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Neurochemical Characterization of a Neuroprotective Compound from *Parawixia bistriata* Spider Venom That Inhibits Synaptosomal Uptake of GABA and Glycine[S]

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ABSTRACT

The major contribution of this work is the isolation of a neuroprotective compound referred to as 2-amino-5-ureidopentanamide (FrPbAII) (M_r = 174) from *Parawixia bistriata* spider venom and an investigation of its mode of action. FrPbAII inhibits synaptosomal GABA uptake in a dose-dependent manner and probably does not act on Na^+, K^+, and Ca^{2+} channels, $GABA_B$ receptors, or γ-aminobutyrate:α-ketoglutarate aminotransferase enzyme; therefore, it is not directly dependent on these structures for its action. Direct increase of GABA release and reverse transport are also ruled out as mechanisms of FrPbAII activities as well as unspecific actions on pore membrane formation. Moreover, FrPbAII is selective for GABA and glycine transporters, having slight or no effect on monoamines or glutamate transporters. According to our experimental glaucoma data in rat retina, FrPbAII is able to cross the blood-retina barrier and promote effective protection of retinal layers submitted to ischemic conditions. These studies are of relevance by providing a better understanding of neurochemical mechanisms involved in brain function and for possible development of new neuropharmacological and therapeutic tools.

GABA is the most predominant inhibitory neurotransmitter in the mammalian central nervous system (Hendry et al., 1987). In addition, it has been well established that a detailed understanding of the GABA pathways and new approaches to identify targets and drugs for the treatment of neural diseases are of relevance (Wong et al., 2003; Beleboni et al., 2004a).

After endogenous synthesis, the buildup of GABA in synaptic vesicles is directed by a Na^+-independent proton-electrochemical gradient generated at the cost of an H^+-ATPase transporter (Christensen et al., 1991). Release of GABA occurs either by classic Ca^{2+}-dependent exocytosis or through a Ca^{2+}-independent mechanism probably involving reverse transport of the neurotransmitter (Agostinho et al., 1994). Once released, GABA acts through specific receptors localized in pre- and postsynaptic membranes, which may be classified as ionotropic ($GABA_A$ and $GABA_C$) or metabotropic ($GABA_B$) receptors (Bormann, 2000; Bowery et al., 2002).

The physiological GABA concentrations in synaptic cleft are maintained by neuronal and glial membrane transporters. GABA transporters are proteins of the Na^+- and Cl^--dependent transporter family, composed of several subfamilies such as the choline, monoamine, taurine, glycine, and betaine amino acid transporters (Worrall and Willians,

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ABBREVIATIONS: FrPb, *Parawixia bistriata* fraction; HPLC, high-performance liquid chromatography; TTX, tetrodotoxin; TEA, tetraethylammonium; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; MS, mass spectrometry; GABA-T, GABA:α-ketoglutarate aminotransferase (EC 2.6.1.19); ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglionar cell layer; LDH, lactic acid dehydrogenase; CNS, central nervous system; m.u., mass units; NO-711, 1-[2-[[(diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3 pyridinecarboxylic acid hydrochloride.

1994). Up to now, the existence of four subtypes of GABA transporters has been postulated. The nomenclature of these proteins is not clear, varying according to the species from which they were cloned: mice, rats, and humans (Sarup et al., 2003).

Spider venoms are useful sources of bioactive molecules and show a wide range of pharmacological effects on synaptic transmission. Neurotoxins from these venoms are evolutionary products that could function to immobilize prey or also serve in self-defense. Several of these molecules show high affinity for ion channels, receptors, and transporters in invertebrates and vertebrates (for review, see Beleboni et al., 2004b). These molecules represent a rich source of useful probes for understanding of synaptic transmission events, for identifying insecticide targets, and as aids for the designing of novel drugs for the treatment of neurological disorders (Harvey et al., 1998; Escoubas et al., 2000).

Parawixia bistriata is a social spider found in the South American "cerrados". The injection of its venom produces irreversible and dose-dependent paralysis in termites (Fontana et al., 2000). Intracerebroventricular injection of the venom as well as of its more purified fractions (including *P. bistriata* fraction AII; FrPbAII), abolishes convulsive tonic-clonic seizures induced by picrotoxin, bicuculline, and pentylenetetrazole in rats (Cairrão et al., 2002). In addition, a previous report has shown that a highly purified component of the venom (*P. bistriata* toxin 1.2.3) (M_r = 437) enhances glutamate uptake by a mechanism that seems to be independent from glutamate receptor activation. In addition, *P. bistriata* toxin 1.2.3 prevents neuronal death during retinal ischemia by enhancing glutamate clearance (Fontana et al., 2003).

Considering the recognized relevance of GABA neurotransmission as well as of spider venoms as a rich source of bioactive substances, the major aims of this work were to identify a novel neuroprotective compound from *P. bistriata* spider venom, FrPbAII, and to investigate its mode of action in synaptosomes. According to our results, FrPbAII could serve as a basis for designing of therapeutic drugs that decrease GABA clearance, hence decreasing the neuronal damage.

Materials and Methods

Materials. Methanol, acetonitrile, and trifluoroacetic acid were of analytical grade, purchased from Merck (Darmstadt, Germany), and deuterated methanol was purchased from Acros Organics (Geel, Belgium). HPLC columns were from Shimadzu Techno-Research, Inc. (Kyoto, Japan), and Millipore filters (0.45-μm porosity) from Millipore (São Paulo, Brazil).

[^{3}H]GABA (89 Ci/mmol), [^{3}H]glycine (23 Ci/mmol), [^{3}H]dopamine (10 Ci/mmol), [^{3}H]serotonin (122 Ci/mmol), [^{3}H]noradrenaline (15 Ci/mmol), and L-[G-^{3}H]glutamate (49 Ci/mmol) were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Reagents for Krebs-phosphate buffer, tetrodotoxin (TTX), α-ketoglutaric acid, and unlabeled neurotransmitters were from SigmaAldrich (St. Louis, MO). Nipecotic acid, tetraethylammonium (TEA), and baclofen were from Sigma/RBI (Natick, MA).

3,5-Diaminobenzoic acid was from Acros Organics (Fairlawn, NJ), and thiopental was from Cristalia Produtos Químicos Farmacêuticos Ltda. (São Paulo, Brazil). Scintillation cocktail ScintiVerse was obtained from Fisher Scientific (Loughborough, Leicestershire, UK). Solutions for histological analyses and all other reagents were from Reagen (Rio de Janeiro, Brazil), Vetec (Rio de Janeiro, Brazil), or Merck.

Spider Collection and Preparation of Venom Extract. *P. bistriata* specimens were collected in the region of Ribeirão Preto, São Paulo State, Brazil. Upon arrival in the laboratory, spiders were frozen and stored at −20°C. The venom sacs were removed, crushed in Milli-Q water at 0–4°C, and the extract boiled for 10 min. Then, the venom extract was cleared by centrifugation at 3000*g* for 10 min, and the supernatant was lyophilized and weighted.

