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Neurobiological activity of Parawixin 10, a novel anticonvulsant compound isolated from *Parawixia bistriata* spider venom (Araneidae: Araneae)

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ABSTRACT

The neurobiological activity of Parawixin 10, isolated from *Parawixia bistriata* spider venom, was investigated. Cannulas were implanted in the lateral ventricles of Wistar rats (200–250 g, $n = 6$–8 per group) to perform anticonvulsant and behavioral assays, and synaptosomes from cerebral cortices of male Wistar rats were used for neurochemical studies. The results indicate that pretreatment with Parawixin 10 prevents the onset of seizures induced with kainic acid, *N*-methyl-D-aspartate, and pentylenetetrazole in a dose–response manner. Lower doses of Parawixin 10 significantly increased the latency to onset of kainic acid-, pentylenetetrazole-, and *N*-methyl-D-aspartate-induced seizures. There were maximum increases of 79% in L-[^{3}H]glutamine uptake and 40% in [^{3}H]glycine uptake; [^{3}H]GABA uptake did not change. The findings demonstrate that this novel compound from *P. bistriata* venom exerts a pharmacological effect on the glutamatergic and glycinergic systems.

1. Introduction

In recent decades, several molecules purified from venom, especially polyamines and peptides, have aided the elucidation of physiological intrinsic properties of ion channels, neurotransmitter receptors, and transporters [1–3]. However, despite their great potential as pharmacological tools, only small parts of these compounds have been studied and the vast majority of molecules remain unexplored.

Parawixia bistriata spider venom has been the object of intense investigation by our laboratory [4,5]. When applied to the rat's central nervous system (CNS), *P. bistriata* crude venom induces limbic seizures [6], whereas deproteinized venom blocks generalized tonic–clonic seizures induced with the GABAergic antagonists bicuculline, picrotoxin, and pentylenetetrazole (PTZ). Moreover, the addition of *P. bistriata* spider venom to synaptosomes from rat cerebral cortex increases L-glutamate (L-Glu) uptake and inhibits GABA uptake [5,7].

Fontana et al. [4] isolated and identified Parawixin 1 (formerly PbTx 1.2.3), which increases L-Glu uptake in rat cortical synaptosomes and protects retinal neurons from ischemic damage. Parawixin 1 promotes the direct and selective enhancement of L-Glu influx by the EAAT2 transporter subtype, but does not interfere with the affinity of the transporter for its co-substrates and Na^+ ions, as demonstrated using cloned liposomes and COS cells [8]. According to Salazar and Fahlke [9] the selectivity and specificity of Parawixin 1 make it a starting point in the design of small molecules to be used in the treatment of pathological conditions caused by alterations of L-Glu.

The fractionation of *P. bistriata* venom also revealed a potent inhibitor of GABA and glycine uptake, namely, FrPbAII (2-amino-5-ureidopentanamide). According to Beleboni and co-workers [5], FrPbAII acts directly on these transporters rather than on ion channels permeable to Na^+, K^+, or Ca^{2+}, GABA transaminase, or reverse transport. Bioassays have shown that FrPbAII (formerly PbTx 2.2.1) blocks tonic–clonic seizures induced with bicuculline [7], PTZ, kainic acid (KA), and pilocarpine [10]. Also, when injected into the substantia nigra and pars reticulata, FrPbAII inhibits generalized seizures induced by GABAergic blockade of the area tempestas in the pyriform cortex, and when injected into the dorsal hippocampus, it exerts anxiolytic effects [11]. FrPbAII is also neuroprotective after intravenous administration in Wistar rats submitted to experimental

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glaucoma in the outer and inner nuclear layers of the retina [5]. When injected into vitreous, it completely inhibits retinal layer neuronal death induced by ischemia and ischemia–reperfusion, being 100 times more potent than nipecotic acid (unpublished data).

Considering that neurotransmitter transporter malfunction is closely associated with severe pathological conditions such as epilepsy, Alzheimer's disease, and ischemia, *P. bistriata* spider venom could be considered an interesting source of probes for novel therapeutic strategies. In the light of all these facts, the aim of this study was to identify a novel compound from *P. bistriata* venom that acts on neurotransmitter transport and inhibits seizures induced with chemoconvulsants.

2. Material and methods

2.1. Spider collection and venom fractionation

Nests of *P. bistriata* collected in the rural areas neighboring Ribeirão Preto, São Paulo, Brazil, were frozen at −4 °C, taken to the laboratory, and kept at −20 °C. Glands and venom reservoirs were removed, homogenized in CH_3CN:H_2O (1:1; v/v), and centrifuged at 8000 *g* for 10 minutes at 4 °C. Supernatants were collected and filtered in membranes with a 3000-Da cutoff (Millipore, Microcon, USA) under centrifugation at 8000 *g* and 4 °C until complete filtration. Next, the extract was lyophilized, weighed, and submitted to fractionation.

Dry extract was dissolved in ultrapure water of Milli-Q grade containing 0.1% TFA. The solution was subjected to reverse-phase high-performance liquid chromatography (RP-HPLC; Shimadzu, Japan) using an ODS C18 column (15 μm, 20×250 mm; Phenomenex-Jupiter, USA) at the flow rate of 8.0 mL/min and a UV light detection system at 214 nm. Initially, an isocratic gradient was run with 1% CH_3CN:H_2O (v/v) containing 0.1% TFA for 10 minutes. Next, a linear gradient from 1 to 60% CH_3CN for 60 minutes was performed. Fractions were collected, lyophilized, weighed, and used in bioassays.

