Comparative neuromuscular blocking actions of presynaptic phospholipase A_2 neurotoxins in chick biventer nerve-muscle preparations

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Abstract

Introduction: Among the major toxic constituents of snake venoms, phospholipases A_2 (PLA_2) represent a highly diverse enzyme family responsible for a variety of pathological effects, including pronounced myotoxicity and neurotoxicity.

Materials & Methods: This study focuses on presynaptically active PLA_2 neurotoxins, β-bungarotoxin (BuTx), taipoxin, crotoxin, notexin, and ammodytoxin C (Amtx). We examined and compared their neuromuscular blocking actions using indirectly stimulated chick biventer cervicis nerve-muscle preparations.

Results: BuTx was tested at three concentrations (47, 4.7, and 0.47 nM), producing time and concentration-dependent neuromuscular blockade. It abolished twitch responses within 60 minutes at 47 nM, 120 minutes at 4.7 nM, and 180 minutes at 0.47 nM. All other toxins tested also induced complete neuromuscular paralysis at the given concentrations. Our findings demonstrate that these neurotoxins impair neuromuscular transmission in a dose-dependent manner, ultimately resulting in irreversible muscle paralysis. Among them, BuTx displayed the highest potency, while Amtx was the least potent. To assess postsynaptic function, contracture responses to exogenous acetylcholine (ACh) (1 mM), carbachol (20 μM), and potassium chloride (40 mM) were recorded

before and after toxin exposure in the absence of nerve stimulation. These responses remained unchanged, indicating that postsynaptic sensitivity was preserved.

Conclusion: A deeper understanding of the mechanisms underlying venom-induced neurotoxicity may improve diagnostic accuracy and support the development of effective treatments for snakebite envenomation, reducing associated morbidity and mortality.

Keywords: β-bungarotoxin, Potency, PLA2, Neurotoxicity, Acetylcholine, Carbachol, Agonist

1. Introduction

Venomous snakebites can cause a range of severe health complications, including respiratory paralysis, coagulopathies, fatal hemorrhages, acute kidney injury, and severe local tissue damage that may result in permanent disability or limb loss. Snakebite envenoming remains a major public health issue, particularly in low and middle-income countries, which account for approximately 95% of all cases. Globally, it is estimated that 4.5 to 5.4 million snakebites occur each year, resulting in 1.8 to 2.7 million cases of envenomation. Tragically, these incidents lead to between 81,410 and 137,880 deaths annually. South Asia bears the highest burden, accounting for nearly 70% of all snakebite-related deaths [1]. Among the most dangerous snake species, many are particularly known for producing potent neurotoxins, key contributors to the severe systemic effects observed in envenomation. These species are responsible for a relatively high number of reported snakebite cases and include elapids such as kraits (*Bungarus* spp.), especially the Taiwan banded krait (*Bungarus multicinctus*) [2], the Australian taipans (*Oxyuranus* spp.) [3], the Australian tiger snakes (*Notechis* spp.) [4], the

European long-nosed viper (*Vipera ammodytes*) [5] and South American rattlesnake (*Crotalus durissus terrificus*).

Bungarus multicinctus is a highly venomous elapid snake, a medically important species due to its potent neurotoxic venom. It is accounts for approximately 7.5% of all recorded snakebites in Taiwan, with reported fatality rates ranging from 7% to 50%. Death following envenomation may occur within 6 to 30 hours [2].

β-bungarotoxin (BuTx), is the major presynaptic PLA_2 neurotoxin of *B. multicinctus* venom. It has two distinct subunits, A and B chains, linked by a disulfide bond. The A chain possesses PLA_2 enzymatic activity and is primarily responsible for both the neurotoxicity and catalytic function of the toxin. In contrast, the B chain, lacks measurable PLA_2 activity. BuTx block synaptic transmission at the neuromuscular junction (NMJ), by inhibition of acetylcholine (ACh) release [6].

Crotalus durissus terrificus (*C.d. terrificus*), is responsible for severe envenomations in South America. This species accounted for 2,503 reported envenomation in 2017. Bites from *C.d. terrificus* often result in pronounced neurotoxicity, severe rhabdomyolysis, coagulopathy, and acute kidney failure [7].

Crotoxin is the primary presynaptic neurotoxin of *C.d. terrificus* snake, composed of two subunits: CA and CB. The CA subunit is lack of toxicity and PLA_2 activity. In contrast, the CB subunit exhibits strong PLA_2 activity and is responsible for the toxic effects of crotoxin [8].

Oxyuranus scutellatus scutellatus, (Papuan taipan), is an Australian elapid snake and the primary cause of severe systemic envenomation, characterized by pronounced neurotoxicity leading to irreversible paralysis, coagulopathy, spontaneous systemic hemorrhages, myotoxicity, cardiac disturbances, and acute kidney injury [8]. Following snakebites, the fatality rates is 14.5% in adults and 25.9% in children [9].

Taipoxin, a potent PLA_2 neurotoxin from taipan venom, composed of three homologous subunits: α, β, and γ [10]. The α-subunit is a weak neurotoxin approximately 500 times lower than that of the intact taipoxin in mice and able to induce significant myotoxicity. The β-subunit is a neutral protein lacking of toxicity and enzymatic activity. The γ-subunit, is non-toxic, and displays weak enzymatic activity [10].

Notechis scutatus scutatus, (tiger snake), accounts for approximately 17% of snakebite cases in Australia. Envenomation by the tiger snake results in a range of clinical effects, including coagulopathy, myotoxicity, and acute kidney injury. Severe complications such as cardiac arrest and major hemorrhage have also been reported [10].

Notexin, is a potent presynaptic PLA_2 neurotoxin and a key myotoxin of tiger snake venom. It is a single-chain with 119 amino acids. Notexin has strong PLA_2 activity. Notexin exerts its lethal effects primarily by disrupting neuromuscular transmission, particularly at the junctions of respiratory muscles, leading to rapid death. Interestingly, chemical modification of notexin using *p*-bromophenacyl bromide (pBPB) abolishes its myotoxic effects, suggesting a crucial role for its enzymatic activity in toxicity [11].