Fractionation of the Venom Extract by Reverse Phase HPLC. HPLC procedures was performed on a Shimadzu LC-6A apparatus with ultraviolet detector SPD-6AV coupled with an autoinjector (SIL-10ADvp; Shimadzu, Kyoto, Japan) or on a Shimadzu LC-6AD apparatus with a diode array detector (SPD-M10Avp; Shimadzu), associated with an autoinjector (SIL-10AF; Shimadzu), both using the CLASS-VP 6.14 software (Shimadzu).

FrPbAII was obtained to homogeneity by two chromatographic steps carried out at room temperature. In the first, Milli-Q water (solvent A) and acetonitrile (solvent B) were degassed before use. The lyophilized venom extract was dissolved in Milli-Q water (40 mg/ml), filtered on Millipore filters, and applied onto a reverse phase HPLC column (PREP-ODS 20 × 250 mm; 5 μm) previously equilibrated with 1% (v/v) of solvent B. This sample was eluted by a linear gradient from 1 to 100% of solvent B (v/v) with a hold of 5 min at 20% (v/v) of solvent B. The flow rate was 8.0 ml/min, and elutes were continuously monitored at 215 nm. Seven fractions were collected in ice bath, lyophilized, dissolved in Krebs-phosphate buffer, and assayed for effects on synaptosomal GABA uptake, as described below.

Next, the active fraction referred to as to FrPbAI (*P. bistriata* fraction AI; retention time of 7.25 min) (1.37 mg/ml) was chromatographed on a Shim-pack CLC-C8 (M) (4.6 × 250 mm; 5 μm) analytical column, coupled to a precolumn Shim-pack, CLC G-C8 (4 × 10 mm), after an isocratic profile of Milli-Q water/methanol/trifluoroacetic acid [99:1:0.1 (v/v)] for 8 min at a flow rate of 1.0 ml/min. Again, eluates were continuously monitored at 215 nm. Five eluted fractions were collected in ice bath, lyophilized, dissolved in Krebs-phosphate buffer, and that fraction able to inhibit synaptosomal GABA uptake referred to as FrPbAII (retention time of 4.1 min).

Electrospray Mass Spectrometry. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was performed on a Quattro-LC instrument (Micromass, Manchester, UK). High-resolution quadrupole time-of-flight ESI-MS spectrum was acquired on an UltrOTOF apparatus (Bruker Daltonics, Billerica, MA). Solutions were infused into the ESI source using a model 1746 (Harvard Apparatus, Holliston, MA) syringe pump, at a flow rate of 10 μl/min. Collision-induced dissociation was performed on the isolated protonated molecule using argon as collision gas.

Nuclear Magnetic Resonance. ^{1}H and two-dimensional heteronuclear multiple quantum coherence, heteronuclear multiple bond correlation, and correlation spectroscopy ^{1}H-^{1}H NMR spectra were recorded at 500 MHz on a Bruker Avance DRX-500. Chemical shifts (δ) were referenced to tetramethylsilane signal.

Preparation of Synaptosomes. Male Wistar rats (200–250 g) were bred at University of São Paulo (Ribeirão Preto/São Paulo, Brazil). Animals were kept on a 12:12-h light/dark cycle, at room temperature, and supplied with food and water ad libitum. Animals were decapitated without the use of anesthetics. All procedures followed the guidelines established by the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Every effort was made to avoid unnecessary stress and pain to the experimental animals.

Cerebral cortex (for GABA, glutamate, glycine, or noradrenaline uptake or release assays), retina (for GABA and glycine uptake assays), and hippocampus or striatum (for serotonin and dopamine uptake assays, respectively) were used to prepare synaptosomes as described previously by Gray and Whittaker (1962). Synaptosomes were resuspended in Krebs-phosphate buffer (124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 0.75 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM

Na_2HPO_4, and 10 mM glucose, pH 7.4) and centrifuged for 20 min at 4°C. Protein content was determined according to Lowry et al. (1951), modified by Hartree (1972).

Neurotransmitter Uptake Assays. Synaptosomes were resuspended in Krebs-phosphate buffer and preincubated for 10 min at 25°C (for GABA uptake assays), for 10 min at 37°C (for glutamate, serotonin, dopamine, noradrenaline uptake assays), and for 15 min at 30°C (for glycine uptake assays) in the absence or presence of increasing concentrations of venom extract or fractions obtained after each chromatographic step. In the retina synaptosomes assays, $MgCl_2$ (1 mM, final concentration) was added to 0.32 M sucrose, during retina enucleating and homogenization, or in Krebs-phosphate buffer.

Uptake assays were initiated, in each case, by adding radiolabeled neurotransmitters (10 nM [^{3}H]GABA, 250 nM [^{3}H]glycine, 5 nM [^{3}H]dopamine, 5 nM [^{3}H]serotonin, 50 nM [^{3}H]noradrenaline, or 100 nM [^{3}H]L-glutamate; all at final concentration) to synaptosomal suspensions (100 μg of protein/ml final concentration), and incubated for 3 min (for GABA and glutamate assays), 5 min (for noradrenaline assays), 10 min (for dopamine and serotonin assays), or 15 min (for glycine assays) at the above-specified temperatures. The time and temperature of incubation were adjusted for 5 min at 25°C, for GABA or glycine uptake assays, when rat retina synaptosomes were used. The uptake rate measured under these conditions was found to be linear over time and amount of tissue used. For [^{3}H]dopamine, [^{3}H]serotonin, and [^{3}H]noradrenaline, ascorbic acid and pargyline (1.7 mM and 80 μM, final concentrations, respectively) were added to Krebs-phosphate buffer to avoid monoamines oxidation or degradation.

To evaluate FrPbAII effects on synaptosomal GABA uptake in the presence of agents that alter $GABA_B$ receptor and ion channel activities, synaptosomes from rat cerebral cortex (100 μg of protein/ml) were incubated for 3 min at 25°C, with 10 nM [^{3}H]GABA in the presence or absence of 24 μg/ml FrPbAII; and at final concentrations of 5 μM TTX, 1.0 mM $CdCl_2$, 5.0 mM TEA, or 0.1 mM baclofen.

Incubations were carried out in triplicate, and reactions were interrupted by centrifugation (3000*g* for 3 min at 4°C). Supernatants were discarded; pellets were washed twice with ice-cold distilled water, homogenized in 10% tricloroacetic acid, and centrifuged at 3000*g* for 3 min at 4°C. Aliquots of supernatants were transferred to scintillation vials containing 5 ml of the biodegradable scintillation cocktail ScintiVerse, and their radioactivity was quantified in a scintillation counter (Beckman LS-6800) with a counting efficiency of 35 to 40% for ^{3}H. Nonspecific uptake was estimated in parallel probes with nipecotic acid (6 mM, final concentration) (for GABA assays) and using nonlabeled neurotransmitters (1 mM, final concentrations for glutamate and glycine) or low temperature (0–4°C for all other assays); values obtained were subtracted from those of the total uptake.

Dose-response curves were fitted to the Hill equation in nonlinear regression analyses using Prism software (ver. 3.02 for Windows; GraphPad Software Inc., San Diego, CA). Results were expressed as averaged percentage of control uptake values with S.E.M. Statistical significance was assessed using Student's t test; $*$, $p < 0.05$ values were considered significant.

