus, ER, and mitochondria; the last two being most relevant to nerve terminals.

When the nerve is repetitively stimulated with a train of action potentials, Ca\textsuperscript{2+} quickly accumulates in the terminal. At the end of the stimulus, the intracellular Ca\textsuperscript{2+} concentration quickly subsides back to resting levels within a matter of seconds. This rapid extrusion of Ca\textsuperscript{2+} has physiological importance because high levels of Ca\textsuperscript{2+} over relatively long periods of time can activate intracellular proteases, which cause nerve terminal damage. However in frog motor nerve terminals treated with \( \alpha \)-LTX, the intracellular Ca\textsuperscript{2+} concentration remains elevated long after transmitter release has subsided and will seldom return to resting levels. For those few terminals in which the toxin-induced Ca\textsuperscript{2+} signal eventually settled back down, the decay time was in the order of tens of minutes. It does not seem likely that any intracellular Ca\textsuperscript{2+} store would be
capable of releasing Ca\textsuperscript{2+} inexhaustibly over a period of 20-30 minutes because they have a finite amount of Ca\textsuperscript{2+}. A more likely scenario is that during toxin action, the Ca\textsuperscript{2+} handling mechanisms in the nerve terminal become compromised. One possibility could be depletion of ATP resulting from mitochondrial dysfunction and/or leak across the plasma membrane (Grasso et al., 1982). This might prevent the buffering of Ca\textsuperscript{2+} by Ca\textsuperscript{2+} ATPases on the plasma membrane. Another possibility is that the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange rate is reduced by the accumulation of Na\textsuperscript{+} documented here.

4.6 ION DEPENDENCE OF TRANSMITTER RELEASE BY \(\alpha\)-LTX

4.6.1 The Ca\textsuperscript{2+} Signal and Transmitter Release

It is reasonable to assume that the rise in intracellular Ca\textsuperscript{2+} is responsible for the rise in transmitter release by \(\alpha\)-LTX because the rise in intracellular Ca\textsuperscript{2+} precedes the rise in transmitter release. However, since BAPTA-AM can buffer the release of Ca\textsuperscript{2+} from mitochondria without affecting transmitter release it appears that the Ca\textsuperscript{2+} released by \(\alpha\)-LTX is not essential. These results suggest that something in addition to Ca\textsuperscript{2+} is responsible for the increase in transmitter release by \(\alpha\)-LTX or that the toxin increases the sensitivity of release to Ca\textsuperscript{2+}. The latter notion is supported by the fact that \(\alpha\)-LTX causes more transmitter release than Ca\textsuperscript{2+}-ionophores or high K\textsuperscript{+} solutions given the same extracellular Ca\textsuperscript{2+} concentration in permeabilized cells (Davletov et al., 1998).

The Ca\textsuperscript{2+} dependence of \(\alpha\)-LTX action on exocytosis still remains controversial. In rat brain synaptosomes and adrenal chromaffin cells, transmitter release by \(\alpha\)-LTX depends on the presence of extracellular Ca\textsuperscript{2+} and a rise in intracellular Ca\textsuperscript{2+}. Ca\textsuperscript{2+}-dependent action of \(\alpha\)-LTX to cause transmitter release is SNARE-dependent because botulinum toxins, which cleave SNARE proteins, are able to block most, but not all of this release (Capogna et al., 1997; Davletov et al., 1998; Rahman et al., 1999). The remaining portion is Ca\textsuperscript{2+}-independent and has been attributed to non-specific leakage (i.e. non-quantal release) of transmitter molecules. However, this is not the case in all cell systems.

At the frog NMJ, the actions of \(\alpha\)-LTX appear to be completely independent of intra- and extracellular Ca\textsuperscript{2+} and release is quantal. Studies on PC12 cells and \(\beta\)-pancreatic cells have also come to similar conclusions using Ca\textsuperscript{2+} fluorescent indicators (Lang et al., 1998; Meldolesi et al., 1984). \(\alpha\)-LTX also works in the absence of
extracellular Ca$^{2+}$ on hippocampal slices, however it has not been determined if there is mobilization of Ca$^{2+}$ from intracellular stores. It is not unreasonable to think that exocytosis by α-LTX can occur by an alternative mechanism different from that used in normal Ca$^{2+}$-regulated secretion. For instance, in systems where synaptotagmin function is impaired by peptide injection or genetic mutation, Ca$^{2+}$-regulated secretion by ionophores and depolarizing agents is impaired, yet transmitter release by α-LTX remains unaffected (Geppert et al., 1994; Thomas and Elferink, 1998). Similarly, munc 13-1, which is a phorbol ester receptor essential for synaptic vesicle exocytosis in glutamatergic neurons, is not required for exocytosis by α-LTX (Augustin et al., 1999). Therefore, there are several indicators that α-LTX can stimulate exocytosis by a mechanism independent of intracellular Ca$^{2+}$.

