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Anticonvulsant and GABA Uptake Inhibition Properties of Venom Fractions from the Spiders *Parawixia bistriata* and *Scaptocosa raptoria*

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Abstract

In this article we describe an *in vivo* anticonvulsant effect from denatured crude venom and partially isolated fractions from two spiders: *Parawixia bistriata* and *Scaptocosa raptoria.* Intracerebroventricular injections of these venoms and fractions abolished rat convulsive tonic-clonic seizures induced by picrotoxin, bicuculline and pentylenetetrazole, and also, inhibited GABA uptake in synaptosomes of rat cerebral cortex. The venoms described in this work seems to be promising tools for the study of the GABAergic system, and may be a potential source for new anticonvulsant drugs.

Keywords: Anticonvulsant, bicuculline, GABA, GABA uptake, *Parawixia bistriata*, pentilenetetrazole, picrotoxin, *Scaptocosa raptoria*, spider venom, rat synaptosomes.

Introduction

Many venom components are useful tools in neuroscience research, since they have remarkable specificity and binding affinities for neurotransmitter receptors, transporters and ion channel subtypes. Disruption of GABAergic neurotransmission has been linked to several psychiatric and neurological diseases (e.g., anxiety and epilepsy; for review, see Krogsgaard-Larsen et al., 2000). Due to this, drug discovery has focused on pharmacological approaches that enhance GABAergic function. Examples of this are tiagabine, which inhibits the uptake of GABA in neurons and glia (Borden et al., 1994) and gabapentin, which decreases brain GABA transaminase activity (Leach et al., 1997) and is a postsynaptic $GABA_B$ subtype receptor agonist in rat hippocampal neurons (Ng et al., 2001).

Parawixia bistriata (Araneidae, Araneae) (Rengger, 1836) is a South American social orb weaver spider (Levi, 1992). *P. bistriata* crude venom inhibits by 73% the GABA uptake in synaptosomes from rat cerebral cortex (unpublished data). In termites, the injection of this venom causes irreversible paralysis (Fontana et al., 2000).

Scaptocosa raptoria (Lycosidae, Araneae) (Lucas, 1836) is a solitary grass hunting spider found mainly in Brazil (Bucherl, 1969). Rats injected with *S. raptoria* crude venom exhibited a period of freezing, which is always followed by procursive-type seizures (running, gyrating, atonic falling and jumping) (Ribeiro et al., 2000).

In this work, we described the use of GABAergic models of acute convulsive chemical seizure induced by pentylenetetrazole (PTZ), bicuculline (BIC) and picrotoxin (PICRO) to evaluate the anticonvulsant effects of these crude venoms and two partially isolated fractions. We also performed preliminary experiments evaluating the role played by these frac-

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tions in the inhibition of [U-^{14}C]-GABA uptake in rat cerebral cortex synaptosomes.

Materials and methods

Spider collection, extraction and purification

P. bistriata specimens were collected in the region of Ribeirão Preto, São Paulo, Brazil. Spiders were sacrificed by freezing at −20 °C. The glands were removed and crushed in Milli-Q water. The extracts were treated either by boiling at 100 °C for 10 min and centrifuging for 3 min at 3000 × g, resulting in the boil denatured *P. bistriata* crude venom (BdPb), or by adding acetonitrile/water (1 : 1) and centrifuging for 12 min at 124.4 × g, producing the acetonitrile denatured *P. bistriata* crude venom (AdPb). Lyophilized BdPb from one thousand glands was dissolved in 1.0 mL of Milli-Q water and applied onto a reverse phase HPLC column (Jupiter ODS – 20 × 250 mm, 5 µm particle size). Milli-Q water was degassed (solvent A) and 65% methanol was filtered and degassed (solvent B). Solvent A was kept isocratic for 60 min, then a linear gradient of solvent B (5–100%) was applied for 70 min, and held for 10 min. The flow rate used was 1.0 mL/min and monitored at 215 nm. The fraction which presented the biological activity in [U-^{14}C]-GABA uptake in synaptosomes was injected on a reverse phase HPLC column (Jupiter ODS – 4.6 × 150 mm, 5 µm) and rechromatographed with a non-linear gradient conducted with zero water-trifluoroacetic acid (TFA) 0.05% up to 100% absolute acetonitrile, flow rate 0.5 mL/min, gradient elution in 60 min, the effluent monitored at 215 nm. Fractions were lyophilized and resuspended in water. An active fraction on [U-^{14}C]-GABA uptake in synaptosomes was named FrPbA2 and tested in anticonvulsant experiments.