Vipera ammodytes (*V. ammodytes*), is the most dangerous snake in Europe and is classified as a medically significant species due to its ability to deliver potentially life-threatening bites [5]. Envenomation by *V. ammodytes* can lead to local pain and swelling, paralysis, thrombocytopenia, coagulotoxicity, myotoxicity, and neurotoxicity [11].

Ammodytoxins (Amtx), is a presynaptic PLA_2 neurotoxin from *V. ammodytes* venom, with three isoforms, A, B, and C, each consist of 122 amino acids and exhibit notable differences in biological activity. Despite these variations, all three isoforms produce presynaptic blockade in chick biventer cervicis and mouse phrenic nerve–diaphragm preparations. In low Ca^{2+} media, they induce a characteristic triphasic

response in mouse neuromuscular preparations, similar to those produced by other presynaptic PLA_2 neurotoxins [8, 14].

The PLA_2 neurotoxins are capable of irreversibly blocking neuromuscular transmission [17]. At the presynaptic level, the motor nerve terminal is responsible for the synthesis, packaging, transport, and release of ACh. When an action potential reaches the nerve terminal, it triggers the opening of voltage-gated Ca^{2+} channels, leading to an influx of Ca^{2+} ions. This rise in intracellular Ca^{2+} concentration initiates the fusion of ACh-containing synaptic vesicles with the presynaptic membrane. The SNARE protein complex mediates this exocytosis process, enabling the release of ACh into the synaptic cleft [18] and bind to postsynaptic nicotinic acetylcholine receptors (nAChRs). This interaction opens ligand-gated Na^+ channels, allowing Na^+ influx, depolarizing the postsynaptic membrane, and ultimately triggering muscle contraction.

Under specific experimental conditions, such as low extracellular Ca^{2+} or elevated Mg^{2+} concentrations, presynaptic PLA_2 neurotoxins disrupt neuromuscular transmission in a characteristic triphasic pattern. An initial depression of ACh release, followed by a transient increase in ACh release, and ultimately terminates in irreversible binding of the toxin. This final phase is marked by structural and functional damage to the motor nerve terminal, including a reduction in synaptic vesicles, mitochondrial injury, and complete blockade of neuromuscular transmission [9,14,15,16]. The mechanisms underlying the diverse actions of these neurotoxins remain incompletely understood. This knowledge gap underscores the need for further research aimed at improving both our mechanistic understanding and the clinical management of neurotoxic envenoming. Therefore, the present study was designed to investigate and compare the presynaptic effects of several PLA_2 neurotoxins; with focus on their capacity to inhibit ACh release at the NMJ by using chick biventer cervicis nerve–muscle preparations. Such findings not only enhance our understanding of toxin action but also may help for development of antivenom and

novel pharmacological applications, including treatments diseases, like neurological disorders [17].

2. Materials and Methods

2.1. Venoms

Taipoxin was purchased from Latoxan and Crotoxin was a gift from Dr Grazyna Faure of the Pasteur Institute, Paris and notexin was kindly provided by Dr André Ménez from DIEP, CEN Saclay, France and was also purchased from Latoxan. Amtx were gifts from Dr. Igor Krizaj from Department of Biochemistry and Molecular Biology, J. Stefan Institute, University of Ljubljana, Slovenia. BuTx was supplied by Sigma Chemical Co. Ltd., Poole, Dorset, England and Latoxan, 20 Rue Leon Blum, 2600 Valence-France. *Naja naja oxiana* venom was supplied by Razi Vaccine & Serum Research Institute, Karaj, Iran.

2.2. Isolated Chick-Biventer Cervicis Nerve-Muscle Preparation

Chicks aged 3 to 14 days were euthanized by exposure to CO_2 inhalation. Following euthanasia, the chick was pinned ventral side down on a dissecting board, and a small glass cylinder was placed under the neck to facilitate dissection. A midline incision was made along the back of the neck, extending toward the base of the skull. After incising the skin, the biventer cervicis muscles and their associated white tendons, which house the nerve, became visible (Figure 1). A suture was tied around the ends of the long tendons to facilitate attachment to an isometric transducer. Additionally, a small loop of thread was tied at the distal end of each muscle to secure the muscle to a hook electrode [19].

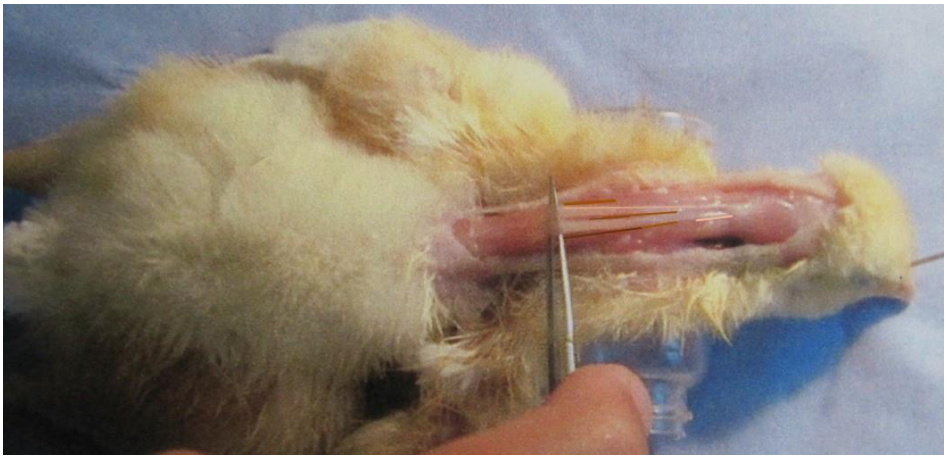

Figure 1. Chick biventer cervicis nerve-muscle preparation.
The forceps have been placed under the bellies of the two muscles to show their location. The long tendons that contain the motor nerves can be seen inserting at the back of the head.

Experiment: Pairs of muscles and their associated nerves were mounted on tissue holders equipped with ring electrodes positioned around the tendons. The muscles were maintained under a resting tension of approximately 1 g in 10 ml tissue baths containing Krebs-Henseleit solution, composed of (mM): NaCl 118.5, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 11.1. The solution was maintained at 34°C, and continuously bubbled with a mixture of 95% O_2 and 5% CO_2 (carbogen). Muscles were stimulated indirectly via electrical stimulation of the nerves within the tendons at a frequency of 0.1 Hz. Stimulation was delivered through ring electrodes with pulse durations of 0.2 msec and voltages greater than that required to produce a maximal twitch response.