4.6.2 The Na$^+$ Signal and Transmitter Release

The block of excited neuromuscular transmission by α-LTX may be due in part to the concomitant rise in intracellular Na$^+$. When α-LTX increases the permeability of the nerve terminal membrane to Na$^+$, intracellular Na$^+$ increases and this could block action potentials. There may also be nerve terminal depolarization, which would inactivate Na$^+$ channels (Nicholls et al., 1982). This depolarization would be expected from the insertion of non-specific cation conductances into the nerve terminal. Terminal K$^+$ conductance would also increase owing to Ca$^{2+}$ stimulation of Ca$^{2+}$-activated K$^+$ channels. The increase in terminal membrane conductance would 'short-circuit' local currents and this would prohibit propagation of action potentials into the terminal. Thus, while Na$^+$ accumulation may be important in blocking action potentials, other mechanisms should also have powerful effects. The block in neuromuscular transmission is much too rapid to be explained by vesicle depletion, which has a time constant of approximately 5 minutes (Longenecker et al., 1970). Despite the block in nerve terminal spike invasion, conduction down nerve fibres remains unchanged (Mallart and Haimann, 1985) and the muscle can still be excited by direct stimulation (Okamoto et al., 1971).

It is possible that Na$^+$ may play a role in transmitter release by α-LTX. Agents such as ouabain (Na$^+$/K$^+$ inhibitor) and monensin (Na$^+$ ionophore), which are known to cause Na$^+$ accumulation in nerve terminals, also cause significant increases in MEPP frequency (Atwood et al., 1983; Baker and Crawford, 1975; Meiri et al., 1981). Even
tetanic stimulation in Ca$^{2+}$-free Ringers solution can increase the frequency of spontaneous transmitter release (Miledi and Thies, 1967). However, the most striking example of Na$^+$-dependent transmitter release comes from another neurotoxin called Brevetoxin-3. Brevetoxin-3 is remarkably similar to $\alpha$-LTX because it too can stimulate transmitter release independently of extracellular Ca$^{2+}$ in frog motor nerve terminals (Meunier et al., 1997). This toxin evidently opens voltage-gate Na$^+$ channels because its actions are completely blocked by TTX. This suggests that a chronic influx of Na$^+$ can produce $\alpha$-LTX-like effects on transmitter release.

In contrast, there is also evidence suggesting that Na$^+$ entry plays no part in transmitter release by $\alpha$-LTX. For instance $\alpha$-LTX still caused vesicle depletion from frog motor nerve terminals bathed in Na$^+$ and CFS (Gorio et al., 1978). Similarly, $\alpha$-LTX-induced release was also observed from synaptosomes in the absence of extracellular Na$^+$ (Storchak et al., 1994). In tissue preparations, it is difficult to get rid of all the extracellular Na$^+$ without the aid of a good Na$^+$ chelator. The bath can be easily contaminated with Na$^+$ from muscle fibres that pump out, or leak Na$^+$ from t-tubules. Also, the acetylcholine receptor channel is Na$^+$ permeable, so Na$^+$ could escape from the muscle directly into the synapse. However in order to get Na$^+$ entry, Na$^+$ would have to build up to millimolar concentrations in the cleft to establish a gradient. This is unlikely because the relatively large bath would rapidly dilute any leak of Na$^+$ from tissues. In contrast, it is possible that the Na$^+$ substitute may be carrying out similar actions to Na$^+$ during $\alpha$-LTX action.