S. raptoria spiders were collected at the University of São Paulo, Campus of Ribeirão Preto, São Paulo, Brazil. Spiders were sacrificed by freezing them. Venom glands were crushed in 0.9% saline and centrifuged for 5 min at 124.4 × g. Acetonitrile was added to the supernatant to a final concentration of 50% and the resulting solution was centrifuged for 5 min at 124.4 × g. This new supernatant was boiled for 10 min, centrifuged (as above) and named acetonitrile and boiled denatured *S. raptoria* crude venom (AbSr).

Also, *S. raptoria* venom glands were crushed in acetonitrile : water (1 : 1, v/v) and centrifuged for 5 min at 124.4 × g. After centrifugation, the venom extract was diluted with cold 0.1% TFA/H_2O to a concentration of 5% acetonitrile. The extract was injected on a reverse phase HPLC column (Jupiter ODS column – 25 × 250 mm, 15 µm). The eluent B was 70% acetonitrile in 0.1% TFA: a linear gradient of 5% to 95% B was conducted for 60 min with flow rate 1.0 mL/min.

Fractions were monitored at 215 nm, lyophilized and resuspended in water. The fraction which presented the biological activity in [U-^{14}C]-GABA uptake in synaptosomes was named SrTx1 and tested in anticonvulsant assays.

P. bistriata venom doses

Hartree's method (1972) was used to determine the protein content of the crude venom: one milligram of crude lyophilized venom corresponds to 0.54 mg of protein. However, for *P. bistriata* venom doses, the results were shown in mg of crude venom, instead of mg of protein.

The concentrations of BdPb and AdPb were 17.5 µg of crude venom/mL and 50 mg of crude venom/mL, respectively.

The concentration of the isolated fraction FrPbA2 was determinated arbitrarily. The value of one optical density at 215 nm was defined as 1000 arbitrary units (AU). The solution of FrPbA2 (1000 µL) absorbed 2.05, so it corresponds to 2050 AU. GABA uptake assays were carried out with 4 doses (1, 10, 25 and 50 µL of the solution, corresponding to 2.05, 20.5, 41 and 82 AU of FrPbA2, respectively). *In vivo* experiments were carried out with 0.212 AU/ 3 µL/ rat, of FrPbA2.

S. raptoria venom doses

Protein content of *S. raptoria* venom was determined by Hartree's method (1972). For seizure tests in rats, 0.8 mg protein/mL were applied of both AbSr and SrTx1. GABA uptake experiments were conducted with SrTx1 concentrations as indicated in Figure 1A.

Rats

Male Wistar rats (n = 123; 200–250 g) were put in groups of three per cage under a 12 : 12 dark/light cycle (lights on at 07/00 h) at 23 ± 1 °C with *ad libitum* access to food and water. All animals were obtained from the animal house of the Campus of Ribeirão Preto at the University of São Paulo. Rats were treated according to the guidelines of use of animals in research approved by The Brazilian Society for Neuroscience and Behavior.

Rat intracerebroventricular injections

Anaesthetized rats (sodium pentobarbital, 50 mg/kg, i.p.) were placed in a stereotaxic frame (Stoelting, USA). A 27-gauge stainless steel guide cannula was placed in the right lateral ventricle (Paxinos & Watson, 1986; coordinates AP: – 1.0 mm, ML: 1.6 mm, DV: 3.3 mm), attached to the skull with acrylic resin and anchored with stainless steel screws. After surgery, animals were allowed a recovery period of 5 days. Microinjections in the lateral ventricle were applied through a 30-gauge stainless steel injection cannula, inserted into the previous implanted guide cannula, in smoothly restricted rats. The injection volume and time was 3 µL and 2 min, respectively.

