NEUROTOXIC EFFECT INDUCED BY *Prasiola crispa* ANTARCTIC-ALGAE METHANOLIC EXTRACT IN COCKROACHES

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Abstract: In this work we have demonstrated the direct neurotoxic effect of *Prasiola crispa* methanolic extract at cockroach neuromuscular junctions. At in vivo cockroach neuromuscular preparations the extract induced a progressive and irreversible neuromuscular blockade, which was preceded by an increase in the muscular tonus with the appearance of spontaneous twitches. The previous application of chloral hydrate, a n-methyl-d-aspartate receptor blocker, increased the time to blockade of the muscle twitches by 50%, whereas it extinguished both the previous increase of tonus and the appearance of spontaneous twitches, before the onset of neuromuscular blockade. Together, these results suggest that *P. crispa* extract induced an insecticide activity by acting mainly at insect neuromuscular junctions. Therefore, the NMDA receptors must be the target of this pharmacological interaction. A further phytochemical and pharmacological investigation will increase the knowledge about the cellular and molecular aspects of *P. crispa* insecticide effect.

Keywords: *Prasiola crispa*, Antarctic Algae, Aeurotoxic Effect, Cockroaches, Neuromuscular Junctions

Introduction

From the biological point of view, the co-evolution between plants and insects are recognized (Ryan & Byrne, 1988) and may explain the selection of a broad spectrum of secondary metabolites used as defense (*i.e.* one or a few plant chemicals antagonistic to insects) (Bown *et al.*, 2006). Nowadays, there is a major motivation to promote research on botanical insecticides with the low environmental cost and minimal ecological impact, and a demand for healthier products. Cockroaches are primitive pest insects, and most of their functional systems are fairly unspecialized. The cockroach nervous system can be used not only to understand the physiological aspects between insect and natural compounds, but also as an important model of biomedical research (Stankiewicz *et al.*, 2012).

The thalloid green alga *Prasiola crispa* is abundant on the upper shores of Antarctica, often near to and on penguin benches. In this habitat, the alga is exposed to a number of stressful conditions, which may give rise to the development of multiple survival strategies, including the production of different secondary metabolites (Harwood & Gushina, 2009; Pereira *et al.*, 2009). The studies targeting *P. crispa* biological activity are scarce, and indeed, our group was the pioneer in investigating the inherent insecticide activity of this alga (Posser *et al.*, 2010). Despite the wealth of literature on the ecological and pesticidal effects, few studies of plant extracts have considered their mechanism of action. Therefore, to the best of our knowledge, the present study is likely to be the first on investigating the cellular aspects involved in the

action insecticide actions of Antarctic algae in cockroaches. The aim of this work was to investigate the mechanism involved in the insecticide effect of *Prasiola crispa* methanolic extract, especially those related to the cellular interactions at insect neuromuscular junctions.

Materials and Methods

Animals

Cockroaches (*Leurolestes circunvagans*) were reared with water and food *ad libitum*, at controlled temperature (22-25°C).

Reagents

All chemicals and reagents used were of the highest purity and were obtained from Sigma, Aldrich, Merck or BioRad.

Plant material

Prasiola crispa (Lightfoot) Kützing (1843) was collected in the ice-free areas near Arctowski Polish Base Region, Admiralty Bay, King George Island (61° 50' - 62° 15' S and 57° 30' - 59° 00' W), Antarctica. The *Prasiola crispa* methanolic extract (PCME) was produced as described elsewhere (Posser *et al.*, 2010). The extracts were dissolved in 100% dimethyl sulfoxide (DMSO) previously to animal administration.

In vivo Cockroach Metathoracic Coxal-Adductor Nerve-Muscle Preparation

We used the *in vivo* cockroach metathoracic coxal-adductor nerve-muscle preparation as biological assay (Full & Stokes, 2008). The animals were previously anesthetized by chilling (-5°C), and fixed ventral side up in Perspex plate recovered with Styrofoam by means of entomologic needles. Isometric recordings were made using a computer-based software model AQDAD (AVS Instruments, São Carlos, SP, Brazil) with a 1g force transducer model AECAD (AVS Instruments). Supramaximal electrical stimuli (5 ms, 0.5 Hz) was delivered at nerve 5 by insertion of a bipolar Ag/AgCl electrode, coupled to a stimulator (AVS Instruments), during 120 min. Drugs (40 µL) were injected with a Hamilton syringe at the third abdominal segment.

Statistical analysis

The results were presented as mean $\pm$ S.E. The ANOVA/MANOVA, was used for significance ($p < 0.05$). The non-parametric Student "t" test was used as a *post hoc*.