Saturation curves were performed as described above, in the presence of increasing concentrations of unlabeled GABA (4.5 nM to 10 μM, final concentration). The kinetic values K_M and V_{max} for synaptosomal GABA uptake were obtained by means of Michaelis-Menten curves. All data are presented as means with S.E.M. In brief, high-affinity GABA uptake assays were initiated by the [^{3}H]GABA (10 nM, final concentration) to synaptosomes (100 μg of protein/ml, final concentration) in triplicate. Statistical analyses of V_{max} and K_M values, obtained in each experiment in the presence or absence of FrPbAII (24 μg/ml, final concentration), were performed using Student's t test for paired data ($*$, $p < 0.05$).

GABA Release Assays. Synaptosomes (3 mg/ml) were preloaded with 0.5 μM [^{3}H]GABA in Krebs-phosphate buffer for 20 min at 25°C. Samples were centrifuged for 3 min at 7200*g* at 4°C, and pellets were washed three times with ice-cold buffer. To assess neurotransmitter release, the final pellet was resuspended in Krebs-phosphate buffer and incubated for 3 min at 25°C in the absence or presence of increasing concentrations of FrPbAII. These concentrations are representative of those used to access the FrPbAII activity in GABA uptake assays. Neurotransmitter release was also measured in the presence of 50 mM KCl and 5 μM TTX, to verify the functional properties of synaptosomal preparations. Reactions were stopped by centrifugation (3000*g* for 3 min at 4°C), and aliquots of supernatants and pellets were separately transferred to scintillation vials containing 5 ml of biodegradable scintillation cocktail; radioactivity was quantified in a scintillation counter. The amounts of released GABA were calculated as percentage of control uptake average. Statistical analyses were performed using Student's t test ($*$, $p < 0.05$).

GABA:α-Ketoglutarate Aminotransferase (EC 2.6.1.19) Activity in the Presence of FrPbAII. To obtain whole brain homogenate preparations, rat brains were removed and homogenized in 5 ml of ice-cold water using a Potter-Elhvejen Labo-Stirrer LS-50-Yamato homogenizer. Protein content was determined by the Lowry method (Lowry et al., 1951), as modified by Hartree (1972). GABA-T activity was measured based on the rate of succinic semialdehyde formation, according to Salvador and Albers (1959). The incubation medium contained 10 mM α-ketoglutaric acid, 50 mM GABA, and 0.2 ml of brain homogenate (150–250 μg of protein/ml) in the presence or absence of 24 μg/ml FrPbAII in a final volume of 1 ml. Samples were incubated for 60 min at 38°C, cooled in an ice bath, and centrifuged for 15 min at 3000*g* at 4°C. Aliquots of 0.3 ml of supernatants were collected and added to 0.3 ml of a 3,5-diaminobenzoic acid solution (0.2 M; pH 6.0), and samples were heated for 60 min at 60°C. After these procedures, the samples were measured in Spectrofluorometer RS-540 (Shimadzu). The excitation and emission wavelength were set to 405 and 505 nm, respectively. For tissue blanks, the incubation at 38°C was omitted.

Effects of FrPbAII in Experimental Glaucoma Model. Male Wistar rats (230–250 g) were intraperitoneally anesthetized with thiopental (50 mg/kg), and their retinas were submitted to ischemia according to Louzada-Junior et al. (1992) and Fontana et al. (2003). Intraocular pressure was increased to 155 mm Hg, by cannulating in the anterior chamber of the eye, with a sterile 27-gauge needle attached to a manometer/pump and an air reservoir. Ischemia was induced for 60 min, after which intraocular pressure was reduced to normal levels for 45 min (reperfusion period). The left retina of each animal was subjected to the experimental conditions, ischemia (n = 5) and ischemia/reperfusion (n = 5), whereas the right retinas served as a nonischemic control (n = 10). Twenty-five microliters of FrPbAII (6 mg/ml) were i.v. injected 15 min before ischemia (n = 5) and ischemia/reperfusion (n = 5) and the right retinas (contralateral retinas) served as a nonischemic control (n = 10). The animals were then sacrificed, and the left and right eyes were rapidly enucleated and fixed in Bouin's solution (75% picric acid, 25% formalin, and 5% acetic acid) for 24 h. After fixation, cornea, aqueous humor, lens, and vitreous humor were removed and the eyecups were dehydrated in 70 to 100% ethanol and embedded in paraffin. Retinas were sectioned at 5 μm, approximately 1 mm from the emergency of the optic nerve, stained with hematoxylin and eosin, and examined using a Zeiss Axiophot microscope. For each experimental group, five microscopic fields (160× or 636 × 474 pixels) of one sagittal section at the superior retina were captured by light microscopy and digitalized with an analogic camera (JVC TK1270) connected to the microscope and a computer. A computer program KS 400 (Carl Zeiss Vision, Aalen, Germany) was used to quantify the cell counts manually (means of cells ± S.E.M.) in established areas (in square millimeters): outer nuclear layer (ONL; 47.16), inner nuclear layer (INL; 40.40), and ganglionar cell layer (GCL; 29.52). Significance of recorded differences between groups were determined using analysis of variance ($*$, $p < 0.05$). Qualitative analysis was performed to char-

acterize histological damage as decreases of cell numbers, cytoplasm vacuolization, edema, disorganization, and pyknotic nuclei.

Results

Effects of the Venom Extract and Purified Venom Fractions (FrPbAI and AII) on Synaptosomal GABA Uptake Are Dose-Dependent. Preliminary experiments assessing the effects of *P. bistriata* boiled crude venom on GABA uptake into cortical synaptosomes, indicated that this venom extract inhibits this process (Fontana et al., 2003). To further explore these observations, we examined the dose dependence of the venom extract on this system. Figure 1a shows the dose-response curve of spider venom extract on GABA uptake. The maximum inhibition (~98.20%) was obtained in the presence of 13,440 μg/ml; the IC_{50} was 1700 ± 130 μg/ml (final concentration).

After these early results, the aqueous extract of venom (40 mg/ml) was applied to a C18 column, resulting in the purification of the first active fraction, referred to as FrPbAI (retention time of 7.25 min) (data not shown). Figure 1b shows the dose-response curve of FrPbAI on GABA uptake. Maximum inhibition (~98.30%) was obtained in the presence of 1344 μg/ml; the IC_{50} was 100 ± 12 μg/ml (final concentration). FrPbAI (1.37 mg/ml) was chromatographed on an analytical C8 column, producing the second active fraction referred to as FrPbAII (retention time 4.1 min).

Figure 1c shows the dose-response curve of FrPbAII on GABA uptake. Maximum inhibition (~96.20%) was obtained in the presence of 320 μg/ml; the IC_{50} was 24 ± 0.019 μg/ml (final concentration). Therefore, IC_{50} values from the venom extract to the FrPbAII followed a profile of increasing affinity.