2.3. Time-matched control experiments on chick biventer cervices

To detect any change in postsynaptic sensitivity, contracture responses to submaximal concentrations of ACh (1 mM), carbachol (20 μM) and KCl (40 mM) were recorded in the absence of nerve stimulation, both prior to toxin application and at the

end of the experiment. The muscles were exposed to ACh and KCl for 30 seconds and to carbachol for 60 seconds, and the heights of the resulting contractures were measured at these time points (Figure 2). As a control sample, the time-matched control experiments were done on this preparation at 34°C prior to testing the activity of toxins. Twitch height and contracture responses were recorded isometrically using Grass Model 79 and Model 7D polygraphs (Grass Instruments), along with FTO3 force-displacement transducers.

2.4. Statistics

The results are expressed as the mean ±S.E.M of at least three experiments unless otherwise noted. The statistical significance was evaluated by the paired and unpaired Student's t-test. Values of $P<0.05$ were taken as significant.

3. Results and Discussion

3.1. Effect of agonists for differentiation of pre- and postjunctional toxin effects

The submaximal concentrations of agonists including ACh (1 mM), carbachol (20 μM) and KCl (40 mM) were recorded in the absence of nerve stimulation, prior to toxin application. Then preparations were washed with fresh solution to remove residual drug for 15 seconds at a flow rate of 7–10 ml/sec and allowed to stabilize for 20–30 minutes with continued stimulation before toxin application [19]. Toxins at various concentrations (see Table 1) were added to the organ bath, and at the end of the experiment muscles were exposed to those agonist ACh and KCl and to carbachol, again. The heights of the resulting contractures were measured at these time points (Figure 2).

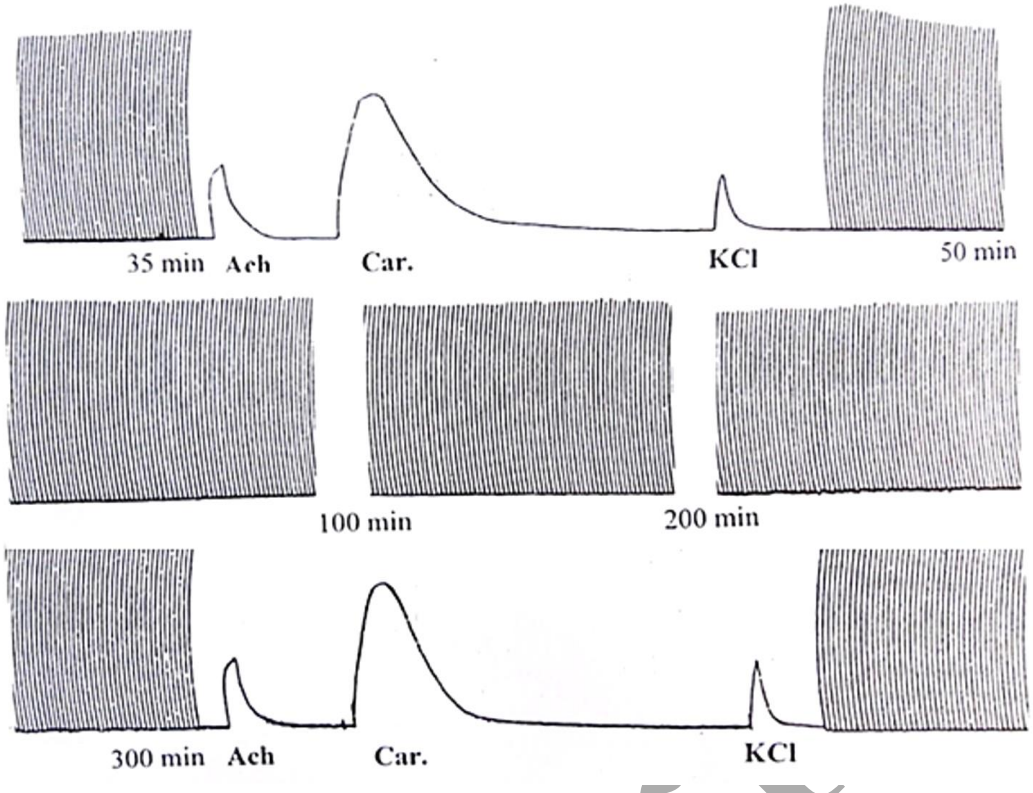

Figure 2. Time-matched control experiment.

The chick biventer cervicis nerve-muscle preparations were stimulated indirectly via the nerve once every 10 second at 34°C with pulses 0.1 ms duration and voltage greater than required to produce the maximum response. Stimulation was turned off during addition of agonists. ACh (1 nM for 30 sec); Car = carbachol (20 μM for 60 sec); and Kcl (40 mM for 30 sec).

Table 1. Summary of effect of neurotoxins BuTx, taipoxin, crotoxin, notexin and Amtx C on twitch response of chick biventer cervicis nerve-muscle preparations.

Neurotoxins	Concentration (nM)	Blocking time (min)	N
BuTx	0.47	180 ± 6	4
BuTx	4.7	120 ± 4	4
BuTx	47	60 ± 3	7
Taipoxin	6.3	275 ± 11	8
Crotoxin	7.2	250 ± 8	6
Notexin	200	230 ± 7	8
Amtx C	3600	275 ± 9	6

The chick biventer cervicis nerve-muscle preparation consists of both focally-innervated twitch fibers (fast muscle fibers) and multiply-innervated contracture-

producing fibers (slow muscle fibers). Electrical stimulation of the nerve attached to the tendon causes twitch responses. Exogenous agonists such as ACh, carbachol and the depolarising salt KCl produce contractile responses of slow fibers.

The results have revealed that The twitches and responses to the addition of exogenous agonists, ACh (1 mM), carbachol (20 μM) and KCl (40 mM) remained stable without marked changes for over 5 hours (Figure 2). Therefore, we can conclude that all these neurotoxins are presynaptic as they have no effect on the nicotinic or muscarinic receptors at the postsynaptic site.

