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Entomotoxic activity of *Rhinella icterica* (Spix, 1824) toad skin secretion in *Nauphoeta cinerea* cockroaches: An octopamine-like modulation

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ABSTRACT

Rhinella icterica is a poisonous toad whose toxic secretion has never been studied against entomotoxic potential. Sublethal doses of *Rhinella icterica* toxic secretion (RITS) were assayed in *Nauphoeta cinerea* cockroaches, in order to understand the physiological and behavioral parameters, over the insect central and peripheral nervous system. RITS (10 μg/g) injections, induced behavioral impairment as evidenced by a significant decrease (38 ± 14%) in the distance traveled ($p < .05$), followed by an increase (90 ± 6%) of immobile episodes ($p < .001$, $n = 28$, respectively). In cockroaches semi-isolated heart preparations, RITS (16 μg/200 μl) induced a significant irreversible dose-dependent negative chronotropism, reaching ~40% decrease in heart rate in 20 min incubation. In in vivo cockroach neuromuscular preparations, RITS (20, 50 and 100 μg/g of animal weight) induced a time-dependent inhibition of twitch tension that was complete for 20 μg/g, in 120 min recordings. RITS (10 μg/g) also induced a significant increase in the insect leg grooming activity (128 ± 10%, $n = 29$, $p < .01$), but not in the antennae counterparts. The RITS increase in leg grooming activity was prevented in 90% by the pretreatment of cockroaches with phentolamine (0.1 μg/g). The electrophysiological recordings of spontaneous neural compound action potentials showed that RITS (20 μg/g) induced a significant increase in the number of events, as well as in the rise time and duration of the potentials. In conclusion, RITS showed to be entomotoxic, being the neuromuscular failure and cardiotoxic activity considered the main deleterious effects. The disturbance of the cockroaches' behavior together with the electrophysiological alterations, may unveil the presence of some toxic components present in the poison with inherent biotechnological potentials.

1. Introduction

Poisonous animals are present worldwide and have representatives from many biological taxa. Animal poisons contain substances with unique biological active molecules that have a variety of molecular targets and biological functions [1]. Among others, anuran amphibians are poisonous animals able to inhabit most regions of our planet, especially tropical areas.

The *Rhinella* genus, which includes *Rhinella icterica* specie, is characterized by the presence of parotoid glands located on the body surface, which are mostly involved in the synthesis and release of a poisonous secretion, used for defense against predators and pathogens [2]. *Rhinella icterica* has a fairly wide area of occurrence, being found in central, southeastern and southern Brazil, including the Pampa Biome. The Pampa Biome spreads to northeastern Argentina and eastern Paraguay, at altitudes ranging from 0 to 1200 m. Due to their coverage area, this specie is found in a great diversity of habitats, from open forests to tropical seasonal zones such as the Cerrado biome including areas with considerable anthropic alterations [3].

Anurans poison detains a wide variety of biological compounds such as: biological amines, alkaloids, peptides, proteins and steroids [4]. Several studies suggest that most of the alkaloids found in amphibians

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are either acquired or produced through bacteria in the digestive system and later stored in the parotoid glands [5–7]. Among all toxins presented in the *R. icterica* toxic secretion (RITS), Oliveira et al. [8] demonstrated a substantial presence of bufalin, a potent calcium channel blocker. Effects of this channel blocker have never been described in insects' nervous system.

For a long time, toxinological studies demonstrating the biotechnological potential of animal poisons have been performed only on vertebrate models. In recent decades, however, suitable animal models have also been sought among invertebrates. Basic principles, involving neurotransmission in insects as well as vertebrate models, follow similar patterns. Among several different neurotransmitters present in the cockroaches' central nervous system (CNS), acetylcholine, dopamine, octopamine, 5-hydroxytryptamine and histamine are the most important and take part in important roles related to the functioning of the organism as well as the behavior of these insects [9,10]. Besides, the simplicity, greater accessibility and an increasing applicability for experimental procedures are the most relevant factors that make cockroaches a suitable model for toxicological studies [11,12].

The aim of this work was to investigate the physiological and behavioral effects induced by the *R. icterica* toxic secretion (RITS), in both central and peripheral nervous system (PNS) of *Nauphoeta cinerea* cockroaches. Hence, the rationale of this study is to determine the sensitivity of insect preparations to *R. icterica* poison as well as the main locus of interaction for entomotoxicity.

2. Materials and methods

2.1. Experimental animals

All experiments were performed on adult *N. cinerea* cockroaches of both sexes (3–4 months after adult molt). The animals were reared with water and food ad libitum at controlled temperature and lighting (± 25 °C and 12-hour light/dark cycles).