All other fractions obtained at each chromatographic step, which include both highly hydrophilic and hydrophobic components, were assayed for their ability to produce inhibition of GABA uptake, having slight or no effect on this system. Uptake assays were regularly accompanied in parallel by lactic acid dehydrogenase (LDH) activity measurement or morphological examination by electron microscopy. No morphological damage or increase in LDH level in the supernatants was detected, indicating that the synaptosomes maintained their integrity in the presence of the venom extract or its active fractions (data not shown).

Structural Elucidation of FrPbAII. FrPbAII was chemically identified as 2-amino-5-ureidopentanamide (Fig. 2) by NMR and ESI-MS analyses. Two multiplets (2 H each) at δ = 1.57 and 1.78 ppm and two triplets at δ = 3.09 (2 H, J = 6.8 Hz) and 3.70 ppm (1 H, J = 6.0 Hz) were observed in ^{1}H NMR. Together with correlation spectroscopy ^{1}H-^{1}H data, these results indicate a saturated carbon chain. Heteronuclear multiple bond correlation and heteronuclear multiple quantum coherence confirmed this part of the molecule and provided ^{13}C chemical shift data suggesting the presence of an amide carbonil (δ = 175.5 ppm) in one edge of the molecule. The other functional groups linked to the carbon chain were proposed based in chemical shifts of ^{1}H and ^{13}C provided by NMR spectra.

Positive ESI-MS scan of FrPbAII showed the most intense signal at m/z 175. ESI-MS/MS experiments undertaken with this peak confirmed that all other peaks present in ESI-MS spectrum were its daughters, formed by in source dissocia-

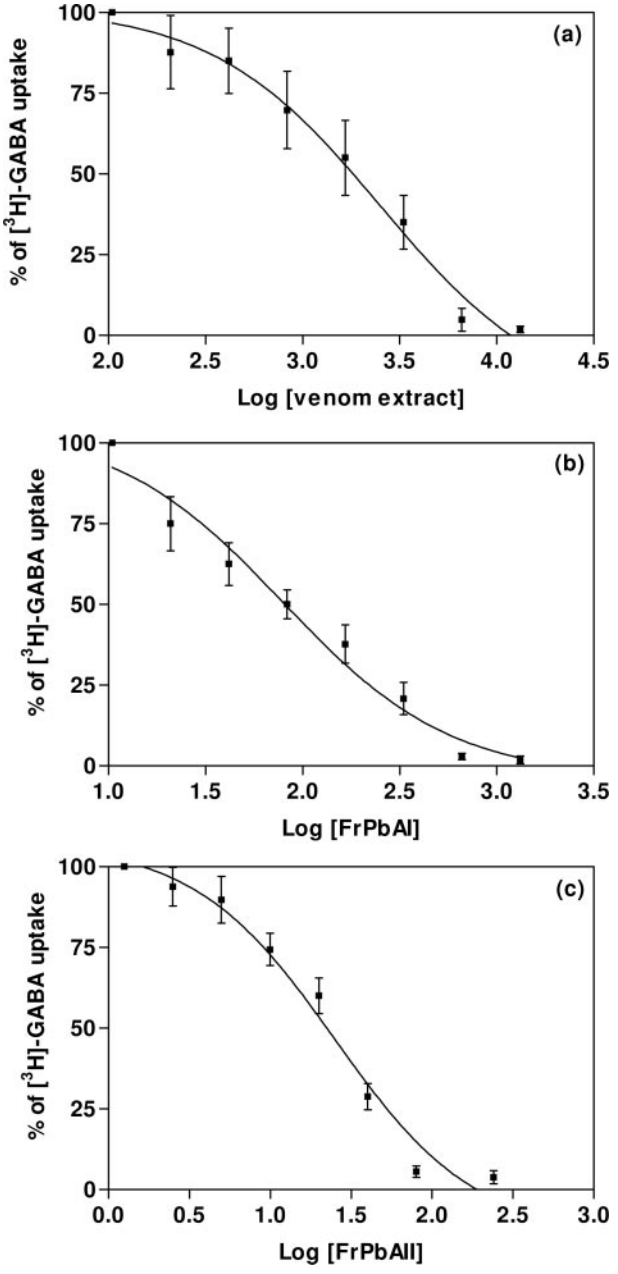

Fig. 1. Dose-response curves for the inhibitory effects of increasing concentrations of *P. bistriata* venom extract (a), FrPbAI (b), or FrPbAII (c) on [^{3}H]GABA uptake in synaptosomes from rat cerebral cortex. Uptake assays were initiated by adding [^{3}H]GABA (10 nM, final concentration). Synaptosomes were preincubated in the presence or absence of venom extract, FrPbAI, and FrPbAII at final concentrations of 210–13,440, 21–1344, and 2.5 to 320 μg/ml, respectively, for 3 min at 25°C. Concentrations of venom extract and fractions are plotted on a logarithmic scale. Higher concentrations than those shown also inhibit GABA uptake, but they were not included in the figure. Data from four to six independent experiments, performed in triplicate, generated an IC_{50} of 1700 ± 130, 100 ± 12, and 24 ± 0.019 μg/ml, respectively, for venom extract, FrPbAI, and FrPbAII in the GABA uptake assays.

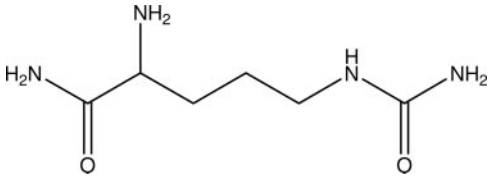

Fig. 2. Chemical structure of FrPbAII.

tion. Therefore, $[M + H]^+$ 175 gives us the M_r = 174 to FrPbAII. In ESI-MS/MS experiments, the following three competitive losses could be observed, which confirmed all the functional groups linked to the carbon chain: loss of NH_3 (17 m.u.), affording *m/z* 158; loss of $CONH_3$ (45 m.u.), giving *m/z* 130; or loss of $NHCONH_2$ (59 m.u.), yielding the more stable ion at *m/z* 116. High-resolution quadrupole time-of-flight ESI-MS analyses (see Supplemental Figure) afforded *m/z* 175.1182 for the protonated parent ion $[M + H]^+$. Together with NMR and MS/MS fragmentation data, this information confirmed the molecular formula $C_6H_{15}N_4O_2^+$ (4.6 ppm error) for $[PbFrAII + H]^+$, since no other reasonable molecular formula is possible within a 50 ppm error.

FrPbAII Does Not Affect Maximum Transport Velocity but Does Alter Apparent Transport Affinities. By measuring the synaptosomal GABA uptake over a range of substrate concentrations, the transport activity was shown to possess both saturability and high affinity. Figure 3 shows the alteration of apparent transport affinities evoked by 24 μg/ml FrPbAII (IC_{50}) (final concentration); it also shows that the V_{max} of GABA transport was not modified. The control values obtained for K_M and V_{max} were 1.17 ± 0.2 μM and 8.19 ± 0.3 pmol/min/mg, respectively. In the presence of FrPbAII, the K_M value was increased to 2.22 ± 0.2 μM and V_{max} was 8.15 ± 0.2 pmol/min/mg, a value very similar to that obtained in control. Therefore, K_M was increased, whereas V_{max} was not significantly changed, suggesting a competitive inhibition.