3.2. Effect of BuTx, on chick biventer cervicis nerve-muscle preparations

To determine the concentration-dependent effects of BuTx, three different concentrations (47, 4.7, and 0.47 nM) of this neurotoxin were tested. BuTx abolished twitch responses in 60 min at 47 nM (n = 7), in 120 min at 4.7 nM (n = 4), and in 180 min at 0.47 nM (n = 4). The results have shown that by increasing toxin concentration, the time of blocking twitch response has decreased. Therefore, the effects of BuTx is concentration-dependent (Figure 3 and Table 1).

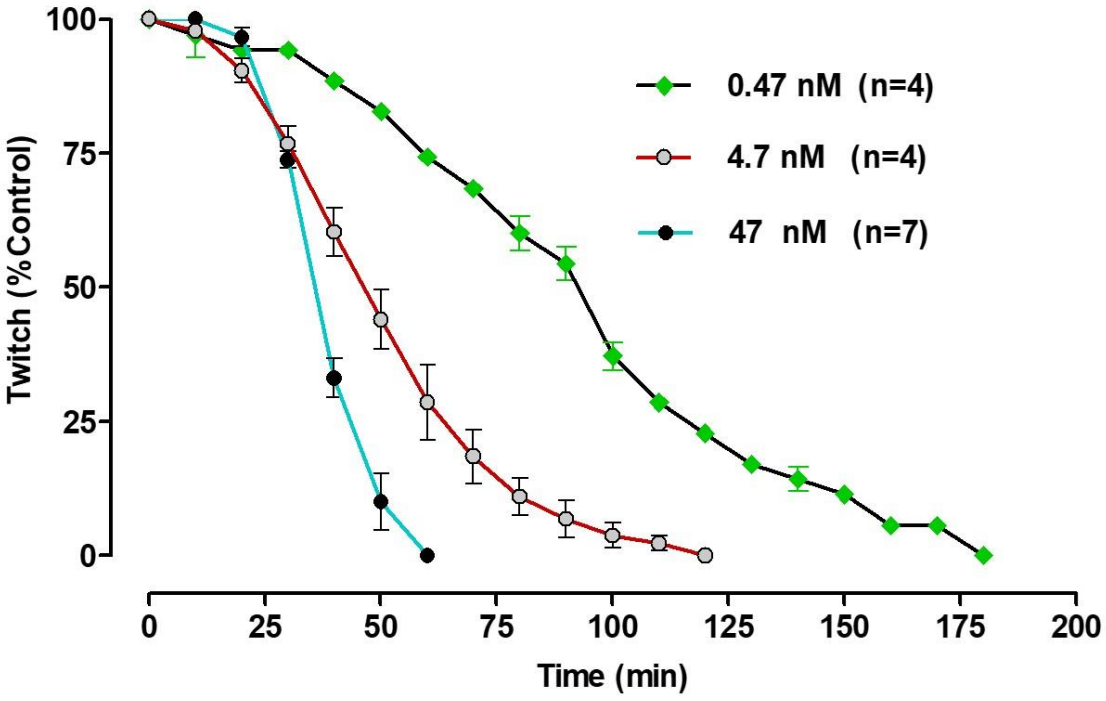

Figure 3. Time course of BuTx effect on twitch response

The effect of BuTx on twitch response of chick biventer cervicis muscles to nerve stimulation. Preparations were stimulated once every 10 second with pulses of 0.2 msec duration and voltage greater than that required producing the maximum response. Each point represents the means ± S. E. M. of 4 - 7 experiments and standard errors are indicated by the bars unless smaller than symbols.

3.3. Comparative effects of BuTx, taipoxin, crotoxin, notexin and Amtx C on chick biventer cervicis nerve-muscle preparations

The activities of BuTx, taipoxin, crotoxin, notexin, and Amtx C were examined using indirectly stimulated chick biventer cervicis preparations. All these toxins caused a slow, progressive decrease in twitch responses to nerve stimulation. Taipoxin at 6.3 nM and Amtx C at 3.6 μM (3600 nM) completely blocked twitch responses within a similar time frame, 275 ± 11 min and 275 ± 9 min respectively. Crotoxin at 7.2 nM, notexin at 0.2 μM (200 nM) and BuTx at 0.47 nM completely blocked twitch responses in 250 ± 8, 230 ± 7 and 180 ± 6 minutes, respectively. The time required to reduce twitch tension to 50% of its maximum value was approximately 100 minutes(Figure 4 and Table 1).