2.2. Purity of fractions

Dry fractions were dissolved in 50% CH_3CN:H_2O (v/v) containing 0.1% formic acid (v/v). Molecular masses were determined by positive electrospray ionization (ESI+) on a high-resolution spectrometer. Fractions were injected with the aid of an infusion pump at a flow of 10 kL/minute. ESI-MS spectra were acquired on an UltrOTOF apparatus (Bruker Daltonics, Billerica, USA) in the continuous acquisition mode, scanning from *m/z* 50 to 2000 with a scan time of 5 seconds. Calibrations were made using intact horse heart myoglobin (Sigma–Aldrich, USA) and its typical cone-voltage-induced fragments.

2.3. Animals and surgery

Male Wistar rats (220–250 g), from the animal house of the Campus Universitarius of the University of São Paulo at Ribeirão Preto were used in the assays. The animals were kept in pairs in wire-mesh cages in a room with a 12-hour dark/light cycle (lights on at 7:00 AM) with water and food cubes ad libitum. Animals were maintained in accordance with the Brazilian Society for Neuroscience and Behavior ethical statements that follow the guidelines for animal care prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council (USA). Luminosity and temperature (22 °C) were kept constant in the housing and experiment rooms. Efforts were made to minimize the potential suffering of experimental subjects. Approval was obtained from the Ethics Committee for Care and Use of Laboratory Animals from Campus Universitarius of the University of São Paulo at Ribeirão Preto (CEUA No. 10.1.619.53.3).

All animals were implanted with stainless-steel guide cannulas (10 mm) in the right lateral ventricle under ketamine (60 mg/kg; Agener União, Brazil) and xylazine (8 mg/kg; Calier, Spain) anesthesia. The coordinates used were 0.9 mm posterior to bregma, 1.6 mm lateral from midline, and 3.4 mm ventral from the surface of the skull according to the atlas of Paxinos and Watson [12]. Next, animals were placed in a stereotactic frame (Stoelting, USA) and injected percutaneously before surgical incision with 0.1 mL of 2% lidocaine hydrochloride containing epinephrine at 1:200.000 (Astra Química, Brazil). Cannulas and stainless-steel screws, for anchoring, were fixed to the skull with dental acrylate. Each cannula was sealed with a stainless-steel wire to avoid obstruction. After surgery, animals were injected with flunixin meglumine (1 mg/kg; Schering–Plough Animal Health, Brazil) to minimize pain. The animals were then allowed to rest for 5–7 days to recover from surgery.

2.4. Anticonvulsant screening

CD_{97} doses of chemoconvulsants were used, that is, the dose producing hindlimb tonic convulsions in 97% of animals. These doses were previously established in dose–response experiments for each convulsant [13].

Before injections, animals were placed in the open field for 10 minutes. Rats were divided into groups (n = 6–8). Three different doses of Parawixin 10 were tested with each convulsant: Parawixin 10 at 0.25, 0.5, 1.0, and 1.5 μg/μL, 10 minutes before KA (0.8 μg/μL); Parawixin 10 at 1.0, 2.0, and 5.0 μg/μL, 10 minutes before NMDA (20 μg/μL); Parawixin 10 at 1.0, 2.0, 3.0, and 6.0 μg/μL, 10 minutes before PTZ (85 mg/kg, 0.2 mL, ip), all injected intracerebroventricularly in a volume of 1 μL. In separate groups of animals, one-milliliter volumes of diazepam (2, 5, and 10 mg/kg) and saline were injected intraperitoneally.

The final volume for drugs delivered by the intracerebroventricular route was 1 μL over a 1-minute period, whereas PTZ was injected in a volume of 0.2 mL into the loose fold of the neck. After drug administration, animals were placed in the arena (open field) and filmed for 30 minutes. Next, rats were packed in individual cages until total recovery.

Kainic acid- and NMDA-induced seizures were scored as follows: 0 = no seizure activity; 1 = jaw movements; 2 = head myoclonus; 3 = hindlimb myoclonus; 4 = elevation; 5 = elevation and fall; 6 = ear and head myoclonus, clonic movements, sequential events of elevation and fall; 7 = rolling, violent jumps, and vocalization; 8 = all behaviors of class 7 followed by periods of hypertonus [14]. Latency to the onset of seizures and percentage of animals protected against score 8 seizures were the parameters used to analyze the anticonvulsant effects.

Seizures induced by PTZ administration were analyzed according to the Lamberty and Klitgaard [15] index, considering behavior of the convulsing animals as well as latency to onset of tonic–clonic seizures.

2.5. Spontaneous locomotor activity and behavioral assays

On the morning of the experiments, Wistar rats (n = 6, 200–250 g) were transferred to the experimental rooms and allowed a 10-minute period for acclimation. Next, they were randomly assigned to treatment groups and injected intracerebroventricularly with either Parawixin 10 (2.5, 5.0, or 10 μg/μL) or saline 15 mM, with the aid of a Hamilton syringe moved by an infusion pump (Insight, Brazil) injecting a volume of 1 μL/1 minute. After 10 minutes, rats were gently placed in an open field consisting of an acrylic arena 60 cm in diameter and 12 cm high. Animals were monitored by a video camera interfaced with a VCR and a monitor placed in the adjacent room for 20 minutes, after which they were returned to their home cages and remained under observation for 2 hours.

Spontaneous locomotor activity of the animals was determined by counting the line crossings in the open field of each animal along four time windows (0–5, 5–10, 10–15, and 15–20 minutes). Moreover, the