Uptake Inhibition Is Not Caused by Alterations in GABA Release or GABA-T Activity. Synaptosomes can also mediate GABA release in a Ca^{+2}-dependent manner, or by means of reverse transport, especially when GABA-T is inhibited (Bernath and Zigmond, 1988). To confirm that GABA release was not directly affected in our experiments, we measured the neurotransmitter release in the presence or absence of FrPbAII, at a representative range of concentrations used in the GABA uptake assay. Figure 4 shows the effect of FrPbAII (20–320 μg/ml, final concentration) on GABA basal release. Spontaneous release of GABA was not altered by FrPbAII. Control samples were incubated in the presence of 50 mM KCl or 5 μM TTX, to verify the functional integrity of preparation. As expected, basal GABA release as increased by KCl, and TTX did not alter the process (data not shown). In addition, our experiments measuring GABA-T activity in the presence of FrPbAII (24 μg/ml, final concentration) did not showed significant alterations (data not shown).

FrPbAII Effects Are Maintained Even in the Presence of Ion Channel Inhibitors or a $GABA_B$ Receptor Agonist. Figure 5 shows that the inhibition of synaptosomal GABA uptake caused by 24 μg/ml FrPbAII (approximately 50%) is maintained even when the experiment was performed using ion channels inhibitors (5 μM TTX, 1.0 mM $CdCl_2$, and 5.0 mM TEA, final concentrations) or a $GABA_B$ receptor agonist (0.1 mM baclofen, final concentration).

FrPbAII Is Selective for GABA and Glycine Systems. Monoamines and glycine transporters are homologous with GABA transporters; glutamate transporters are similar but also are involved in ischemic damage. To verify the selectivity of the action of FrPbAII, synaptosomal uptake of [^{3}H]glycine, [^{3}H]serotonin, [^{3}H]dopamine, [^{3}H]noradrenaline, and [^{3}H]L-glutamate was studied in the presence or absence of increasing concentrations of FrPbAII (20–320 μg/ml), at same range used in the [^{3}H]GABA uptake in this assay. Figure 6 shows that FrPbAII is a selective inhibitor of [^{3}H]GABA and [^{3}H]glycine uptake, having slight or no effect on other homologous or glutamate transporters.

Neuroprotective Effects of FrPbAII in Experimental Glaucoma. To investigate the effect of FrPbAII in vivo, rat retinas were submitted to experimental glaucoma. In these experiments, rats received i.v. injections of 25 μl of FrPbAII (6 mg/ml) or saline 15 min before ischemic treatment. Figure 7 shows micrographs of nonischemic control retinas, subjected to ischemia or to ischemia followed by reperfusion. Marked histological alterations could be observed when com-

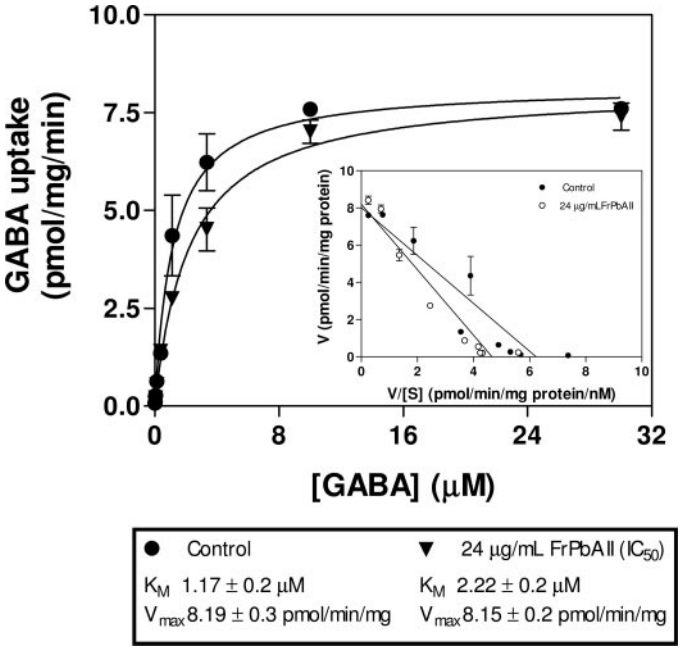

Fig. 3. Kinetic analysis of high-affinity GABA uptake by synaptosomes preincubated in absence (■) or presence (▼) of IC_{50} of FrPbAII (24 μg/ml, final concentration). Uptake was measured in the presence of increasing concentrations of unlabeled neurotransmitter (from 4.5 nM to 30 μM, final concentration) and [^{3}H]GABA (10 nM, final concentration). Data are means ± S.E.M. of four independent experiments, performed in triplicate. Inset, Eadie-Hofstee plot.

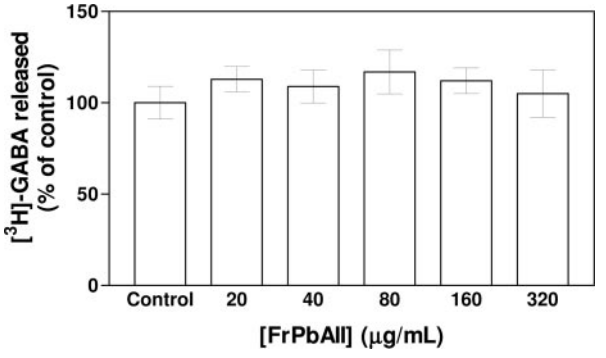

Fig. 4. Effects of final increasing concentrations of FrPbAII on [^{3}H]GABA release. Synaptosomes were preloaded with 0.5 μM [^{3}H]GABA for 20 min at 25°C. Release, expressed as percentage of neurotransmitter released over the control, was initiated by the addition of FrPbAII from 20 to 320 μg/ml. No significant difference was observed between control and experimental treatments (*, $p < 0.05$). Each column is a mean ± S.E.M of three independent experiments, performed in triplicate.

paring nonischemic control (Fig. 7a), ischemic (Fig. 7b), and ischemic/reperfused retinas (Fig. 7c).

Compared with nonischemic control retinas, ischemic retinas (Fig. 7b), showed cytoplasm vacuolization, pyknotic nuclei, and a decrease in cell number in the GCL. The INL displayed greater edema, pyknotic nuclei, and cellular disorganization. The ONL exhibited decreased cell number, compared with control retinas. Edema was also observed in the inner plexiform layer.

In the ischemia/reperfusion retinas (Fig. 7c), the GCL showed lower cell density and increased vacuolization and number of pyknotic nuclei as well as cellular disorganization. The INL also had fewer cells, and those remaining had a greater amount of pyknotic nuclei and cytoplasm vacuolization as well as enhanced edema and cellular disorganization. There were also fewer cells in the ONL.

Compared with ischemic and ischemic/reperfused retinas (Fig. 7, b and c), retinas of animals treated with FrPbAII (Fig. 7, d–f) displayed normal cellular morphology and a remarkable reduction in cell death in all layers. Figure 7d represents another control, a nonischemic retina of animal treated with FrPbAII that showed no signs of cell damage. Figure 7e shows ischemia-induced retinas treated with FrPbAII. No degeneration, pyknotic nuclei, or disorganization of the cells but some cell loss was observed. Figure 7f shows ischemic/reperfused retinas treated with FrPbAII, where protection was observed in all layers, and there was a decrease in cell loss.