Results

The administration of DMSO alone did not interfere with the muscle strength ($n = 6$) during 120 min recordings. The injection of PCME (100, 200, 400 and 800 µg/g of animal weight) induced a dose and time-dependent neuromuscular blockade in 120 min recordings. When the minimum dose of 100 µg/g was assayed there was $80 \pm 5\%$ blockage of twitches in 120 min recordings ($n = 6$, $p < 0.05$). The injection of (800 µg/g) PCME induced 100% blockade of twitch tension in 10min ($n = 6$, $p < 0.05$). In all doses tested, before the onset of extract neurotoxic effect, there was a previous increase of muscular tonus followed by a blockade of the twitches. The appearance of spontaneous post-twitches, during the first 30min recordings was also noticed. The addition of Chloral hydrate (40 µg/g), a *n-methyl-d-aspartate* receptor antagonist, 15 min previously to PCME (400 µg/g), increased the time required to 50% blockade of the twitches (from 70 ± 2 min to 110 ± 3 min, $n = 5$, $p \leq 0.05$). In this protocol, there was also an inhibition of the tonus increasing effect.

Discussion

In this work we have dealt with the cellular aspects of the insecticide activity induced by *Prasiola crispa* methanolic extract. In this respect, we have confirmed this latter effect and, in addition, we observed a direct interaction of the algae biocompounds at the cockroach neuromuscular junctions.

A number of plants have been ascribed as natural insecticides and their active constituents isolated and characterized (Pavela, 2005). Such plants, synthesize several chemical constituents which act by inhibiting insect growth, feeding and by altering other important physiological parameters (Viegas Junior, 2003). In cockroaches, the neuromuscular transmission releases glutamate as chemical neurotransmitter to promote the muscle twitches by activation of the insect *n-methyl-d-aspartate* (NMDA) receptor (Peoples & Weight, 1998). According to our results, PCME induced a progressive inhibitory effect of insect muscle twitch tension that resulted in an irreversible paralysis of neuromuscular activity. Neurotoxic insecticides act in different manners in the insect nervous system. Natural and synthetic piretroids for example, act by causing a persistent activation of muscle sodium channels, inducing an irreversible and lethal depolarizing effect

(Soderlund, 2012). Others, like insect venoms, prefer the central nervous system as target, inducing a decrease in the release of the neurotransmitter dopamine to cause lethargy and motion weakness (Weisel-Eichler *et al.*, 1999). In the case of PCME, it seems that the algae biocompounds are activating the NMDA receptors at the insect end plate. The first clue for this theory comes from the observation that chloral hydrate, an NMDA blocker, is able to reduce the potency of the neuromuscular blockade induced by the algae extract. Therefore, having followed this treatment, no increase in muscular tonus was seen, indicating that NMDA receptors were not activated. In addition, the

spontaneous twitches were extinguished after chloral hydrate, which demonstrates that the depolarizing action of PCME is caused possibly by the activation of NMDA receptors. The results are of notorious importance because it reinforces the potential biotechnological application of *Prasiola crispa*. To note, NMDA-receptor subunits are particularly involved in several CNS pathologies including acute and chronic pain, stroke, head trauma, drug-induced dyskinesias, and dementia in Alzheimer's disease and Parkinson's disease (Dingledine *et al.*, 1999). The evidence that *P. crispa* possess secondary metabolites that interact with NMDA receptors not only demonstrates the cockroach

Figure 1. Effect of *Prasiola crispa* methanolic extract (PCME) on the *in vivo* cockroach metathoracic coxal-adductor nerve-muscle preparation. Panel **A**, shows the graph of the effect of different concentrations of *P. crispa* extract on 120 min muscle twitch tension recordings. Panel **B** shows the increase of muscular tonus and the followed appearance of spontaneous twitches. In **C**, the graphical expression of the time required to 50% blockade of twitches before and after chloral hydrate (40 µg/µl). In Panel **D**, complete inhibition of neuromuscular twitches by PCME (40 µg/µl) after 5 min recordings. *significance at $p \leq 0.05$; • increase of muscular tonus; ▲ spontaneous twitches; ▼ complete neuromuscular blockade.

neuromuscular models as suitable for investigating the mechanism of insecticide interactions, as well as, showed its yield potential for the development of NMDA-receptor-selective therapeutic agents.

Conclusion

Prasiola crispa methanolic extract induces neuromuscular blockade in *Leuolestes circumvagans*. The latter effect is probably evoked by activation of insect NMDA receptors at the motor end plate. The results also confirm the presence of potent insecticide chemical constituents in *Prasiola crispa* extract as future candidates for the development of NMDA-receptor-selective therapeutic agents.

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