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A revive of α-latrotoxin

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I. Introduction

α-Latrotoxin is a black widow spider (Latrodectus mactans) venom toxin causing massive neurotransmitter release. Venomous animals use a highly complex cocktails of proteins, peptides and small molecules to subdue and kill their prey. Structure–function studies of spider toxins are leading to the discovery of novel therapeutic routes for neuromuscular diseases, pain and to a variety of other pathological conditions. This review presents an overview of α-latrotoxin to help in Proteomic analysis of α-latrotoxin for uncovering its therapeutic potential. For more than three decades, the venom of the black widow spider and its principal active components, latrotoxins, have been used to induce release of neurotransmitters and hormones and to study the mechanisms of exocytosis. α-latrotoxin studies have contributed to the widespread acceptance of the vesicular theory of transmitter release. Presynaptic receptors for α-latrotoxin – neurexins, latrophilins and protein tyrosine phosphatase σ – and their endogenous ligands have now become centerpieces of their own areas of research, with a potential of uncovering new mechanisms of synapse formation and regulation that may have medical implications. However, any future success of α-latrotoxin research will require a better understanding of this unusual natural tool and a more precise dissection of its multiple mechanisms.There are at least seven highly homologous LTXs. The best characterized of these is the vertebrate-specific toxin, α-LTX (Grasso 1976; Frontali et al. 1976). The venom also contains five insect-specific toxins, called latroinsectotoxins α, β, γ, δ and ε (Grishin 1998), and one crustacean-specific protein, α-latrocrustatoxin (Krasnoperov et al. 1990; Volynskii et al. 1999). α-Latrotoxin induces neurotransmitter release by stimulating synaptic vesicle exocytosis via two mechanisms: (1) A Ca^{2+}-dependent mechanism with neurexins as receptors, in which α-latrotoxin acts like a Ca^{2+}-ionophore, and (2) a Ca^{2+}-independent mechanism with CIRL/Latrophilins as receptors, in which α-latrotoxin directly stimulates the transmitter release machinery. α-Latrotoxin is a large protein composed of an N-terminal region containing disulfide bonds, and a C-terminal region containing 22 ankyrin repeats (Südhof, 2001, Ushkaryov et al., 2008).

1. Black widow spider (Latrodectus mactans): Latrodectus mactans, more commonly known as the southern black widow, are identified by their red and black coloring and are native to North America (Upadhyay & Ahmad, 2011). They have historically been known as one of the most abundant toxin-bearing species of spiders in the US (Upadhyay & Ahmad, 2011). Widow spiders are known for the severe potency of the neurotoxic venom, which contains a cocktail of various forms of the protein latrotoxin including α-latrotoxin, α-latrocrustatoxin, and α- latroinsectotoxin (Garb et al., 2004). Widow spiders (genus Latrodectus) have been documented as having a distribution that spans multiple continents and oceanic islands (Garb et al., 2004). The genus Latrodectus consists of approximately 30 species and includes species known as the black widow, the Australian red-back spider, and the cosmopolitan brown widow (Garb et al., 2004).Females are darker, more venomous, and significantly larger than males (leg spans of 30 to 40 mm compared with 16 to 20 mm). Males also are capable of biting but rarely inflict severely envenoming bites. Most females are dark gray or black with red or orange hourglass or geometric patterns, There are more than 30,000 species of spiders, most of which cannot inflict serious bites to humans because of their delicate mouthparts and impotent or prey-specific venoms. However, some spiders produce toxic venoms that can cause skin lesions, systemic illnesses, and neurotoxicity. One of the more common bites is inflicted by the widow spiders (Latrodectus species). A bite from a widow spider results in muscle spasms and rigidity starting at the bite site within 30 minutes to two hours. Latrodectus spiders are most abundant and active during the warmer months. Systemic toxicity from widow spider bites (i.e., latrodectism) is caused by α-latrotoxin, a neurotoxic component of Latrodectus venom that causes massive presynaptic release of most neurotransmitters, including acetylcholine, norepinephrine, dopamine, and glutamate.