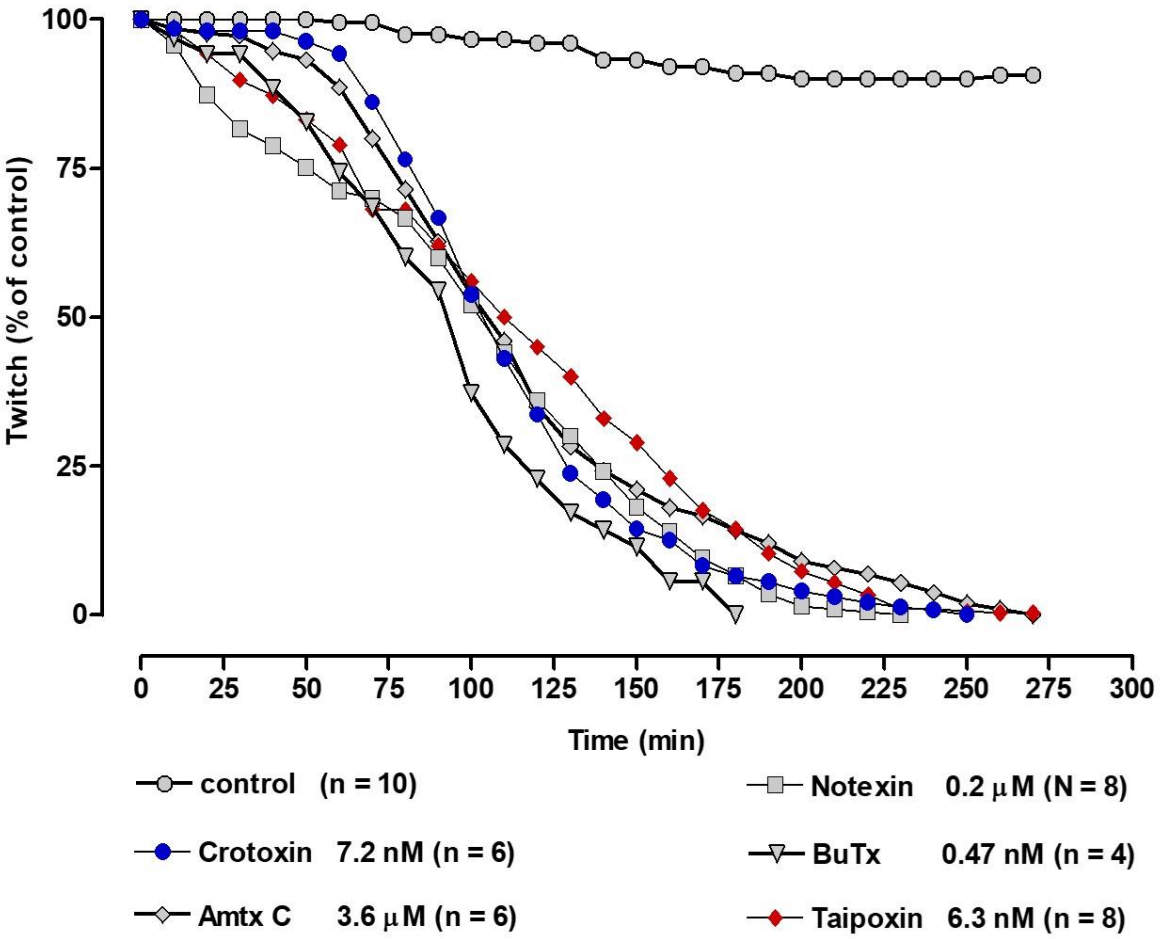

Figure 4. Neuromuscular blockade by tested PLA_2 neurotoxins
Concentration- and time-dependent neuromuscular blockade caused by BuTx, crotoxin, Amtx, notexin, and taipoxin on twitch response of chick biventer cervicis muscles to nerve stimulation. Preparations were stimulated once every 10 s with pulses of 0.2 ms duration and voltage greater than that required to produce the maximum response. Each point represents the mean of 4–8 experiments. SEMs are not shown for clarity but were <1.6% for control, <2.5% for BuTx, <5% for taipoxin, <3.7% for notexin, <4% for crotoxin, and <4.4% for Amtx.

The potency index (PI) for toxins, which reflects their effectiveness based on the blocking time of the twitch response and toxin concentration (Table 1), was empirically calculated as an indirect measure derived from experimental observations of concentration- and time-dependent toxin effects, using the following general formula (Table 2):

PI = 1 / (Concentration (nM) × Time (min) to block)

Table 2. Comparative analysis of the potency index (PI) of toxins assessed under identical experimental conditions.

Toxin	Concentration (nM)	Time to block (min)	PI
BuTx	0.47	180	**0.0118**
Taipoxin	6.3	275	**0.000577**
Crotoxin	7.2	250	**0.000556**
Notexin	200	230	**0.0000217**
Amtx	3600	275	**1.01×10^{-6}**

Note: Higher PI values indicate greater functional potency, corresponding to lower toxin concentrations and shorter times required to achieve twitch blockade.

The comparative analysis performed under identical experimental conditions (Figure 4) demonstrates that BuTx exhibits the highest functional potency, as reflected by its markedly higher PI. Crotoxin and taipoxin display similar potencies, both lower than BuTx. In contrast, notexin and Amtx show substantially lower potencies, requiring higher concentrations and longer exposure times to achieve twitch blockade (Table 2).

3.4. Responses to exogenous ACh, Carbachol and KCl

The effects of toxins on the responses of preparations to exogenous ACh (1 mM), Carbachol (20 µM) and KCl (40 mM) were investigated. Agonists were applied prior to and following toxin exposure. Among the toxins, only notexin had significant effects on responses to ACh and in addition to its presynaptic effect, showed a mild myotoxic effect on the preparation, while BuTx and crotoxin had small but significant effects on KCl responses (Figure 5) (* $P< 0.05$). The results clearly demonstrated the ability of these PLA_2 neurotoxins to abolished twitch responses when muscle stimulated indirectly. In the absence of muscle stimulation, appearance responses to exogenous agonists showed that nicotinic receptors at the postjunctional site were not blocked by toxin and ACh can bound and produce its effect. Also, muscarinic receptors at the postjunctional site were not blocked by toxin and carbachol can bound to both receptors and produce its effect.

Carbachol has been shown longer effect because it is not hydrolysis by acetylcholine esterase. KCl is a depolarising agent and produced its effect as an action potential (Figures 2 & 5). Based on the exogenous agonist responses, we can concluded that these PLA_2 neurotoxins acting presynaptically.