The numbers of cells in control, ischemic, and ischemic/reperfused retinas with or without treatment with FrPbAII are presented in Table 1. There was a decrease in number of cells in all layers after ischemia and in those that were reperfused after ischemia. The cell numbers were significantly decreased by 38% in the ONL, 54% in the INL, and 51% in the GCL after ischemia and were decreased by 40% in the ONL, by 63% in the INL, and 58% in the GCL in the

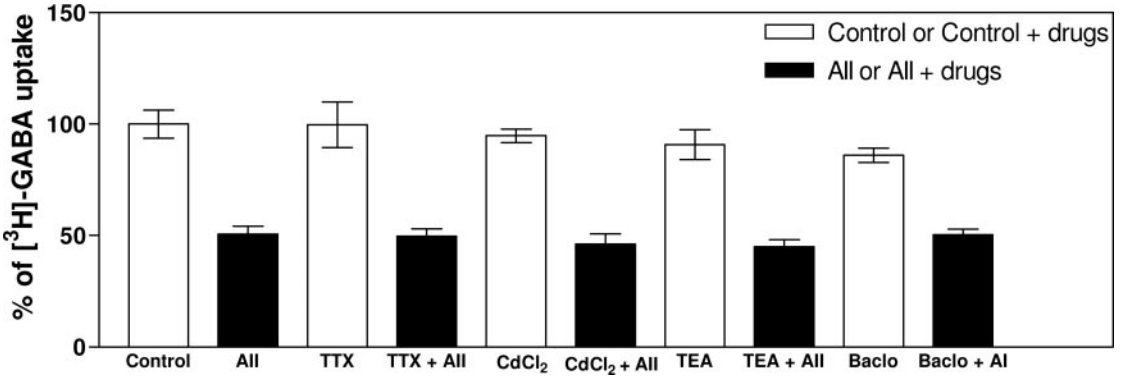

Fig. 5. GABA uptake inhibition caused by 24 μg/ml FrPbAII (approximately of 50%) is maintained even in presence of Na^+ (5 μM TTX), Ca^{2+} (1 mM $CdCl_2$), and K^+ (5 mM TEA) channels inhibitors or the $GABA_B$ receptor agonist baclofen (0.1 mM) at final concentration. Each column is a mean ± S.E.M of five independent experiments, performed in triplicate. AII, FrPbAII; Baclo, baclofen.

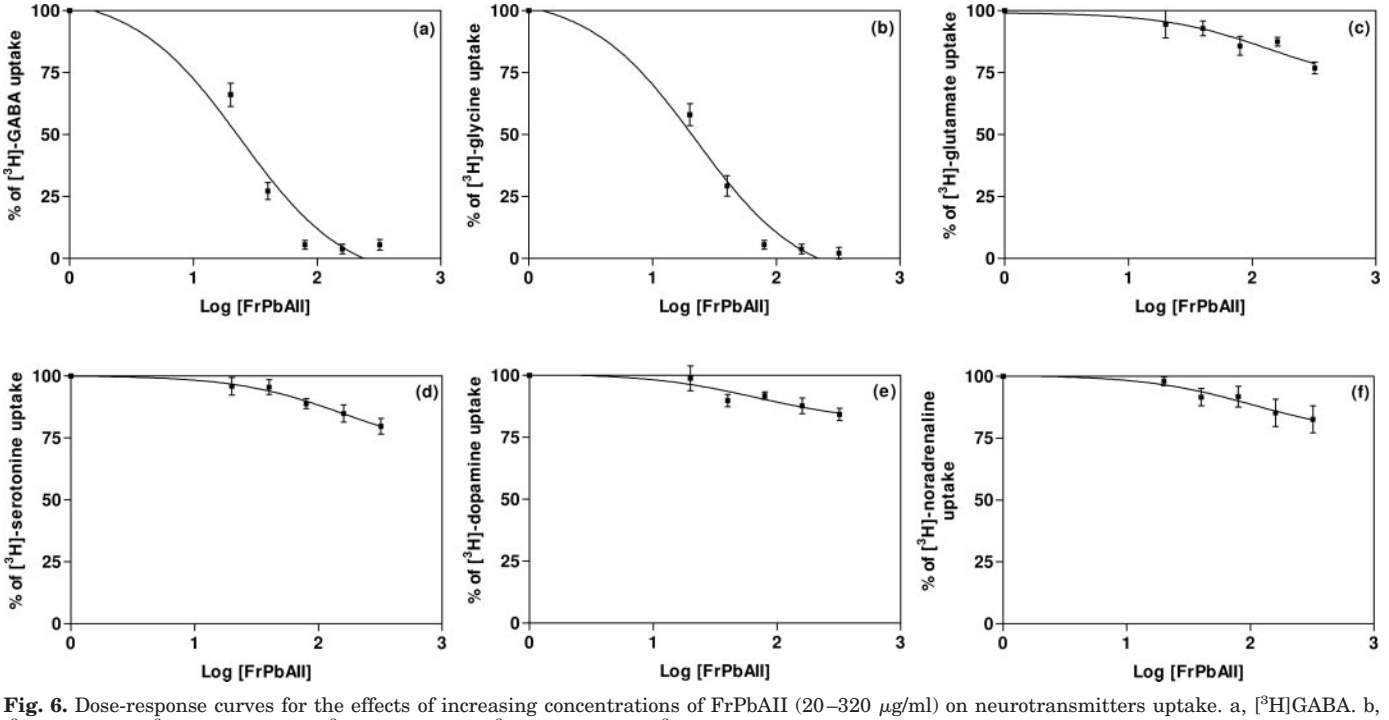

Fig. 6. Dose-response curves for the effects of increasing concentrations of FrPbAII (20–320 μg/ml) on neurotransmitters uptake. a, [^{3}H]GABA. b, [^{3}H]glycine. c, [^{3}H]glutamate. d, [^{3}H]serotonin. e, [^{3}H]dopamine. f, [^{3}H]noradrenaline uptake. Data are means ± S.E.M. of three independent experiments, performed in triplicate.

retinas subjected to ischemia/reperfusion compared with the nonischemic control retinas (*, $p < 0.05$). In contrast, retinas of animals treated with FrPbAII, showed significant protection of 27% in ONL, 57% in INL, and 50% in GCL after ischemia and of 20% in ONL, 81% in INL, and 44% in GCL in ischemia/reperfusion compared with ischemic and ischemic/reperfusion conditions (*, $p < 0.05$), respectively (Table 1).

FrPbAII Is Also Able to Inhibit GABA and Glycine Uptake in Synaptosomes from Rat Retinas. Synaptosomes from rat retina were used to verify the direct correlation between neuroprotection and inhibition of GABA and glycine uptake caused by FrPbAII. In these experiments, FrPbAII also caused a dose-dependent inhibition of neurotransmitter uptake (Fig. 8), as it was demonstrated by our assays using synaptosomes from rat cerebral cortex (Fig. 1c). IC_{50} values for FrPbAII in GABA and glycine uptake were 13.80 and 13.10 μg/ml, respectively.