If Na$^+$ entry is not important for transmitter release by $\alpha$-LTX, then depolarization and swelling of the nerve terminal are probably not as important as well. The former is supported by the fact that $\alpha$-LTX still caused release in isotonic K$^+$ solutions (Rubin et al., 1978) and the latter is supported by the fact that in Ca$^{2+}$- and Na$^+$-free saline, $\alpha$-LTX still caused vesicle depletion without nerve terminal swelling (Gorio et al., 1978). Furthermore, MEPP frequency increases in hypertonic solutions, which causes nerve terminals to shrink in diameter (Blioch et al., 1968; Longenecker et al., 1970).
4.7 THE α-LTX RECEPTOR

Localization of the α-LTX receptor using CL1 antibodies agrees well with the site of α-LTX action. CL1 immunoreactivity was predominantly found at nerve terminals where the α-LTX presumably binds and stimulates neurotransmitter release. No CL1 immunoreactivity was found in unpermeabilized preparations (data not shown) suggesting that the antibody was raised against an intracellular epitope of the receptor molecule. This staining pattern corresponds to observations made by Ceccarelli’s group using anti-α-LTX antibodies at the frog NMJ (Valtorta et al., 1984), however there are subtle differences. Unlike α-LTX immunoreactivity, which was only found on the edges of nerve terminals, CL1 immunoreactivity was found both in the periphery and in a banded pattern that closely resembled active zone staining. The spacing of the bands appeared to be ~4 μm apart, which is uncharacteristic of active zone staining by SNARE protein antibodies, which are normally found about 1-2 μm apart. The bands appeared to be located at every other active zone, which is more characteristic of Schwann cell finger staining revealed by P(0) antibodies (Georgiou and Charlton, 1999). However, Schwann cells do not show any α-LTX immunoreactivity when nerve terminals are denervated (Valtorta et al., 1984). Ceccarelli’s group may not have detected the banded pattern of staining because they used an epifluorescence microscope, which cannot resolve images to the same extent as a confocal microscope. If this was the case, there should have been a diffuse staining pattern along the middle of the terminal, but there was not. Another possibility is that the antibody was detecting an epitope on a different active zone protein, which was exposed during permeabilization.

In both studies, very little staining was found elsewhere at the NMJ. Schwann cells, capillaries, muscle cells, and unmyelinated pre-terminal branches were consistently negative for any α-LTX or CL1 immunoreactivity. This finding is consistent with reports that CL is predominantly expressed in nervous tissue (Matsushita et al., 1999). There has been a recent report that CL1 mRNA is found ubiquitously but at substantially lower amounts relative to brain (Sugita et al., 1998). CL2 mRNA, however, is ubiquitously distributed at relatively high levels. Therefore, one would expect to find α-LTX all over the tissue preparation. CL2 may not have been detected using anti-α-LTX antibodies because its affinity for α-LTX is 14 times less than CL1 (Ichtchenko et al., 1999). Furthermore, if CL2 is not found in regions of high density like
CL1, then detection by immunofluorescence becomes very difficult. Although CL3 is also found in nervous tissue, it does not appear to be involved in α-LTX action since over expression of CL3 does not sensitize PC12 cells to α-LTX unlike CL1 (Sugita et al., 1998). It is unlikely that the CL1 antibody would cross-react with CL2 or CL3 because the intracellular C-terminal domain, which the antibody is directed against, is the most variable region among the α-LTX receptor family.

4.7.1 CL1 Required for α-LTX Action

α-LTX must bind to its receptor before triggering the release of neurotransmitters from synaptic terminals. α-LTX binds to its receptor with high affinity ($K_D \approx 0.5$ nM) due to a fast association rate and a slow dissociation rate (Meldolesi, 1982). Dose-response studies on synaptosomes and PC12 cells using radioactively labelled α-LTX show a parallel correlation between toxin binding and secretion (Meldolesi, 1982; Meldolesi et al., 1983). If there is no receptor or if binding is prevented, then transmitter release is unaffected by α-LTX. For instance, α-LTX cannot stimulate transmitter release in the presence of concanavalin A at the frog NMJ (Rubin et al., 1978). This lectin, which binds to sugar residues high in mannose, has been shown to non-specifically reduce the amount of α-LTX binding to PC12 cells by steric hindrance (Grasso et al., 1978). α-LTX binding and release was also reduced if intact cells were treated with a protease such as trypsin, which cuts extracellular proteins at lysine and arginine residues (Meldolesi et al., 1983). And finally, transfection of CL1 mRNA into chromaffin cells or injection of rat brain mRNA into oocytes can sensitize these cells to α-LTX (Bittner et al., 1998; Filippov et al., 1990). Together, these results suggest that α-LTX binding to high affinity receptors is the first step in α-LTX action.