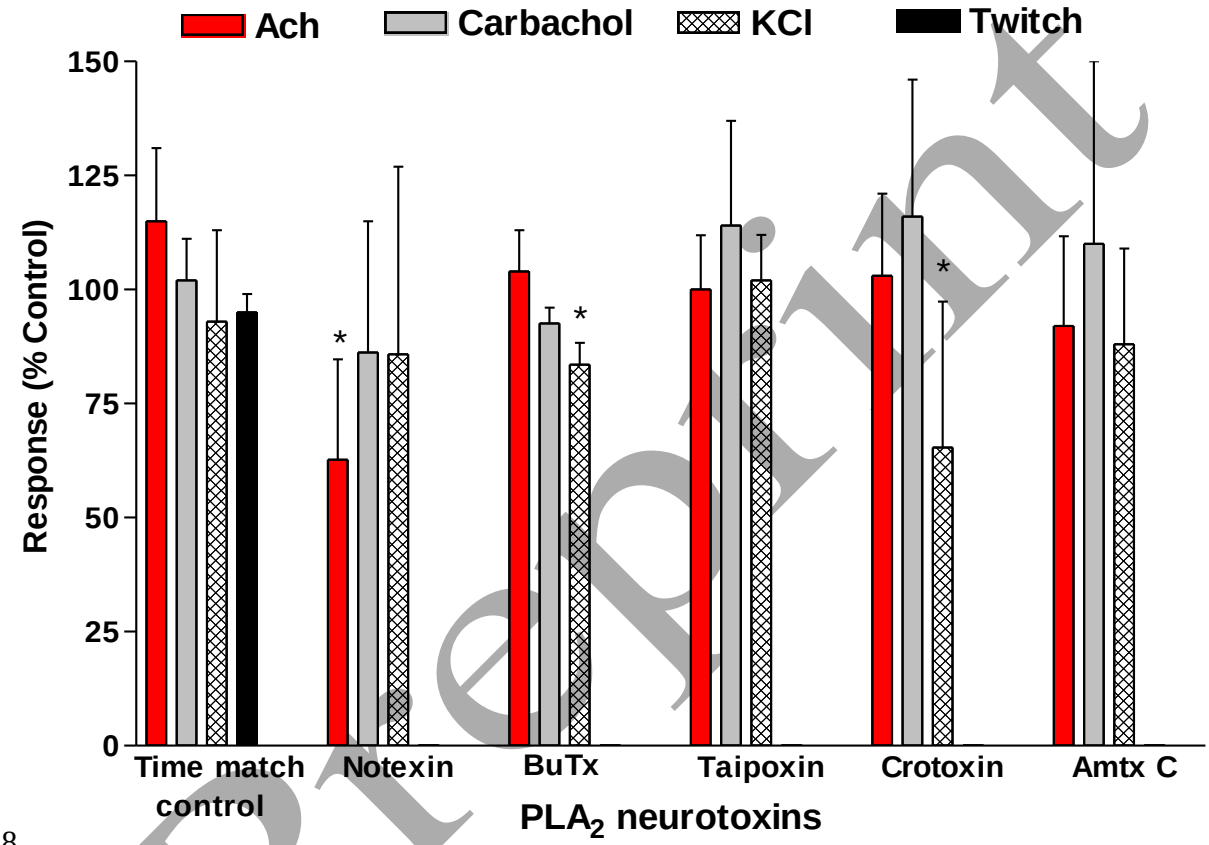

Figure 5. Effects of PLA_2 neurotoxins on responses of chick biventer cervicis muscle to agonists

Responses of chick biventer cervicis nerve–muscle preparations to acetylcholine (ACh, 1 mM), carbachol (20 μM), and potassium chloride (KCl, 40 mM) were measured with indirect muscle stimulation. The concentrations of toxins used were the same as in the experiments shown in Fig. 4. Agonist responses were tested after the twitch responses had been abolished. Each measurement represents the mean of 4–8 experiments (*P < 0.05).

3.5. In vivo effects of Iranian snake *Naja naja Oxiana* venom in mice

To investigate the dose-dependent effects of neurotoxic venom, we examined *Naja naja oxiana* venom administered via intraperitoneal injections at doses of 2 and 4 mg/kg. At a dose of 2 mg/kg (n=8), all mice succumbed within an average of 34 minutes. In contrast, the higher dose of 4 mg/kg (n=6) resulted in 100% mortality within an average of 28 minutes (data not shown).

Snake venoms containing potent PLA_2 neurotoxins are of significant importance in public health and biomedical research, including toxicology, physiology, and pharmacology. These neurotoxins act primarily via PLA_2 enzymes, which irreversibly impair neuromuscular transmission, leading to acute neuromuscular paralysis with respiratory involvement, a condition that can be life-threatening following a snakebite [18]

Clinical reports indicate that approximately 27–87% of patients experiencing neurotoxicity develop respiratory muscle weakness, often requiring mechanical ventilation [3]. The specific factors contributing to the development of respiratory muscle weakness in certain individuals remain unclear. However, it is thought to depend on the specific snake species and the quantity of venom delivered.

Supporting this, our in vitro study demonstrated that BuTx elicits a concentration-dependent neuromuscular blockade: at the highest concentration tested (47 nM), complete blockade occurred within 120 minutes, whereas at the lowest concentration (0.47 nM), full inhibition was observed at 230 minutes. These findings suggest that higher systemic concentrations of BuTx may lead to more rapid and severe neuromuscular impairment in clinical settings. Similarly, the present in vivo experiments using *Naja naja oxiana* venom, which also contains PLA_2 neurotoxins [20], produced dose-dependent lethality. Intraperitoneal injection of 2 mg/kg resulted in 100% mortality within an average of 34 minutes, while a dose of 4 mg/kg reduced average survival time

to 28 minutes. However, caution should be exercised when extrapolating these results to human cases of envenomation, as species-specific physiological differences may significantly influence the clinical effects of venom exposure.

In this study, we demonstrated the fatal effects of PLA_2 neurotoxins at the NMJ using an in vitro model with indirectly stimulated chick biventer cervicis nerve-muscle preparations. Our primary goal was to assess the potency of various PLA_2 neurotoxins in inhibiting ACh release. We also compared their relative efficacy and confirmed that their inhibitory effects are concentration-dependent. Although the precise molecular mechanisms underlying PLA_2-induced presynaptic toxicity remain incompletely understood, existing literature suggests that these neurotoxins can impair ACh release via multiple presynaptic mechanisms, including disruption of synaptic vesicle recycling, membrane phospholipid degradation, and interference with Ca^{2+}-dependent exocytosis.

Rowan and Harvey [21] found that PLA_2 neurotoxins did not significantly alter Ca^{2+} currents when K^+ channels were blocked, suggesting that their inhibitory effect on neurotransmitter release is unlikely to be mediated by altered Ca^{2+} influx or changes in cytoplasmic Ca^{2+} levels. Expanding on this, Ueno and Rosenberg [22] proposed that the phosphorylation of synapsin, a key synaptic vesicle-associated protein, is essential for vesicle mobilization. They suggested that BuTx inhibits the phosphorylation of synapsin, GAP-43, and MARCKS, thereby inhibiting vesicle mobilization and neurotransmitter release.