2.2. Rhinella icterica toxic secretion (RITS)

Toads were collected at Derrubadas (27° 15′ 57″ S, 53° 51′ 45″ W), a region located in the northwest of Rio Grande do Sul, Brazil, with prior approval of the Brazilian Biodiversity Information and Authorization System (SISBIO): collector license n° 24,041–2. Poison extraction was performed by manual compression of the parotoid glands and the resulting secretion was dissolved in methanol followed the solvent evaporation and lyophilization, as demonstrated in previous studies [13–15]. The resulting extract was than dissolved in insect saline solution previous to the biological assays.

2.3. Reagents and solutions

All chemicals and reagents used were of the highest purity available and were obtained from Sigma-Aldrich, Merck, Roche, Life Technologies or BioRad. Test-solutions were prepared daily by dilution in insect saline immediately before use. The insect saline is a buffered solution prepared with the following composition in mM: NaCl, 214; KCl, 3.1; CaCl2, 9.0; sucrose, 50; HEPES buffer, 5.0 and pH 7.2 [16]. Except when stated otherwise, all drugs were injected into the abdominal hemocoell, in 10 µl volumes, by means of a Hamilton syringe.

2.4. Locomotory activity

The influence of *R. icterica* poison on the locomotory activity of *N. cinerea* was assayed in animals randomly selected and placed individually in a white polystyrene box (25 cm in length × 15 cm in width × 7 cm in height) (Fig. 1A). Their behavior was recorded during 10 min by a logitech® HD WEBCAM (Fig. 1B). Behavioral parameters were automatically measured with video-tracking software (IDtracker, Stoelting, CO, USA). Locomotory activity was assessed through computational analysis (Fig. 1C), by using the software ID tracker following an ad-hoc script developed at Matlab® software (30 days free-trial license). To ensure standard experimental conditions, all experiments were performed during the same period of the day (from 10:00 a.m. to 4:00 p.m.).

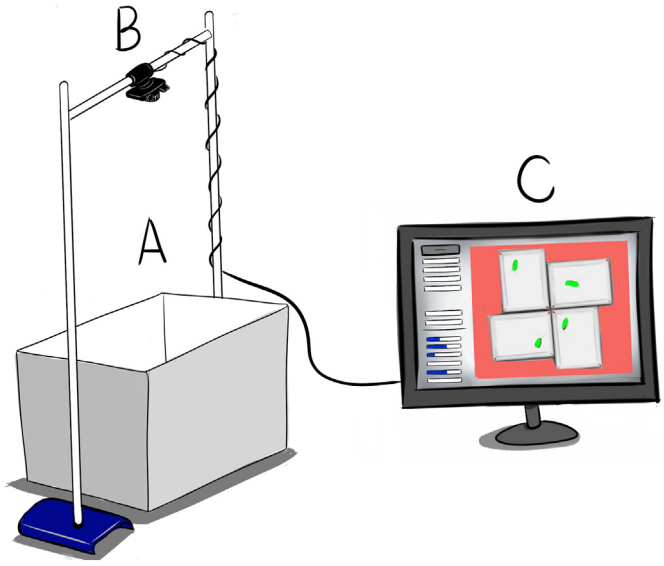

Fig. 1. Schematic representation of the setup for locomotory behavior recordings. (A) the set of boxes where the animals were kept during the video monitoring. (B) webcam mounted above the system. (C) computer-based system for recording, retrieving and posterior analysis of the videos.

2.5. Semi-isolated cockroach heart preparation

A semi-isolated cockroach' heart bioassay was assembled essentially as described by Rodríguez et al. [17]. Briefly, adult cockroaches were anesthetized by chilling (5–7 min), until immobile and placed ventral side up on a dissection plate. The lateral margins of the abdomen were cut along each side, and the ventral abdominal body wall was pulled out to show the viscera. After moving the viscera carefully aside, the heart was exposed still contracting, while attached to the dorsal body wall. The heart preparations were washed by bathing it, in 200 µl of insect saline solution at room temperature (21–24 °C). After 5 min of heart beat stabilization, the treatments were delivered by exchanging the bathing solution. The beats/min average in the first 5 min was taken as a reference. Heart beat frequency was monitored under a stereoscopic microscope, for 30 min. Four cockroaches were used in each group of treatment. In the control group, only the saline solution was used to bath the heart.

2.6. In vivo cockroach metathoracic coxal-adductor nerve-muscle preparation

To analyze the effect induced by RITS on insect neuromuscular junctions the in vivo cockroach metathoracic coxal-adductor muscle preparation was used, essentially as described by Martinelli et al. [18]. Briefly, the animals were immobilized by chilling and mounted, ventral side up, in a plate covered with 1.0 cm soft rubber. The animal was then firmly attached, using entomologic needles, and the metathoracic coxal was firmly tied to an isometric transducer. The left leg was then tied at the medial joint with a dentistry suture line connected to a 1.0 g force isometric transducer (AVS Instruments, São Carlos,SP, Brazil). The transducer was coupled to a manipulator, which allowed muscle length adjustment. A bipolar electrode was inserted onto nerve 5, which includes the motor axon to the muscle, to provide electrical stimulation.