2. Spider Venom: Widow spiders (genus Latrodectus) possess neurotoxic venom that varies in potency among species. α-latrotoxin is the main protein in widow venom that affects vertebrates, including humans. The black widow spider (genus Lactrodectus) has horrified humans for millennia – mostly by its very painful (sometimes

fatal; Bogen and Loomis 1936) bite, but not least by its striking coloration and enigmatic behavior. Spider venoms are known to be complex multicomponent mixtures of biologically active substances (Vassilevski et al., 2009). Venom composition is species-specific and is dependent on factors that include sex, nutrition, habitat condition, and climate such that the spider dispersing the venom carefully calculates an affective dosage based on components of its victim (Vassilevski et al., 2009). Venom is used in both protection and to aquire prey (Vassilevski et al., 2009). Spider venoms include various substances of different chemical nature(Vassilevski et al., 2009). These substances can be divided by molecular mass into three different groups which are low molecular weight substances, small peptides, and high molecular weight substances including enzyme and neurotoxin proteins (Vassilevski et al., 2009). Latrotoxins are the functional high molecular weight neurotoxin proteins found in the venom of all widow spiders (Kiyatkin et al., 1990). Spiders belonging to the genus Latrodectus are the most clinically significant group of spiders in the world due to the severe symptoms caused by their envenomation (Graudins et al., 2012). Due to the toxicity of their venom and their common occurrence in places where people frequent or live, members oft his genus are some of the only spiders that cause medically significant bites (Garb et al., 2004).

3. Latrotoxin: Latrotoxin is responsible for symptoms of envenomation known as latrodectism (Graudins et al., 2012). Physiological affects to prey are caused by latrotoxin selectively binding to presynaptic nerve endings and triggering an immense release of neurotransmitters (Upadhyay & Ahmad, 2011). The spiders of the genus Latrodectus possess multiple latrotoxin proteins that each target specific taxonomic groups including vertebrates (α-latrotoxin), crustaceans (α- latrocrustatoxin), and insects (α-latroinsectotoxin) (Vassilevski et al., 2009). A comparison of the structure of the different latrotoxin proteins revealed an average of 30%, identical amino acid residues. This high homology suggests these proteins likely evolved by gene duplication (Vassilevski et al., 2009). All latrotoxins, α-latrotoxin included, trigger neurotransmitter release in organisms they are active in (Südhof, 2001).

4. α-latrotoxin: α-latrotoxin is a large ~130 kDa hydrophilic protein that targets neural and neuroendocrine nerve terminals to cause large amounts of spontaneous neurotransmitter release (Graudins et al., 2012). It's been proposed that α-latrotoxin is synthesized as a protein precursor in the venom gland, where it is cleaved by endoproteases to generate the mature toxin, which is composed of four different domains (Südhof,2001). Domain I is a cleaved signal peptide. Domain II is a conserved N-terminal domain that is composed of 431 amino acid residues containing two hydrophobic sequences, each of 20-26 amino acids, and three invariant cysteine residues (Südhof, 2001). Domain III is a central domain composed of 22 imperfect ankyrin-like repeats. These repeats are characterized by 33-residue patterns that are made up of two alpha helices separated by loops covering 745 amino acids (Südhof, 2001). Domain IV is a C-terminal domain containing 206 amino acid residues. This domain is less conserved between latrotoxins and is most likely cleaved during the maturation of α-latrotoxin (Südhof, 2001). The structure of α-latrotoxin has recently been studied extensively in Latrodectus tredecimguttatus to better understand its neurotoxic effect on vertebrates after the stimulation of neurotransmitter release (Ushkaryov et al., 2004). Two closely related species, the European black widow (Latrodectus tredecimguttatus) and the red-back spider (Latrodectus hasseltii), are the only two species that have been studied for genetic variation of α-latrotoxin (Graudins et al., 2012).

5. Target cells: The toxin induces strong and sustained release of neurotransmitters and hormones from secretory cells capable of regulated exocytosis (Rosenthal et al., 1990). The effects of α-LTX on neurosecretion were first described on a cellular level in the 1970's at frog neuromuscular junctions (NMJ) (Longenecker et al., 1970; Frontali et al. 1976), and later in mouse brain slices (Tzeng et al., 1978), in synaptosomes (isolated brain nerve terminals) from rat (Grasso et al. 1978), dog (Tzeng and Siekevitz 1979) and guinea pig (Nicholls et al., 1982), primary cerebellar granule cell cultures (Grasso and Mercanti-Ciotti 1993) and in hippocampal organotypic cultures (Capogna et al. 1996). In addition, catecholamine-secreting chromaffin cells (Picotti et al., 1982) and PC12 cell line (Robello et al., 1987), insulin-secreting pancreatic β-cells in primary or derived cell cultures (Lang et al., 1998), oxytocin- and vasopressin-secreting neurohypophysis cells (Hlubek et al., 2003) and luteinising hormone-secreting rat gonadotropes (Tse and Tse 1999) were also used to characterise the toxin's effects on secretion from non-neuronal excitable cells. A secretory cell not sensitive to α-LTX still remains to be found.