However, Rowan et al. [23] observed that spontaneous neurotransmitter release persisted in nerve-muscle preparations exposed to PLA_2 neurotoxins, indicating that synaptic vesicles remained available for docking and exocytosis. These findings suggest that PLA_2 neurotoxins do not completely deplete the vesicle pool, but may interfere with activity-dependent vesicle mobilization or recycling. Dixon and Harris [14], using

electron microscopy and immunocytochemistry, demonstrated that BuTx depletes synaptic vesicles and degenerates motor nerve terminals, while leaving the postjunctional membrane largely intact. Similar ultrastructural changes were reported by Harris et al. [9] following exposure to notexin and taipoxin. They proposed that the observed vesicle loss results from enhanced neurotransmitter release combined with impaired vesicle recycling, potentially due to toxin internalization or activation of intracellular signaling pathways. However, because direct evidence for toxin internalization was lacking, the latter mechanism appeared more likely.

Montecucco and Rossetto [24] later suggested that PLA_2 neurotoxins may be internalized during synaptic vesicle endocytosis. Once inside the vesicle, the toxins hydrolyze lumenal phospholipids, producing lysophospholipids and free fatty acids. This disrupts vesicle membrane integrity, leading to ion gradient collapse, Ca^{2+} influx, terminal depolarization, and the activation of second messenger pathways, such as Ca^{2+}-dependent proteases, that ultimately impair vesicle recycling and neurotransmitter release. Supporting this mechanism, Harris et al. [9] and subsequent studies [25,26] confirmed that Ca^{2+} influx through voltage-gated Ca^{2+} channels is essential for the neurotoxic effects of PLA_2 toxins.

The toxic effects of BuTx appear to be independent of its phospholipase enzymatic activity. Replacing extracellular Ca^{2+} with Sr^{2+} or removing Ca^{2+} altogether does not prevent its neurotoxic effects, suggesting that PLA_2 activity is not essential for the initial phase of toxicity. Furthermore, BuTx does not alter Na^+ or Ca^{2+}-activated K^+ currents in motor nerve terminals, indicating that its action does not involve these ion channels and instead follows a more specific, targeted mechanism.

Prasarnpun et al. [27] demonstrated that BuTx induces Ca^{2+} influx through voltage-gated Ca^{2+} channels and facilitates ACh release via SNARE-dependent exocytosis,

ultimately leading to synaptic vesicle depletion. Tedesco et al. [28] showed that nerve terminal degeneration begins with elevated intracellular Ca^{2+} levels, which disrupt organelle function and initiate terminal damage. While Rigoni et al. [29] proposed that neurotoxins may enter nerve terminals directly, earlier evidence by Simpson et al. challenges this idea. Specifically, blocking endocytosis did not prevent the neurotoxic effects of PLA_2 toxins, suggesting that internalization is not required for their presynaptic action.

Presynaptic PLA_2 neurotoxins hydrolyze membrane phospholipids into lysophospholipids and free fatty acids, destabilizing nerve terminal membranes, disrupting ion gradients, and increasing intracellular Ca^{2+} influx. These changes activate second messenger pathways that ultimately result in nerve terminal degeneration and impaired neurotransmitter release. The consistent outcomes observed with multiple toxins, including BuTx, taipoxin, notexin, and textilotoxin, support the existence of a shared pathogenic mechanism centered on phospholipid hydrolysis and membrane destabilization [24].

However, our findings indicate that, beyond this common mechanism, there are notable differences in the potency of individual neurotoxins. BuTx exhibited the highest potency, while Amtx was significantly less effective in blocking neuromuscular transmission (Figure 4)

In conclusion, this investigation supports previous findings indicating that these neurotoxins share similarities in their composition, particularly the presence of PLA_2 enzyme, and their biological activities, such as inducing paralysis at the NMJ. Improving our understanding of venom composition and its neurotoxic mechanisms is essential for better diagnosis and treatment, ultimately reducing snakebite-related morbidity and mortality worldwide.

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Authors' Contribution
Study concept, design Supervision: **A.L.H,** Study concept, Acquisition of data, and Supervision: **E.G.R,** Experimental, Data curation performance, writing original draft, review and editing: **B.F** Preparing, drafting and revising the article for submission: **A.M**

Ethics
We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest
The authors declare that they have no conflict of interest.

Data Availability
Data will be made available on request from the corresponding author.

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References:

[1] Chippaux J-P. Snakebite envenomation turns again into a neglected tropical disease! J Venom Anim Toxins Incl Trop Dis. 2017;23:38. [DOI: 10.1186/s40409-017-0127-6]

[2] Mao YC, Liu PY, Chiang LC, Liao SC, Su HY, Hsieh SY, et al. Bungarus multicinctus multicinctus snakebite in Taiwan. Am J Trop Med Hyg. 2017;96(6):1497-1504. [DOI: 10.4269/ajtmh.17-0005]

[3] Lalloo DG, Trevett AJ, Korinhona A, Nwokolo N, Laurenson I, Paul M, et al. Snake bites by the Papuan taipan (Oxyuranus scutellatus canni): paralysis,

hemostatic and electrocardiographic abnormalities, and effects of antivenom. Am J Trop Med Hyg. 1995;52:525–531.[DOI: 10.4269/ajtmh.1995.52.525]

[4] Isbister GK, O'Leary MA, Elliott M, Brown SGA. Tiger snake (Notechis spp) envenoming: Australian Snakebite Project (ASP-13). Med J Aust. 2012;197:173–177. [DOI: 10.5694/mja11.11300]

[5] Di Nicola MR, Pontara A, Kass GE, Kramer NI, Avella I, Pampena R, et al. Vipers of Major clinical relevance in Europe: Taxonomy, venom composition, toxicology and clinical management of human bites. Toxicology. 2021;453:152724. [DOI: 10.1016/j.tox.2021.152724]

[6] Strong PN, Goerk J, Oberg SG, Kelly RB. ß-Bungarotoxin a presynaptic toxin with enzymatic activity. Proc Natl Acad Sci USA. 1976;73:178-185. [DOI: 10.1073/pnas.73.1.178]

[7] Boldrini-França J, Corrêa-Netto C, Silva MMS, Rodrigues RS, Torre PDL, Pérez A, et al. Snake venomics and antivenomics of Crotalus durissus subspecies from Brazil: assessment of geographic variation and its implication on snakebite management. J Proteomics. 2010;73(9):1758-76. [DOI: 10.1016/j.jprot.2010.06.001]

[8] Sampaio SC, Hyslop S, Fontes MRM, Prado-Franceschi J, Zambelli VO, Magro AJ, et al. Crotoxin: Novel activities for a classic β-neurotoxin. Toxicon. 2010;55(6):1045–1060. [DOI: 10.1016/j.toxicon.2010.01.011]