4.7.2 Role for CL1 in α-LTX-Stimulated Transmitter Release

The discovery of CL1 stimulated great interest in the α-LTX field because it might constitute a novel synapse-specific G-protein-linked receptor with a function related to exocytosis. This was a reasonable hypothesis considering that the CL1 co-purified with the t-SNARE, syntaxin (Krasnoperov et al., 1997). Furthermore, direct stimulation of exocytosis by G-proteins has also been shown to directly control exocytosis in chromaffin and insulin secreting cells (Gasman et al., 1997; Lang et al., 1995). If CL1
activation stimulated transmitter release directly, it would provide a ready explanation for the actions of the toxin in the absence of extracellular Ca\(^{2+}\).

Although \(\alpha\)-LTX binding is required for transmitter release, it appears that signal transduction via CL1 is not required according to recombinant receptor studies (Krasnoperov et al., 1999). When part of the cytoplasmic C-terminus was truncated, \(\alpha\)-LTX was still able to stimulate transmitter release. Furthermore, the amount of secretion by the toxin was unaffected when 6 of the 7 transmembrane segments were removed. Without these transmembrane segments, the receptor would be unable to activate G proteins. Thus, \(\alpha\)-LTX is probably using CL1 only as a landing marker in order to exert its actions at nerve terminals.

Does CL1 have a physiological role directly participating in synaptic transmission? If the receptor serves as a target for \(\alpha\)-LTX, a potent secretagogue, it is likely that the receptor is positioned appropriately for the regulation of exocytosis. The fact that CL1 binds to syntaxin, which is localized to active zones (Boudier et al., 1996), almost assures that this is the case. It has been suggested that CL1 may play a modulatory role in synaptic transmission because overexpression of CL1 in chromaffin cells appears to depress \(\alpha\)-LTX- and Ca\(^{2+}\)-stimulated secretion (Bittner et al., 1998). In other words, it has been hypothesized that CL1 may act as a molecular 'brake' on secretion that can be released by \(\alpha\)-LTX. Conversely, transfection of CL1 has no affect on Ca\(^{2+}\)-stimulated secretion in PC12 cells (Sugita et al., 1998), thus the role of CL1 in exocytosis still remains controversial.

### 4.7.3 Role for CL1 in \(\alpha\)-LTX-Stimulated Ca\(^{2+}\) Mobilization

The coupling of CL1 to G-proteins makes the receptor ideally suited for signal transduction into the cell. CL1 interacts with the G\(\alpha_0\) and G\(\alpha_{\q11}\) class of G-proteins in rat brain synaptosomes. It is well established that the former inhibits adenylate cyclase and the latter activates PLC. It has been shown in synaptosomes that activation of PLC and release of stored Ca\(^{2+}\) is responsible for transmitter release by \(\alpha\)-LTX (Davletov et al., 1998). This conclusion remains controversial because other studies on synaptosomes have shown that: (1) PKC inactivation has no effect on \(\alpha\)-LTX action (Storchak et al., 1998), (2) the main second messengers, cAMP, cGMP, and IP\(_3\) do not control synaptic vesicle exocytosis and do not play a major role in \(\alpha\)-LTX action in
CFS (Grasso et al., 1980), and (3) recombinant α-LTX can bind and stimulate PIP$_2$ hydrolysis similar to wild-type α-LTX, without significant stimulation of exocytosis (Ichtchenko et al., 1998). There is no doubt that Ca$^{2+}$ release by CL1 activation would have some effect on exocytosis, however more experiments have to be completed to determine if this is the true physiological function of the receptor.

Studying G-protein-coupled receptors using the frog NMJ preparation is not a trivial task because there is a lack of specific pharmacological agents. Pertussis toxin (PTX) was the only inhibitor of G-protein function that I tested on the frog NMJ. This toxin specifically disrupts G-protein function by inhibiting the G$_{\alpha_o}$ class of G-proteins. Ca$^{2+}$ mobilization induced by α-LTX was not affected when PTX was added at 4 μg/ml (data not shown), which is a concentration known to block Ca$^{2+}$ mobilization in Schwann cells (Robitaille, 1998). This data does not say much about the mechanism of Ca$^{2+}$ mobilization by α-LTX since there are G$_{\alpha_o}$ proteins that are insensitive to PTX. It only tells us that Ca$^{2+}$ mobilization by α-LTX at the frog NMJ is not working by a pertussis toxin-sensitive G protein. Furthermore, the ineffectiveness of U-73122, the putative inhibitor of PLCβ only provides indirect evidence against the involvement of G$_{\alpha_q/11}$, the other G-protein that co-precipitates with CL1. Without positive data, it is difficult to make interpretations because the functional links of G protein-coupled receptors can be complex and difficult to parse out (Selbie and Hill, 1998). It still remains to be determined if the G protein function is required at all for α-LTX-induced Ca$^{2+}$ mobilization at the frog NMJ.