Histological examination of coronal sections containing lateral ventricle was done at the end of the experiment to confirm the correct position of the cannula. Rats were sacri-

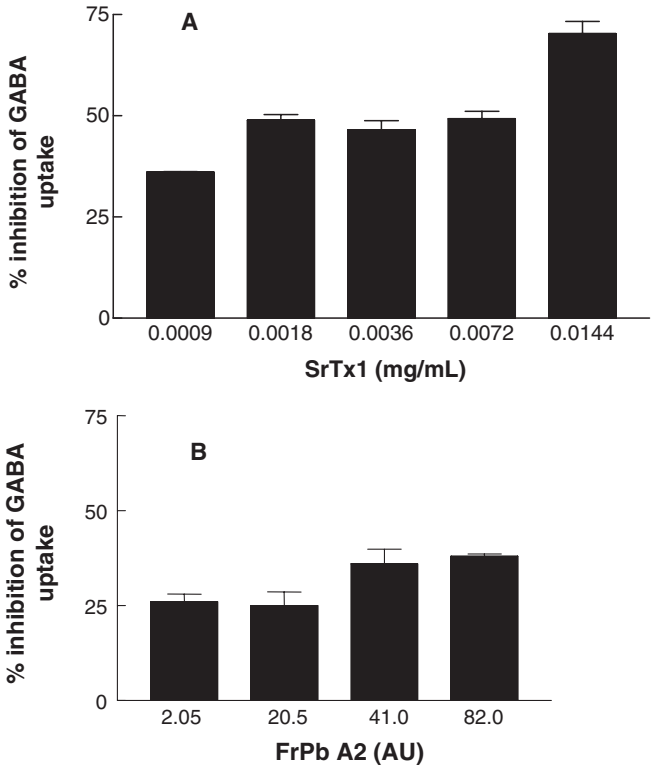

Figure 1. (A) Inhibition of GABA uptake by different concentrations of the SrTx1 isolated from *S. raptoria* venom. (B) Inhibition of GABA uptake by different concentrations of the FrPbA2 isolated from *P. bistriata* venom. All experimental groups were compared to synaptosomes incubated with Krebs phosphate buffer, that represents zero inhibition of GABA uptake. Results are expressed as percent inhibition ± SEM of control uptake. Both fractions inhibit significantly the GABA uptake at all tested concentrations (one-way ANOVA, $p < 0.05$).

ficed with ether and perfused transaortically with 0.9% saline and 4% formol solution. After at least 24 h of fixation, the brains were embedded in paraffin, cut into sections, and stained with cresyl violet. Some animals, after sacrifice and perfusion, received i.c.v. injection of dye (bromophenol blue), their brains were removed and frozen in −20 °C for 2 h, then cut manually to see if the lateral ventricle was stained.

Anticonvulsant assays

Convulsant doses (PTZ 100 mg/kg, i.p.; PICRO: 249 µg/3 µL/rat, i.c.v.; BIC: 9 µg/3 µL/rat, i.c.v.) were standardized in order to provoke tonic-clonic seizures in 100% of injected control animals in less than 30 min. Animals (n = 113) were injected with 0.9% saline (controls), venoms or fractions (experimental group) by the intracerebroventricular (i.c.v.) route. After 20 min, rats were injected with the convulsant agents (PTZ i.p., PICRO or BIC, i.c.v.). The rat's behavior was recorded on videotapes for 30 min, and the presence or absence of tonic-clonic seizures or death was evaluated.

Synaptosomal preparation

Male Wistar rats (n = 10) were decapitated and their brains were rapidly removed for the preparation of synaptosomes as previously described (Gray & Whittaker, 1962; Coutinho-Netto et al., 1980). The synaptosomal fraction was resuspended in Krebs-phosphate buffer (in mM: 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 0.75 $CaCl_2$, 1.2 $MgSO_4$, 20 Na_2HPO_4, 10 glucose, pH 7.4, gassed with O_2 prior to resuspension) and centrifuged for 20 min at 4 °C. Synaptosomal integrity was determinated by assaying lactate dehydrogenase (LDH) activity (Bergmeyer, 1983).

GABA uptake assays

Biological effects of the venom and isolated fractions were verified on GABA uptake as follows: synaptosomal pellets were resuspended in Krebs-phosphate buffer and pre-incubated for 15 min at 25 °C (Iversen & Neilsen, 1968). In uptake experiments, synaptosomal suspension was added to Eppendorf tubes (100 µg protein/mL) containing [U-^{14}C]-GABA (21 nM final concentration and specific radioactivity 238.0 µCi/mmol, NEN, Boston, MA, USA) and Krebs-phosphate buffer (control group) in presence or absence of isolated spider venom fractions (FrPbA2 or SrTx1) in several concentrations. All experimental groups were compared to synaptosomes incubated with Krebs phosphate buffer, which represent zero inhibition of GABA uptake.

