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Anxiolytic activity and evaluation of potentially adverse effects of a bradykinin-related peptide isolated from a social wasp venom

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ABSTRACT

Anxiety disorders are major health problems in terms of costs stemming from sick leave, disabilities, healthcare and premature mortality. Despite the availability of classic anxiolytics, some anxiety disorders are still resistant to treatment, with higher rates of adverse effects. In this respect, several toxins isolated from arthropod venoms are useful in identifying new compounds to treat neurological disorders, particularly pathological anxiety. Thus, the aims of this study were to identify and characterize an anxiolytic peptide isolated from the venom of the social wasp *Polybia paulista*. The peptide was identified as Polisteskinin R, with nominal molecular mass $[M + H]^+ = 1301$ Da and primary structure consisting of Ala-Arg-Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg-OH. The anxiolytic effect was tested using the elevated plus maze test. Moreover, adverse effects on the spontaneous behavior and motor coordination of animals were assessed using the open field and rotarod tests. Polisteskinin R induced a dose-dependent anxiolytic effect. Animals treated with the peptide and diazepam spent significantly more time into the open arms when compared to the groups treated with the vehicle and pentylenetetrazole. No significant differences in spontaneous behavior or motor coordination were observed between the groups, showing that the peptide was well tolerated. The interaction by agonists in both known BK receptors induces a variability of physiological effects; Polisteskinin R can act on these receptors, inducing modulatory activity and thus, attenuating anxiety behaviors. The results of this study demonstrated that the compound Polisteskinin R exerted potent anxiolytic effects and its analogues are promising candidates for experimental pharmacology.
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1. Introduction

As a group, anxiety disorders are the most prevalent mental health condition in both developing and developed regions around the world, affecting one-eighth of the global population [1]. These disorders place a significant burden on both individuals and the healthcare system, since they are a prominent symptom of many psychiatric disorders and other comorbid conditions [2–5]. As such, pathological anxiety is a major health problem in terms of costs stemming from sick leave, disabilities, healthcare, and premature mortality [6,7].

Pathological anxiety has become a focal point for psychopharmacology research in recent decades [8–11]. However, some anxiety disorders are still resistant to treatment and first-line therapy can be ineffective for some patients ([10], for revision see [12]). According to Sartori and collaborators, novel anxiolytic classes with an improved pharmacological profile are unavailable and preclinical and clinical trials in this regard are still in the early stages [13,14].

In this respect, several toxins isolated from arthropod venoms are useful in identifying new compounds to treat neurological disorders, particularly anxiety disorders [9, for revision see 15]. In the central nervous system (CNS), these molecules can be highly selective, targeting different neuronal receptors, transporters and ion channels, in both excitatory and inhibitory neurotransmission [16–18]. Chemical identification and biological characterization of wasp venom peptides have garnered considerable attention from researchers for decades. Neuroactive peptides isolated from these venoms show potent anticonvulsant, anxiolytic, antipanic and antinociceptive effects when applied directly to the CNS of mammals [15,19–22].

The main class of neuroactive peptides present in wasp venom is Kinins, specifically bradykinin (BK) and its analogue peptides [23–27]. In general, these peptides exhibit 9–18 amino acids residues, containing a bradykinin-like sequence (Arg-Pro-Pro) at the N-terminal [28,29]. Threonine^6-bradykinin (Thr^6-BK) is one of the most widely known kinin-related peptides. It acts presynaptically, blocking excitatory nicotinic transmission by activating the inhibitory GABAergic system and

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delaying the irreversible block of synaptic transmission in the insect CNS [30,31]. Moreover, this peptide showed a potent antinociceptive effect similar to morphine, caused by interaction with B2-receptors [32]. However, this class contains a number of that have yet to be pharmacologically evaluated and biologically characterized.

As such, this study aimed to investigate the anxiolytic effect of a bradykinin-related peptide isolated from the venom of the social wasp *Polybia paulista* in rats placed in an elevated plus maze (EPM), an animal anxiety model. In addition, the peptide was evaluated for possible adverse effects, assessing the spontaneous behavior (open field test) and motor coordination of animals (rotarod test).

2. Methods

2.1. Biological material and extract preparation

Polybia paulista colonies were collected in Distrito Federal, Brazil authorized by Chico Mendes Institute for Biodiversity Conservation in Brazil (ICMBio - License number 21723-1) and the Access and Remittance Authorization for the Genetic Patrimony Component Use for Scientific Research was obtained from the National Council of Technological and Scientific Development (CNPq n° 010476/2013-0).

Entomologist Prof. Dr. Fernando Barbosa Noll (UNESP, Brazil) kindly identified the wasp specie. The wasps' nest was immediately placed in a cooler with ice. To euthanize the wasps, in the lab, the nest was stocked at −20 °C for 24 h. The venom reservoirs were removed by micro dissection using surgical micro-scissors from 8000 venom sacs. They were macerated in a 1:1 acetonitrile (ACN)/deionized water solution. The supernatant of the macerated material was centrifuged using an ultra-filter Microcon® (Merck KGaA, Germany) with a 3 kDa cut-off for 30 min at 10,000 ×g. The resulting material constituted by compounds with molecular weight of up 3000 Da, was lyophilized, weighed and then resuspended in water (2%) and ACN with trifluoroacetic acid (TFA, 0.07%).