### 4.7.4 Role for CL1 in α-LTX-Stimulated Cation Influx

In every cell system, α-LTX increases the permeability of the plasma membrane to cations. At the frog NMJ, the plasma membrane is permeable to both Na$^+$ and Ca$^{2+}$. This raises the possibility that CL1 may be linked to a non-specific cation channel on the plasma membrane. Opening of cation channels by activation of receptors coupled to the G$_{\alpha_q}$ class of G-proteins have been discovered in Drosophila. However, CL1 mutants lacking any signal transduction potential because of transmembrane deletions do not prevent (but slightly impairs) the increase in membrane permeability by α-LTX (Krasnoperov et al., 1999). This suggests that CL1 function is not necessary for α-LTX to increase membrane permeability.
4.8 PROPOSED MECHANISM OF $\alpha$-LTX ACTION

There have been three major theories to explain the underlying mechanism of $\alpha$-LTX-induced transmitter release in the absence of extracellular Ca$^{2+}$. The first proposed that $\alpha$-LTX acts as an ionophore by creating pores in the membrane and that transmitter release increased as a result of changes in ion concentrations. The second proposed that $\alpha$-LTX binding to its receptor activated a signal transduction pathway, which triggered transmitter release via second messengers. The third proposed that $\alpha$-LTX binding to its receptor increased transmitter release directly through protein-protein interactions. None of these hypotheses, which are not mutually exclusive, have been ruled out at the frog NMJ (see Figure 4.1 for a summary). Each mechanism is supported by a large amount of data, suggesting that they all may be participating in the actions of $\alpha$-LTX. Evidence for multiple effects of $\alpha$-LTX action has been provided in frogs (Gorio et al., 1978) and also chromaffin cells (Bittner et al., 1998).

After binding to CL1, $\alpha$-LTX probably inserts itself into the membrane and creates a large conductance cation pore with several other toxin molecules at the frog motor nerve terminal. The hypothesis that $\alpha$-LTX molecules can associate in clusters to form pores is not new and has been suggested previously in Xenopus oocytes (Filippov et al., 1990; Filippov et al., 1994). This has been supported by recent structural data showing that $\alpha$-LTX can form tetramers in the membrane with a pore diameter of about 25 Å (Rahman et al., 1999). However, it is not clear yet whether tetramerization occurs before or after CL1 binding but it may be a contributing factor to the relatively long latency period. A previous study suggests that the binding domain of $\alpha$-LTX is separate from the pore-forming domain because monoclonal antibodies to $\alpha$-LTX can block Ca$^{2+}$ uptake by $\alpha$-LTX without affecting toxin binding (Cattaneo and Grasso, 1986).

The formation of large conductance pores in the membrane raises the possibility that a factor, which normally inhibits transmitter release may be leaving the nerve terminal. One candidate is glutathione, which is the major determinant of the oxidation-reduction state of the cytosol and the ER. This tripeptide serves to prevent the formation of disulfide bonds in the cytosol and to catalyse their formation in the ER. Oxidizing agents such as diamide have been shown to stimulate release from nerve terminals independently of extracellular Ca$^{2+}$ by oxidizing intracellular glutathione (Carlen et al., 1976). Similarly, the oxidation potential of the cytosol could increase if the intracellular
levels of glutathione, which are normally kept at about 3 mM (Mcllwain, H. Biochemistry and the Central Nervous System, 3rd ed., Churchill, London, 1966, pg 136), were to decrease. This would occur if glutathione 'leaked' out of the nerve terminal through pores created by \( \alpha \)-LTX in the plasma membrane. In addition to increasing transmitter release, increased oxidation may be linked to the \( \text{Ca}^{2+} \) signal because it facilitates the opening of the MPT pore. Furthermore, it may also explain the destructive effects of \( \alpha \)-LTX because neurons become more susceptible to nitric oxide, which can react with reactive oxygen species (superoxide and free radicals) to form peroxynitrite, a highly toxic species capable of damaging protein, lipid, and nucleic acid molecules. The only caveat is that glutathione is a negatively charged peptide that wouldn't be expected to pass through the cation selective pore. However, the large conductance pores created by \( \alpha \)-LTX in planar lipid bilayers may be characteristically different than the large conductance pores created when a receptor is present. In other words, assembly of the pore in intact cells could give rise to a structure that passes ions and molecules without bias.