6. Site and mode of action: From the earliest description of toxin's actions on neuronal systems, it emerged that α-LTX affects specifically the presynaptic element, from which it causes massive neurotransmitter release (Longenecker et al., 1970). The toxin has no major enzymatic activities (Frontali et al., 1976). Crucially, α-LTX has been discovered to create Ca^{2+}-permeable channels in lipid bilayers (Finkelstein et al., 1976), and a large body of evidence shows that Ca^{2+} influx through membrane channels induced by α-LTX in the presynaptic

membrane accounts for a major part of its effect. Pore formation occurs in all the biological systems mentioned above, but the features of α-LTX-triggered release cannot be fully explained by the toxin pore.The effect of α-LTX at NMJs is usually delayed and develops fully after ~10 min, although synaptosomes react much faster. It is detected electrophysiologically as an increase in the frequency of spontaneous miniature postsynaptic potentials at NMJs (Longenecker et al. 1970; Misler and Hurlbut 1979; Ceccarelli and Hurlbut 1980;Tsang et al., 2000) and excitatory or inhibitory postsynaptic currents at central synapses (Capogna et al., 1996). In addition, α-LTX affects action potential-evoked, synchronous release, and does this in a time-dependent manner: initially, it enhances evoked potentials, but eventually inhibits (Capogna et al., 1996) or blocks them (Longenecker et al., 1970; Hurlbut and Ceccarelli 1979; Liu and Misler 1998). Finally, when used in higher concentrations, α-LTX can cause morphological deformation and cell death, as reviewed by Sudhof (2001).However, the most surprising feature of the α-LTX-evoked secretion is that it can occur both in the presence and absence of Ca^{2+}.

6.1 Ca^{2+}-independent release: The ability of α-LTX to trigger neurotransmitter exocytosis in the absence of extracellular Ca^{2+} remains particularly interesting and inexplicable to the field (Longenecker et al., 1970; Ceccarelli et al., 1979; see also Sudhof (2001) and Ushkaryov et al., (2004) for review). This is clearly different from depolarisation-induced exocytosis, which is Ca^{2+}-dependent, but not unlike the effect of hypertonic sucrose. The possibility that α-LTX-induced release involves an unknown, Ca^{2+}-independent mechanism which may also occur during normal synaptic activity has provided the casus belli for many a quest for α-LTX structure and receptors that could trigger neurotransmission via intracellular mechanisms.In neurones, the Ca^{2+}-independent secretion is restricted to small synaptic vesicles, as demonstrated by synaptosomal and NMJ experiments, where glutamate, GABA and acetylcholine are released in the absence of Ca^{2+}, while catecholamines or peptides are not (Matteoli et al., 1988; Davletov et al., 1998; Khvotchev et al., 2000). Ca^{2+}-independent release does not normally occur in endocrine cells (Grasso et al., 1980;Michelena et al., 1997; Silva et al., 2005), although in some cultured cells it does (Meldolesi et al., 1983; Lang et al., 1998; Tse and Tse 1999).The characteristics of Ca^{2+}-independent release are peculiar: it requires the presence of divalent cations, such as Mg^{2+}, which can be added or removed in succession, causing respective bouts of secretion or its cessation (Misler and Hurlbut 1979). In the absence of Mg^{2+}, this release can be supported by slightly hypertonic sucrose, by itself insufficient to cause secretion. The Ca^{2+}-independent release can be blocked by millimolar La^{3+} (Rosenthal et al., 1990) or concanavalin A (Grasso et al., 1978; Boehm and Huck 1998). It may involve release of Ca^{2+} from mitochondria, as observed in peripheral (Tsang et al., 2000) and not central synapses (Adam-Vizi et al., 1993), but it is unclear if stored $Ca^{2+}{}_i$ itself can trigger release.As Ca^{2+} is important for endocytosis, the sustained secretory activity in its absence eventually depletes NMJ terminals (Longenecker et al., 1970; Ceccarelli and Hurlbut 1980), but not synaptosomes (Watanabe and Meldolesi 1983), of all vesicles. Electrophysiological recordings at NMJs show that in the absence of Ca^{2+} α-LTX causes a large but slow rise in the frequency of spontaneous exocytotic events, which then slow down and cease altogether; bursts of miniatures are never observed.