2.2. Fractionation and purification

The solution (compounds <3000 Da) was chromatographed on a reverse-phase high performance liquid chromatography (RP-HPLC Prominence, Shimadzu, Japan) using a C_{18} semi-preparative column (C_{18} ODS, 15 μm, 20 × 250 mm, Phenomenex®, USA) using the solution A (0.1% TFA/H_2O) and the solution B (0.1% TFA/ACN) and elution with 5% from solution B for 20 min, following with 5% until 60% from the solution B in 40 min, and flow rate of 2 mL/min.

The active fraction was re-chromatographed with an analytics column (C_{18} Synergi 4 μm, Hydro-RP, 250 × 4.6 mm, Phenomenex®, USA) under conditions similar to those cited above, but with elution with 5% until 45% of the solution B in 80 min and in a flow rate of 1 mL/min. Effluents from both columns were monitored photometrically at 214 nm. Finally, the fraction corresponding to the peptide of interest was lyophilized, dried and reconstituted in physiological solution (saline) for the bioassays.

Matrix-assisted laser desorption and ionization-time of flight mass spectrometry (MALDI-TOF MS) was used to attest the purity of the peptides isolated by chromatography and to characterize the amino acid sequence of the peptide. The analysis of the molecular mass of the peptide was performed using a MALDI TOF-TOF (UltraFlex III, Bruker Daltonics, Germany) according to the procedures described by Bemquerer and collaborators in 2012 [33]. Matrix solution was prepared using 5.0 mg of α-cyano-4-hydroxy-cinnamic acid (HCCA, Bruker Daltonics, Germany) and solubilized with 250 μL of ACN, 200 μL of deionized water, and with 50 μL of an aqueous TFA solution (at 3% by volume). Then, 3 μL of the saturated matrix solution were mixed with 1 μL of the resuspended sample solution and the mixture was spotted. Fragmentations for manual de novo peptide sequencing were performed by MALDI-TOF/TOF using the LIFT™ method. Sequencing was manually performed by using the PepSeq software (MassLynx 4.0, Waters, USA). Database (Swiss-Prot) searches for sequence similarities were performed using protein BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PA- GE1 ⁄ 4Proteins).

2.3. Animals

Male Wistar rats (*Rattus novergicus*) weighing 220–250 g, from the animal house of the University of Brasília – Brazil, were used. They were kept in wire-mesh cages in a room on 12/12 h light/dark cycle (7:00 am–7:00 pm lights on) with free access to food and water throughout the experiment. The animals were maintained at constant conditions of luminosity, temperature (25 ± 2 °C) and humidity (55%) in the housing and experiment rooms.

All experimental procedures were approved by the Committee for Ethics in Animal Use (CEUA) of the University of Brasília, under the license number 45.810/2009. Animals were preserved in accordance with the Brazilian Society for Neuroscience and Behavior ethical statements, which follows the guidelines for animal care prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA and Brazilian Arouca Law (n° 11.794/2008). Likewise, every effort was made to minimize the number and potential suffering of the experimental animals. According to detailed in CEUA protocol, we used 84 animals in all experiments.

2.4. Surgery

The animals were anesthetized intraperitoneally (i.p.) with ketamine hydrochloride (60 mg/kg, Syntec, Brazil) and xylazine hydrochloride (10 mg/kg, Syntec, Brazil) and fixed in a stereotactic frame (Insight Equipments®, Brazil). After a subcutaneous (s.c.) local injection (0.05 mL) of lidocaine hydrochloride (30 mg/mL) with norepinephrine hemitartrate (0.04 mg/mL) (Lidostesim® 3%, Dentsply, Brazil), the cranium was exposed for implantation of a stainless steel guide cannula (10 mm) in the right lateral ventricle. The following coordinates were used: 0.8 mm posterior to bregma, 1.6 mm lateral from the midline and 3.4 mm ventral from the surface of the skull according to the atlas of Paxinos and Watson [34]. At the end of surgery, we put a stainless steel wire was introduced into the guide cannula to protect it from obstruction and fixed it to the skull with acrylic resin. All animals were allowed 5–7 days to recover from surgery before performing the anxiolytic assays.

2.5. Anxiolytic activity

Independent groups of rats (n = 8–12) were injected intracerebroventricularly (i.c.v.) with the peptide in different concentrations (6, 3 and 1.5 ηmol/animal) and vehicle (saline 150 mM) with a microsyringe (Hamilton®, USA) and a infusion pump (BI-2008, AVS Projects, Brazil). The animals were placed in the center of an EPM ten minutes after treatment. The EPM was made of wood and it had two open arms and two enclosed arms (50 cm in length and 10 cm in width) connected by a central connecting square. The closed arms were protected by walls with 40 cm in height (EP 151, Insight Equipments®, Brazil). To avoid falls from the apparatus, the two open arms were fenced by an acrylic raised flap. The maze was elevated 50 cm above the floor. The animal's behavior was recorded with a video camera positioned above the maze. The signal was relayed to a monitor in an adjacent room, which allowed the identification of all forms of behavior. The maze was cleaned with alcohol 70% after each trial. In addition to the vehicle group, two other control groups were employed: positive control, in that the anxiolytic Diazepam (DZP; 2 mg/kg, i.p.) was injected and the animals were placed in the EPM 30 min later, and negative control, in which the anxiogenic substance Pentylenetetrazole (PTZ; 30 mg/kg, s.c.) was injected and the animals were placed in the EPM 15 min later. The animals were placed

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