Tubes were incubated in a 25 °C bath for 10 min using shaking. Uptake was finished by centrifugation for 3 min at 3000 × g. The pellet was washed in cold Krebs phosphate and 0.5 mL of 10% tricloroacetic acid (TCA) was added. After another centrifugation for 3 min at 3000 × g, 0.2 mL of supernatant was transferred to scintillation vials and their radioactivity was measured in a liquid-scintillation counter (LS-6800, Beckman) with an 85% counting efficiency. Protein content was determined by Hartree's method (1972).

For each experimental condition, blank values (unspecific uptake) were obtained from samples prepared and incubated as above, but in a sodium-free buffered medium where NaCl was replaced by isosmotic choline chloride. The nonspecific radioactivity was subtracted from the total radioactivity.

Statistical analysis

GABA uptake inhibition results were analyzed by one-way ANOVA. The presence or absence of tonic-clonic seizures or deaths was analyzed by Fisher exact test. For both statistical tests, 5% of significance level ($p < 0.05$) was considered.

Results

Behavioral anticonvulsant assays

All control animals exhibited tonic-clonic seizures (Table 1) followed by death (Table 2) after injection of all GABAergic antagonists (PTZ, PICRO and BIC). In the experimental

Table 1. Anticonvulsant effect of *P. bistriata* venom (BdPb, AdPb and FrPbA2) and *S. raptoria* venom (AbSr and SrTx1) in the PTZ, PICRO and BIC models of acute seizure induction in rats. Number of animals presenting (S) or not presenting (NS) tonic-clonic seizures in PTZ, PICRO and BIC models.

	PTZ		PICRO		BIC	
Group	S	NS	S	NS	S	NS
Control Pb	10 (100)	0 (0)	7 (100)	0 (0)	11 (100)	0 (0)
BdPb	4 (40)	6 (60)*	–	–	–	–
AdPb	–	–	2 (33.3)	4 (66.6)*	1 (20)	4 (80)**
FrPbA2	–	–	–	–	2 (28.6)	5 (71.4)**
Control Sr	10 (100)	0 (0)	6 (100)	0 (0)	6 (100)	0 (0)
AbSr	7 (53.8)	6 (46.1)*	6 (75)	2 (25)	1 (12.5)	7 (87.5)**
SrTx1	–	–	–	–	3 (50)	3 (50)

(**) $p < 0.01$ and (*) $p < 0.05$: two-sided Fisher exact test. Control Pb: control animals used in *P. bistriata* experiments. Control Sr: control animals used in *S. raptoria* experiments. S: presence of tonic-clonic seizure. NS: no presence of tonic-clonic seizures. –: Not determined. Numbers in parentheses are the percentage of animals.

Table 2. Number of animals dead (D) or not dead (ND) after BIC, PICRO or PTZ injection.

	BIC		PICRO		PTZ	
Group	D	ND	D	ND	D	ND
Control Pb	9 (81)	2 (19)	7 (100)	0 (0)	–	–
AdPb	1 (20)	4 (80)*	–	–	–	–
BdPb	–	–	0 (0)	6 (100)*	–	–
FrPbA2	2 (28.6)	5 (71.4)**	–	–	–	–
Control Sr	6 (100)	0 (0)	6 (100)	0 (0)	13 (100)	0 (0)
AbSr	0 (0)	7 (100)**	2 (25)	6 (75)**	5 (38.5)	8 (61.5)**
SrTx1	2 (33.3)	4 (66.6)$^{\#}$	–	–	–	–

(**) $p < 0.01$ and (*) $p < 0.05$: two-sided Fisher exact test. ($^{\#}$) $p < 0.05$: one-sided Fisher exact test. Control Pb: control animals used in *P. bistriata* experiments. Control Sr: control animals used in *S. raptoria* experiments. D: number of dead animals. ND number of animals that did not die. –: Not determined. Numbers in parentheses are the percentage of animals.

group, the incidence of tonic-clonic seizures decreased significantly in several treatments. For PTZ, BdPb (60%) and AbSr (46.1%) were effective; after PICRO injection, AdPb (66.6%); for BIC, AdPb (80%), FrPbA2 (71.4%) and AbSr (87.5%) were effective (Table 1). Nevertheless, AbSr (25%) and SrTx1 (50%) protection was not statistically significant after PICRO and BIC injections, respectively (Table 1). Some treatments also reduced the mortality of experimental animals. With PTZ, AbSr was effective (61.5%); with PICRO, BdPb (100%) and AbSr (75%); with BIC, AdPb (80%), FrPbA2 (71.4%), AbSr (100%) and SrTx1 (66.6%) were effective (Table 2).