6.2 Ca^{2+}-dependent release: In the presence of $Ca^{2+}{}_e$, α-LTX also causes a slow and large increase in the frequency of miniature events, overlaid by bursts of release (Ceccarelli et al., 1979). As in the absence of Ca^{2+}, depletion of vesicles and block of exocytosis also occurs, but because vesicles are able to recycle for some time, the total number of quanta released is twice higher in Ca^{2+} than in its absence (Fesce et al., 1986; Auger and Marty 1997). It appears that the gradual rise and fall of the frequency of vesicle fusion events is a common feature of α-LTX-induced release both with and without $Ca^{2+}{}_e$, and that Ca^{2+}adds an extra component, increasing the total release.Influx of Ca^{2+} through α-LTX pores might explain Ca^{2+}-dependent secretion, but not the bursts of miniatures. These may be due to Ca^{2+} waves caused by activation of phospholipase C (PLC) (Vicentini and Meldolesi 1984; Davletov et al., 1998) and release of intracellular Ca^{2+} ($Ca^{2+}{}_i$). In fact, U73122 (blocks PLC activation by G proteins), thapsigargin (depletes Ca^{2+} stores) and 2-APB (inhibits activation of Ca^{2+}stores) block α-LTX action (Davletov et al., 1998; Ashton et al., 2001; Capogna et al., 2003), implicating a G protein cascade in Ca^{2+}-dependent toxin-induced release.

7. The structure of α-LTX (Sequence analysis) α-LTX is synthesised as a 157 kDa polypeptide (Kiyatkin et al., 1990) by free ribosomes in the cytosol of secretory epithelial cells of the venom glands (Cavalieri et al., 1990). These cells disintegrate and expel toxin into the gland lumen together with various proteases (Duan et al., 2006). Here, it is cleaved at both termini by a furin-like protease (Volynski et al., 1999), producing an active α-LTX of ~131 kDa (Kiyatkin et al., 1990; Ichtchenko et al., 1998). The most striking feature of the α-LTX primary structure is a series of 22 ankyrin repeats that account for about two thirds of the sequence (Kiyatkin et al., 1995). Ankyrin repeats are found in a wide variety of proteins and generally mediate protein-protein contacts but, unlike other protein binding motifs, they take part in a wide range of interactions and do not have a specific

target (Sedgwick and Smerdon 1999). The N-terminal third of α-LTX shows no significant homology to other proteins. This region contains three conserved cysteines important for structural stability and activity of all LTXs (Kiyatkin et al., 1995; Ichtchenko et al., 1998).α-LTX is a mostly hydrophilic protein that does not have a classical hydrophobic signal peptide or clear transmembrane regions (TMRs).

8. Receptor interaction: Although α-LTX is able to insert into pure lipid membranes (Finkelstein et al., 1976), reconstituted receptors greatly enhance the rate of insertion (Scheer et al., 1986). Biological membranes seem even more refractive to the toxin: when cells do not possess α-LTX receptors, no pore formation can be detected (Hlubek et al., 2000; Van Renterghem et al., 2000; Volynski et al., 2000), whereas expression of exogenous receptors allows abundant α-LTX insertion and concomitant channel formation. Receptors, thus, confer specificity to the pore-mediated effects of α-LTX. It is not clear whether receptors are directly involved in membrane insertion, simply concentrate toxin near the membrane or organise membrane lipid domains to make them accessible to α-LTX. Importantly for some aspects of toxin-evoked secretion, interaction with one of the receptors, neurexin (NRX), is Ca^{2+}-dependent, while interaction with the other two, latrophilin 1 (LPH1) and protein tyrosine phosphatase σ (PTPσ), is completely Ca^{2+}-independent. The role of the structural domains of α-LTX in receptor interaction has been studied by mutagenesis. Ichtchenko et al. (1998) have generated three point-mutants by replacing conserved cysteines in the N-terminal domain with serines. These mutants (C14S, C71S, C393S) are inactive in release, probably because they do not bind α-LTX receptors. Accordingly, chemical reduction of disulphide bonds in α-LTX abolishes receptor binding. Thus, proper folding of the N-terminal domain, aided by disulphide bonds, is essential for toxin-receptor interactions. In another work, Li et al. (2005) have deleted the C-terminal ankyrin repeats 15 to 22, comprising the head and part of the body, and found that this mutant fails to bind LPH1 but still interacts with NRX Iα. This suggests that α-LTX complex with LPH1 requires an intact C-terminal quarter of the toxin molecule. Whether this sequence binds LPH1 directly or affects the conformation of other α-LTX regions that actually interact with LPH remains to be addressed. Clearly, the C-terminal region is not necessary for binding of NRX Iα.