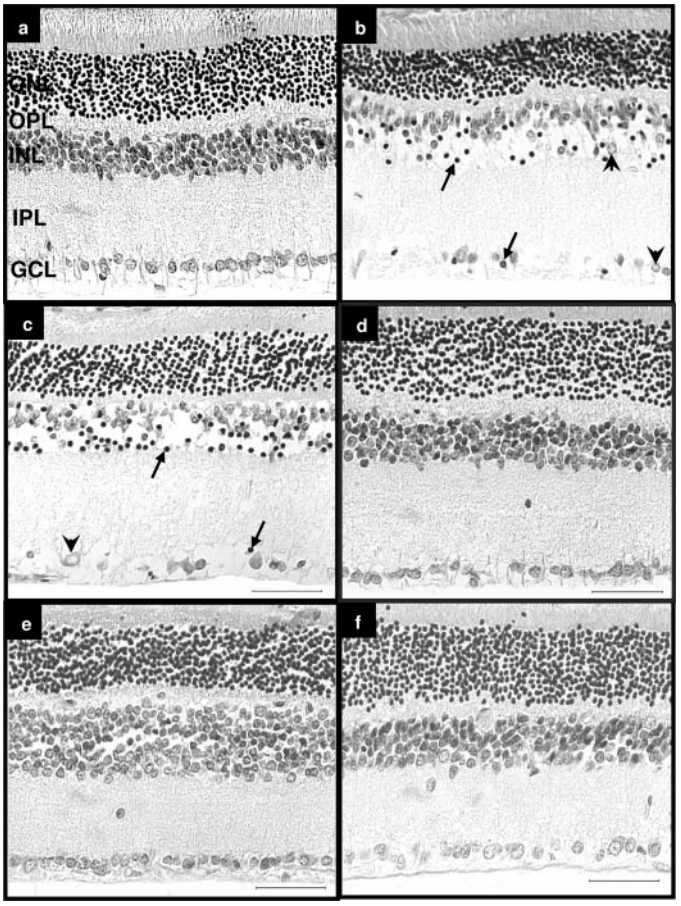

Fig. 7. Effects of the intravenous injection of 25 μl of FrPbAII (6 mg/ml) in ischemic and ischemic/reperfused rat retinas. Retinal sections were stained with hematoxylin and eosin. Arrowheads denote areas of vacuolization and arrows, pyknotic nuclei. a, control. b, ischemic. c, ischemic/reperfused. d, control retina of animal pretreated with FrPbAII. e, ischemic pretreated with FrPbAII. f, ischemic/reperfused pretreated with FrPbAII. IPL, inner plexiform layer; and OPL, outer plexiform layer. Scale bars, 50 μm.

Discussion

In this work, we show the novel neuroprotective compound from *P. bistriata* venom extract (FrPbAII) inhibits synaptosomal GABA uptake in a dose-dependent manner (Fig. 1c) and probably by a competitive antagonism (Fig. 3). In addition to this neuroprotective property, FrPbAII has previously been reported as a significant anticonvulsant against seizures induced in rats (Cairrão et al., 2002).

Synaptosomes are recognized as a useful model to neurochemical studies because they retain all machinery for the uptake, storage, and release of neurotransmitters and for ionic conductance (Gray and Whittaker, 1962; Bicalho et al., 2002, Wang and Sihra, 2003). In our studies, morphological examination of the synaptosomal preparations by electron microscopy demonstrated that membrane integrity was maintained, and levels of LDH were not changed when synaptosomes were incubated with either venom extract, FrPbAI, or FrPbAII (results not shown). These results indicate that the observed uptake inhibition was not due to plasma membrane disruption or pore formation.

Synaptosomes mediate residual GABA release during the uptake process. If FrPbAII was elevating basal release, it could enhance the unlabeled/labeled GABA ratio in the assay buffer producing an apparent and unreal decrease of neurotransmitter transport. Therefore, we also examined the effect of FrPbAII on GABA efflux. The synaptosomal release of neurotransmitter was stimulated by K^+ and was not altered by TTX, confirming the functional integrity of the preparation (data not shown). FrPbAII did not affect the levels of basal GABA release (Fig. 4), an indication that it does not act indirectly by altering tonic or depolarization-dependent GABA release or even via membrane pore formation.

$GABA_B$ receptors are present on neural terminals throughout the CNS, acting as autoreceptors when localized on the presynaptic membrane. In this case, their activation inhibits release of further synaptic vesicles through the suppression of high-threshold Ca^{2+} channels (Harayama et al., 1998).

Upon the uptake process, GABA can be catabolized by the action of GABA-T. The enzyme inhibition increases GABA concentration in the brain, especially by reverse transport, decreasing susceptibility to convulsions and epileptic conditions (Sherif and Ahmed, 1995). This process is distinguished from exocytotic release by being nonvesicular and indepen-

TABLE 1
Number of cells in retinal layers

	ONL	INL	GCL
		%	
Control	953 ± 43	259 ± 9	37 ± 4
Ischemia	590 ± 15 (38)[a]	119 ± 6 (54)[a]	18 ± 2 (51)[a]
Ischemia/reperfusion	574 ± 30 (40)[a]	96 ± 13 (63)[a]	16 ± 1 (58)[a]
FrPbAII/ischemia	748 ± 28 (27)[b]	186 ± 10 (57)[b]	27 ± 2 (50)[b]
FrPbAII/ischemia/reperfusion	688 ± 23 (20)[c]	174 ± 9 (81)[c]	23 ± 1 (44)[c]

[a] Percentage of cell losses compared with control retinas in nonischemic conditions.
[b] Percentage of decrease in cell losses with FrPbAII treatment compared with ischemic conditions (protection percentage).
[c] Percentage of decrease in cell losses with FrPbAII treatment compared with ischemic/reperfusion conditions (protection percentage).

dent of Ca^{2+} influx via selective voltage-dependent channels (Agostinho et al., 1994; Beleboni et al., 2004a).

If FrPbAII is able to inhibit GABA-T, K^+ channel activity, and $GABA_B$ receptor function or to enhance Na^+ or Ca^{2+} channel activity, these effects also could be responsible for an apparent and unreal decrease of neurotransmitter uptake, ruling out the possibility of a direct effect of FrPbAII on the GABA transporter.

To evaluate whether inhibition of GABA uptake by FrPbAII is mediated by an indirect mechanism through $GABA_B$ receptors or ion channels, we investigated whether channel and receptor agonists or antagonists could modify its effects on GABA uptake. These agents included TTX, a Na^+ channel blocker; cadmium chloride, a Ca^{2+} channel blocker; TEA, a K^+ channel blocker; and baclofen, a $GABA_B$ receptor agonist.