Another possibility is that \( \alpha \)-LTX stimulates exocytosis directly in CFS after binding to CL1 and inserting into itself into the membrane. Ankyrin-like repeats in the toxin molecule suggests that \( \alpha \)-LTX could interact with intracellular components. In cultured spinal cord neurons (Rubin et al., 1978) and the frog NMJ (Clark et al., 1972), gross structural changes occur if the toxin is applied in high concentrations. Alternatively, \( \alpha \)-LTX could be interacting with proteins involved in secretion like synaptotagmin, which functions as a molecular 'brake' for \( \text{Ca}^{2+} \)-stimulated exocytosis. Therefore \( \alpha \)-LTX may increase transmitter release independently of \( \text{Ca}^{2+} \) by disrupting the physiological function of synaptotagmin. However, direct stimulation of transmitter release via protein-protein interactions does not explain the \( \text{Ca}^{2+} \)-dependence of \( \alpha \)-LTX action in neuroendocrine cells. In other words, this hypothesis implies that there should not be any ion dependence of \( \alpha \)-LTX action. It may be the case that these cell types just lack synaptic components required for a \( \text{Ca}^{2+} \)-independent mechanism of transmitter release.
4.9 FUTURE RESEARCH

Implication of mitochondria in action of α-LTX has opened new doors in this field of research. Chronic release of Ca\(^{2+}\) from mitochondria is normally associated with mitochondrial dysfunction. The result of this dysfunction may be the activation of the permeability transition pore located on the inner mitochondrial membrane. Once opened, this channel permits non-specific leakage of ions, small molecules, and matrix proteins. The idea of a factor leaking out from mitochondria to either directly/indirectly stimulate transmitter release is both interesting and appealing. One likely candidate is cytochrome c, which is known to activate cell-destructive caspases once it enters the cytosol. Antibodies against cytochrome c are commercially available and can be used to detect release of cytochrome c by immunocytochemistry. Cell damage may also be caused by intracellular proteases, which are activated by a rise in intracellular Ca\(^{2+}\). If the increase in transmitter release by α-LTX is due to the destruction of vital proteins, then application of caspase or protease inhibitors should be able to block the effect of the toxin. Impaired mitochondrial function can also lead to the generation of free radicals (such as superoxide and hydroxyl radicals). Free radicals are reactive species that can cause widespread cellular damage. Free radical scavengers, such as vitamin E, are known to protect the cells against free radicals and may effect the actions of α-LTX if free radicals are involved. Chronic Ca\(^{2+}\) release from mitochondria may also stimulate the production of nitric oxide. This molecule may stimulate transmitter release directly or indirectly by combining with superoxides to form the destructive radical peroxynitrite. A variety of inhibitors of nitric oxide synthesis are readily available and can be used as potential pharmacological blockers of α-LTX action. This is interesting because it implicates the receptor in mitochondrial dysfunction and cell death. This research is important because it may reveal new insights into synaptic health and disease.
Figure 4.1. Proposed models for the action of α-LTX at frog motor nerve terminals in the absence of Ca\(^{2+}\).

Models A and B have been separated to make the diagrams a little more clearer. It is possible that mechanisms from both diagrams overlap. (A) α-LTX binding to its receptor either activates a non-selective cation channel or forms a pore complex to allow Na\(^{+}\) to enter the terminal. The increase in intracellular Na\(^{+}\) causes Ca\(^{2+}\) to be released from mitochondria. Some known factors, which might stimulate exocytosis, include Ca\(^{2+}\), Na\(^{+}\), α-LTX, or CL. (B) α-LTX binding to CL could activate an intracellular messenger to cause the release of Ca\(^{2+}\) from mitochondria. CsA can block the release of Ca\(^{2+}\). Unknown molecules leaking out from mitochondria or the nerve terminal might also be involved in stimulating exocytosis.
5 REFERENCES


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