9. Receptors: The three currently known receptors for α-LTX - NRX, LPH (or CIRL) and PTPσ - were all isolated from brain extracts by affinity chromatography on immobilised α-LTX (Petrenko et al., 1990; Davletov et al., 1996; Krasnoperov et al., 1996;Krasnoperov et al., 2002a). Surprisingly, they were purified only as a result of three separate efforts several years apart. In part, this piecemeal discovery of α-LTX receptors reflects differences in purification procedures; in part, it is due to the researchers' inability to explain all the actions of the toxin by the features of each newly found receptor, warranting further attempts at isolating the "ultimate" target of α-LTX. While new proteins interacting weakly/transiently with the toxin and mediating some of its activities may still be found, there is no doubt that NRX Iα, LPH1 and PTPσ exhaust the repertoire of major α-LTX receptors. As these proteins are discussed in detail below, it is important to remember that, with any receptor, wild-type α-LTX can insert into the membrane and form pores (or engage in other interactions), causing strong direct effects that make definitive conclusions regarding receptor signalling, or lack of it, difficult. Only when membrane insertion and pore formation are blocked (as, for example, in $\alpha\text{-LTX}^{N4C}$ mutant), can the observed effects be attributed to the action of one or the other receptor.

9.1. Neurexin: The first α-LTX receptor to be identified was isolated from solubilised bovine brain by toxin-affinity chromatography in the presence of Ca^{2+} (Petrenko et al., 1990) and termed neurexin (NRX) (Ushkaryov et al., 1992) .

9.2. Latrophilin: Because NRX Iα requires Ca^{2+} to bind α-LTX, it cannot mediate the toxin's effects in the absence of Ca^{2+}. The quest for a Ca^{2+}-independent receptor continued, and a major protein was eventually isolated by α-LTX-chromatography of solubilised brain proteins and termed latrophilin (LPH) (Davletov et al., 1996; Lelianova et al., 1997), or Ca^{2+}-independent receptor of α-LTX (CIRL) (Krasnoperov et al., 1996;Krasnoperov et al., 1997). LPH binds toxin in the presence or absence of divalent cations (Davletov et al., 1996). It was not discovered earlier because its 120-kDa toxin-binding fragment was confused on SDS gels with α-LTX, while the second (65 kDa) fragment was lost due to its quantitative precipitation upon boiling with SDS.

9.3. Protein tyrosine phosphatase σ: In the search for a Ca^{2+} independent α-LTX receptor, affinity chromatography in the absence of Ca^{2+} was used to isolate brain proteins with any affinity for α-LTX. Sequencing of all proteins in the α-LTX column elute (Krasnoperov et al., 1996; Krasnoperov et al., 2002b) has revealed that, in addition to LPH1, PTPσ can also bind to the column as a set of two minor bands.

II. Conclusions

Even though the effect of toxin application in various systems is obvious and well documented, the molecular machinery underlying its mode of action are still highly debated and controversial. α-LTX is a naturally occurring highly poisonous agent which is used by black widow spiders to inflict excruciating pain and scare away its enemy (a vertebrate animal). It is, therefore, not surprising that the toxin's effects are so powerful and diverse, while its mechanisms (including its targeting of multiple receptors and several modes of actions) are so complex. Nevertheless, the α-LTX studies have led to the formulation of extremely important scientific concepts. It now appears obvious that neurotransmitter release occurs via exocytosis, but in the early days of α-LTX research, it was a hotly debated topic. Toxin studies have also led to the discovery of several presynaptic receptors and their ligands (in particular neurexins/neuroligins and latrophilin/Lasso). Surprisingly, after all the years of α-LTX use in synaptic research, the mechanism/s of its Ca^{2+}-independent action are still unknown. Novel toxin mutants and unconventional approaches will have to be employed to address this question. α-LTX receptors are now being studied in-depth. Several questions in this field that require more work: What are the physiological functions of these receptors? What are the consequences of their interactions with their respective endogenous ligands? How do the different toxin receptors interact with each other? Why is the structure of latrophilin so unusual and why can this receptor exchange fragments with other aGPCRs? Finally, what are the signaling mechanisms of each α-LTX receptor? There is no doubt that studies of α-LTX and its receptors should continue, but at a new, modern level.

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