[9] Harris JB, Grubb BD, Maltin CA, Dixon R. The neurotoxicity of the venom phospholipases A_2, notexin and taipoxin. Exp Neurol. 2000;161:517–526. [DOI: 10.1006/exnr.1999.7275]

[10] Harris JB, Maltin CA. Myotoxic activity of the crude venom and the principal neurotoxin, taipoxin, of the Australian taipan, Oxyuranus scutellatus. Br J Pharmacol. 1982;76:61-75. [DOI: 10.1111/j.1476-5381.1982.tb09191.x]

[11] Chang CC, Su MJ. Presynaptic toxicity of the histidine-modified, phospholipase A_2-inactive, ß-bungarotoxin, crotoxin and notexin. Toxicon. 1982;20:895-905.[DOI: 10.1016/0041-0101(82)90077-0]

[12] Gopcevic K, Karadzic I, Izrael-Zivkovic L, Medic A, Isakovic A, Popovic M, et al. Study of the venom proteome of Vipera ammodytes ammodytes (Linnaeus, 1758): A qualitative overview, biochemical and biological profiling. Comp. Biochem. Physiol. Part D Genom. Proteom. 2021;37:100776. [DOI: 10.1016/j.cbd.2020.100776]

[13] Ivanović SR, Rešetar Maslov D, Rubić I, Mrljak V, Živković I, Borozan N, et al. The Venom of Vipera ammodytes ammodytes: Proteomics, Neurotoxic Effect

and Neutralization by Antivenom. Vet. Sci. 2024;11:605. [DOI: 10.3390/vetsci11120605]

[14] Dixon RW, Harris JB. Nerve terminal damage by ß-bungarotoxin, its clinical significance. Am J Pathol. 1999;154:447-455. [DOI: 10.1016/s0002-9440(10)65291-1]

[15] Fathi HB, Rowan EG, Harvey AL. Suramin inhibits the early effects of PLA_2 neurotoxins at mouse neuromuscular junctions: A twitch tension study. J Venom Res. 2011; 2:6-10. https://pubmed.ncbi.nlm.nih.gov/21544175 [PMID: 21544175]

[16] Fathi HB, Rowan EG, Harvey AL. The effect of temperature on the effects of the phospholipase A_2 neurotoxins b-bungarotoxin and taipoxin at the neuromuscular junction. Toxicon. 2013;70:86–89. [DOI: 10.1016/j.toxicon.2013.04.016]

[17 Ranawaka UK, Lalloo DG, de Silva HJ. Neurotoxicity in Snakebite-The Limits of Our Knowledge. PLoS Negl Trop Dis. 2013;7(10):e2302. DOI: 10.1371/journal.pntd.0002302

[18] Fagerlund MJ, Eriksson LI. Current concepts in neuromuscular transmission. Br J Anaesth. 2009;103:108–114. [DOI: 10.1093/bja/aep150]

[19] Harvey AL, Barfaraz A, Thomson E, Faiz A, Preston S, Harris JB. Screening of snake venoms for neurotoxic and myotoxic effects using simple in vitro preparations from rodents and chicks. Toxicon. 1994;32:257-265. [DOI: 10.1016/0041-0101(94)90078-7]

[20] Trento MVC, Sales TA, de Abreu TS, Braga MA, Cesar PHS, Marques TR, et al. Exploring the structural and functional aspects of the phospholipase A_2 from Naja spp. Int J Biol Macromol. 2019;140:49-58. [DOI: 10.1016/j.ijbiomac.2019.08.125]

[21] Rowan EG, Harvey AL. Potassium channel blocking actions of beta-bungarotoxin and related toxins on mouse and frog motor nerve terminals. Br Pharma. 1988;94:839-847. [DOI: 10.1111/j.1476-5381.1988.tb11595.x]

[22] Ueno E, Rosenberg P. beta-Bungarotoxin blocks phorbol ester-stimulated phosphorylation of MARCKS, GAP-43 and synapsin I in rat brain synaptosomes. Toxicon. 1995;33(6):747-62. [DOI: 10.1016/0041-0101(95)00020-m]

[23] Rowan EG, Pemberton KE, Harvey AL. On the blockade of acetylcholine release at mouse motor nerve terminals by β-bungarotoxin and crotoxin. Br J Pharma. 1990;100:301-304. [DOI: 10.1111/j.1476-5381. 1990. tb 15799.x]

[24] Montecucco C, Rossetto O. How do presynaptic PLA_2 neurotoxins block nerve terminals? Trends Biochem Sci. 2000;25(6):266-70. [DOI: 10.1016/s0968-0004(00)01556-5]

[25] AlShammari AK, Abd El-Aziz TM, Al-Sabi A. Snake Venom: A Promising Source of Neurotoxins Targeting Voltage-Gated Potassium Channels. Toxicon. 2004;16:12. [DOI: 10.3390/toxins16010012]

[26] Harris JB, Scott-Davey T. Effects on the Peripheral Neuromuscular System with Comments on the Role of Phospholipases A_2 in Disorders of the CNS and Their Uses in Industry. Toxins. 2013;5:2533-2571. [DOI: 10.3390/toxins5122533]

[27] Prasarnpun S, Walsh J, Harris JB. Beta-bungarotoxin-induced depletion of synaptic vesicles at the mammalian neuromuscular junction. Neuropharmacology. 2004;47:304–314. [DOI: 10.1016/j.neuropharm.2004.04.012]

[28] Tedesco E, Rigoni M, Caccin P, Grishin E, Rossetto O, Montecucco C. Calcium overload in nerve terminals of cultured neurons intoxicated by alpha-latrotoxin and snake PLA_2 neurotoxins. Toxicon. 2009;54(2):138-44. [DOI: 10.1016/j.toxicon.2009.03.025]

[29] Rigoni M, Paoli M, Milanesi E, Caccin P, Rasola A, Bernardi P, et al. Snake phospholipase A_2 neurotoxins enter neurons bind specifically to mitochondria, and open their transition pores. J. Biol. Chem. 2008;283(49):34013–34020. [DOI: 10.1074/jbc.M803243200]