FrPbAII-inhibited GABA uptake was unchanged by the blockade of voltage-dependent Na^+ channels, Ca^{2+} or K^+ channels, when these blockers were tested at concentrations known to produce effective inhibition of channel activity (Fig. 5). Despite of its potential effects on neuronal GABA concentration, baclofen did not alter the ability of FrPbAII to inhibit GABA uptake into synaptosomes, suggesting that these effects are not dependent on $GABA_B$ receptor inhibition (Fig. 5). Moreover, GABA-T activity is maintained in the presence of FrPbAII, indicating that reverse transport is not involved in FrPbAII effects (data not shown). Therefore, FrPbAII effect cannot be directly dependent on Na^+, K^+, or Ca^{2+} channels, $GABA_B$ receptors, or GABA-T for its action. These results associated to those demonstrated in Fig. 4 rule out the possibility that FrPbAII could act by inhibition or activation of these structures, indicating its selectivity of action.

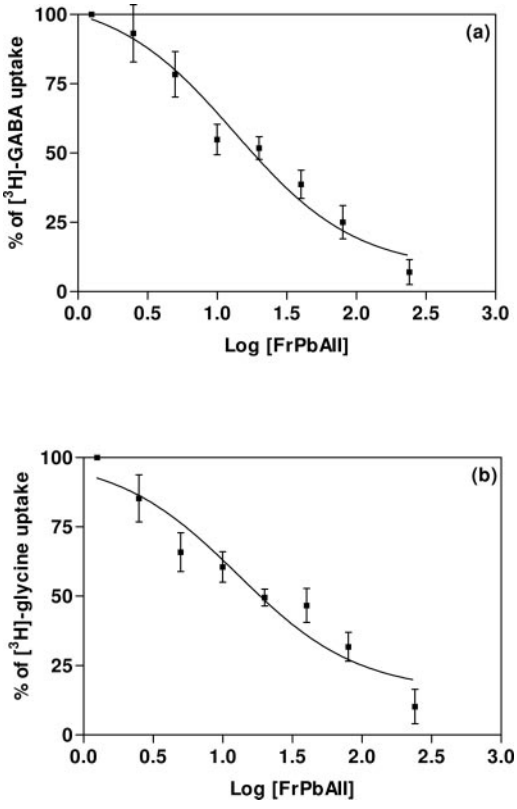

Fig. 8. Dose-response curves for the inhibitory effects of increasing concentrations of FrPbAII on [^{3}H]GABA (a) or [^{3}H]glycine (b) uptake in synaptosomes from rat retinas. Uptake assays were initiated by adding [^{3}H]GABA or [^{3}H]glycine (at, respectively, 10 and 250 nM final concentrations). Synaptosomes were preincubated for 3 min at 25°C in the presence or absence of FrPbAII at final concentrations of 2.5 to 320 μg/ml, the same range used in assays with synaptosomes from cerebral cortex. Concentrations are plotted on a logarithmic scale. Data from four independent experiments, performed in triplicate, generated an IC_{50} of 13.80 and 13.10 mg/ml, respectively, for FrPbAII in the GABA and glycine uptake assays.

As demonstrated in Fig. 6, FrPbAII is highly selective for GABA and glycine transporters, having slight or no effect on homologous (serotonin, dopamine, and noradrenaline) or glutamate transporters. These results reinforce the concept of a remarkable selectivity of FrPbAII, a desirable attribute for future development of a new therapeutic drug or pharmacological tool.

GABA and glycine are relevant inhibitory transmitters in retinal synapses, because it was well established in the CNS, and many reports suggest a heterogeneous distribution of GABA/glycine receptors and transporters in the vertebrate retina (Honda et al., 1995; Wässle et al., 1998; Gadea et al., 1999). The organization and accessibility of the retina has made it the best-characterized system for examining the physiology and function of amino acid transporters, and it is an excellent model to study the effects of drugs on ischemia/reperfusion in the CNS (Louzada-Junior et al., 1992; Eliasof et al., 1998).

It is well established that drugs acting on GABA and glycine uptake may provide an effective means for protecting the brain against neuronal injury and for treating epilepsy, as illustrated by the clinical use of tiagabine (Meldrum, 1997; Fisher and Bogousslavsky, 1998). Ichinose and Lukasiewicz (2002), using the selective GABA transporter-1 blocker NO-711, showed that blockade of GABA uptake resulted in increased activation of $GABA_C$ receptors. This compound has proved to be effective as an anticonvulsant in animal models and as a neuroprotector against ischemia of CA1 pyramidal neurons in gerbil (O'Connell et al., 2001). Thus, to obtain insight into the possible neuroprotective actions, consequent to inhibition of GABA and glycine uptake, we examined FrPbAII effects during retinal ischemia and ischemia followed by reperfusion, using the experimental glaucoma model. In our experiments, FrPbAII was found to protect neurons from injury in all retinal layers, particularly in the INL (Fig. 7; Table 1).

Thus, inhibition of GABA and glycine uptake by FrPbAII can increase GABA and glycine levels on the synaptic cleft, promoting an activation of their receptors. These processes hyperpolarize neurons, leading to a reduced transmitter release and/or action potential firing, effects that can act in the ischemic cascade by promoting a neuroprotective effect of the retina in the experimental glaucoma model. Similar conclusions could be taken to explain our previous results, indicating a marked anticonvulsant activity presented by FrPbAII (Cairrão et al., 2002).

The neuroprotective effects of FrPbAII are observed in both ischemic and ischemic/reperfused retinas. Louzada-Junior et al. (1992) have shown that there is a higher percentage of glutamate release, and subsequent neuronal death during the reperfusion period than during ischemia; nevertheless, we still observed protection when FrPbAII was administered in this condition.

FrPbAII is also able to inhibit GABA and glycine uptake in synaptosomes prepared from rat retina (Fig. 8). This result

confirms the view that the neuroprotective action of FrPbAII is due to inhibition of GABA and glycine uptake. Moreover, an enhancement of glutamate uptake could be discarded as a mechanism by which FrPbAII could act as a neuroprotective compound, because no effect on glutamate transport is observed when synaptosomes were incubated with FrPbAII (Fig. 6). A direct FrPbAII interaction with $GABA_A/GABA_C$ receptors on GABA binding site is also ruled out as a neuroprotective mechanism, because only a very high concentration of FrPbAII is able to induce [^{3}H]GABA displacement on binding assays (Zukin et al., 1974; data not shown).

Although a number of classic GABA analogs are useful as pharmacological tools in research epilepsy and ischemic cerebral damage, they were shown to be inefficient in therapy because of their low permeability of the blood-brain barrier (Krogsgaard-Larsen et al., 1998). FrPbAII protects retinas against neuronal damage when administered intravenously, suggesting that this compound is able to cross the blood-retina barrier, which is structurally and functionally similar to the blood-brain barrier (Steuer et al., 2005).

To summarize, our results together provide insight into the effects of a new neuroprotective compound from *P. bistriata* spider venom that acts primarily and directly on GABA and glycine transporters. Indeed, the significant inhibition of GABA and glycine uptake caused by FrPbAII associated with its selectivity and blood-retina barrier permeability strongly suggest its potentially high value in studies of transport mechanisms and the role of uptake during inhibitory neurotransmission. Moreover, an understanding of the structure and activity of active compounds from arthropod venoms may provide "proof of principle" for a new class of neuroprotective and anticonvulsant drugs that act by inhibiting GABA and glycine clearance.

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