GABA uptake assays

The isolated fraction SrTx1 inhibited significantly GABA uptake in rat synaptosomal preparations. The percentage of inhibition at doses of 0.0009, 0.0018, 0.0036, 0.0072, and 0.0144 mg protein/mL were 36.0, 48.9, 46.5, 49.3 and 70.3%, respectively (Fig. 1A). Also, FrPbA2 inhibited significantly GABA uptake. The percentage of inhibition at doses of 2.05, 20.5, 41.0, and 82.0 AU were 26.0, 25.0, 36.0 and 38%, respectively (Fig. 1B). Synaptosomal integrity was considered viable due to the absence of LDH activity (data not shown).

Discussion

In this study, we present the anticonvulsant effects of denatured crude venoms from *P. bistriata* and *S. raptoria* spiders, and two isolated fractions (FrPbA2 and SrTx1, respectively) in acute GABAergic models of seizure induction. Also, FrPbA2 and SrTx1 inhibited significantly GABA uptake in the synaptosomal preparation.

One possible interpretation for this effect is that increasing the availability of the neurotransmitter GABA by an

effective blockade of neuronal or glial GABA uptake, venoms could have overcome the antagonists. So, it could be expected that venoms should be more effective in protecting animals from competitive GABA antagonists (e.g., BIC) in contrast to uncompetitive ones (e.g., PICRO). This hypothesis could explain why AdPb protected only 66.6% of the animals injected with PICRO, in contrast to 80% of animals injected with BIC. AbSr was effective in seizures and death protection only in rats that received BIC and PTZ, being inefficient with the PICRO group (Table 1). Notably, the biochemical anticonvulsant mechanism of these spider venoms and isolated fractions is not thoroughly understood and requires future investigation. As a further follow-up to these preliminary studies, specific GABAergic transporter blockers (e.g., tiagabine, nipecotic acid, and guvacine) could be tested. The pharmacological interpretation that drugs capable of neuronal or glial GABA uptake blocking could be used as antiepileptic agents has been extensively studied (Krogsgaard-Larsen et al., 2000).

Spider venoms are comprised mainly of proteins and polypeptides, and also low molecular weight components, such as acylpolyamines (Usherwood & Blagbrough, 1991; McCormick & Meinwald, 1993; Uchitel, 1997). One possibility is that the active anticonvulsant agents from *P. bistriata* and *S. raptoria*, described in the present work, could be acylpolyamines. Jackson and Parks (1990) demonstrated that acylpolyamines (AG2) isolated from *Agelenopsis aperta* protected rats against PICRO-, BIC- and kainic acid-induced seizures. Also, it was described that acylpolyamines paralyzes insects (Usherwood, 1994), and previous results from our laboratory showed that BdPb (Fontana et al., 2000) and the crude *S. raptoria* venom (unpublished observations) paralyzes termites. However, the presence of acylpolyamines in *P. bistriata* and *S. raptoria* spider venoms and its relation with the anticonvulsant effect requires additional investigations.

The mechanism of action expected for acylpolyamines should, as suggested by the present work, be interaction with GABA transporters rather than GABA receptors. The anion selective ion channel gated by a recombinant GABA (A) receptor is not blocked by an acylpolyamine isolated from venom of *Philanthus triangulum* (Jackson & Usherwood, 1988), and this ion channel is rather inhibited allosterically by an acylpolyamine (Brackley et al., 1990). It is described that mammalian endogenous polyamines (putrescine, spermidine and spermine) inhibit 15 to 40% of the GABA uptake on rat brain synaptosomes (Law et al., 1984).

The synaptosomal preparation was important in helping purification steps, selecting the active fractions to be tested in the anticonvulsant experiments. Moreover, it helped in the partial elucidation of the mechanism of action of FrPbA2 and SrTx1. Using this methodology, our group recently demonstrated inhibition of GABA uptake by the wasp *Agelaia vicina* venom (Pizzo et al., 2000).

The preliminary anticonvulsant results with *P. bistriata* and *S. raptoria* venoms and isolated fractions deserve further study, due to the need for of new anticonvulsant drugs and pharmacological tools.

In conclusion, the isolated fractions FrPbA2 and SrTx1 apparently exhibited an anticonvulsant effect *in vivo* and GABA uptake inhibition *in vitro*. To our knowledge, this is the first study describing an anticonvulsant effect of the *P. bistriata* and *S. raptoria* venoms.

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