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Purification of a neuroprotective component of *Parawixia bistriata* spider venom that enhances glutamate uptake

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1 In this study, we examined the effects of crude venom from the spider *Parawixia bistriata* on glutamate and GABA uptake into synaptosomes prepared from rat cerebral cortex. Addition of venom to cortical synaptosomes stimulated glutamate uptake and inhibited GABA uptake in a concentration-dependent manner.

2 The venom was fractionated using reverse-phase high-performance liquid chromatography on a preparative column. The fraction that retained glutamate uptake-stimulating activity was further purified on a reverse-phase analytical column followed by ion-exchange chromatography.

3 The active fraction, referred to as PbTx1.2.3, stimulated glutamate uptake in synaptosomes without changing the K_M value, and did not affect GABA uptake. Additional experiments showed that the enhancement of glutamate uptake by PbTx1.2.3 occurs when ionotropic glutamate receptors or voltage-gated sodium and calcium channels are completely inhibited or when GABA receptors and potassium channels are activated, indicating that the compound may have a direct action on the transporters.

4 In an experimental model for glaucoma in which rat retinas are subjected to ischemia followed by reperfusion, PbTx1.2.3 protected neurons from excitotoxic death in both outer and inner nuclear layers, and ganglion cell layers.

5 This active spider venom component may serve as a basis for designing therapeutic drugs that increase glutamate clearance and limit neurodegeneration.
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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-propionate; CNS, central nervous system; EAAT1–5, excitatory amino-acid transporter subtypes 1–5; ED_{50}, dose of drug that produces effect under investigation in 50% of the population; FAB/MS, fast atom bombardment/mass spectrometry; GCL, ganglion cell layer; GLAST, glutamate and aspartate transporter; GLT-1, glutamate transporter 1; h.p.l.c., high-performance liquid chromatography; SG-209, *N*-[2-(acetoxy)ethyl]-3-pyridinecarboxamide; INL, inner nuclear layer; IPL, inner plexiform layer; λ, wavelength; NBQX, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide; NMDA, *N*-methyl-D-aspartate; OD, optical density; ODS, octadecylsilane; ONL, outer nuclear layer; OPL, outer plexiform layer; PbTx, *Parawixia bistriata* toxin; PDC, L-*trans*-pyrrolidine-2,4-dicarboxylic acid; PhTx4, peptide isolated from *Phoneutria nigriventer* spider venom; PTZ, pentylenetetrazole; TFA, trifluoroacetic acid; TTX, tetrodotoxin; u, unit of toxin based on $\lambda = 215_{nm}$; i.v., intravenous injection

Introduction

The termination of neurotransmission is achieved by rapid uptake of the released neurotransmitter by neuronal and astrocytic sodium-dependent transporters. Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and its uptake is critical for preventing glutamate excitotoxicity (for reviews see Gegelashvili & Schousboe, 1997; Danbolt, 2001). This process is driven by and coupled to ion gradients (Szatkowski *et al*., 1990; Zerangue & Kavanaugh, 1996; Seal & Amara, 1999; Amara & Fontana, 2002). To date, five structurally distinct subtypes of glutamate transporters have been cloned from animal and human tissue: GLAST or EAAT1 (Storck *et al*., 1992) and GLT-1 or EAAT2 (Pines *et al*., 1992), both present in astroglial cells (Rothstein *et al*., 1994); EAAC1 or EAAT3, present in neurons (Kanai & Hediger, 1992); EAAT4, found in the cerebellum (Fairman *et al*., 1995; Gegelashvili & Schousboe, 1998); and EAAT5, expressed in vertebrate retina (Arriza *et al*., 1997).

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L-Glutamate has been implicated in many important physiological processes such as developmental plasticity and long-term potentiation, as well as in pathological conditions like cerebral ischemia, amyotrophic lateral sclerosis (Rothstein *et al*., 1992), epilepsy (Coutinho-Netto *et al*., 1981; Rothstein *et al*., 1996; Tanaka *et al*., 1997), Alzheimer's disease (Hardy *et al*., 1987), motor neuron disease (Rothstein *et al*., 1992) and Huntington's disease (Massieu & Garcia, 1998). Glutamate excitotoxicity is also implicated in ischemic retinal damage. For example, in rats, injection of L-glutamate into the intravitreal chamber causes degeneration of inner nuclear and ganglion cells layers (Sisk & Kuwabara, 1985). Louzada Jr *et al*. (1992) also demonstrated an increase of L-glutamate concentration in retinas of rabbits exposed to ischemia and ischemia/reperfusion.

Spider venoms are a useful source of bioactive substances and show a wide range of pharmacological effects on synaptic transmission (Jackson & Usherwood, 1988; Uchitel, 1997). Spider venoms are comprised of proteins, polypeptides, low molecular weight acyl-polyamines and several other compounds (Meinwald & Eisner, 1995; reviewed by Rash & Hodgson, 2002). Some purified neurotoxins are directed against essential components of the nervous system, such as glutamate receptors and ion channels (Anis *et al*., 1990; Usherwood & Blagbrough, 1991; Blagbrough *et al*., 1992; Green *et al*., 1996). Acylpolyamines from wasp venom have been shown to inhibit glutamate uptake in insect neuromuscular synapses (van Marle *et al*., 1984; 1986) and from a spider venom to increase glutamate uptake (van Marle *et al*., 1989). The ω-agatoxins and the ω-conotoxins, peptides isolated from *Agelenopsis aperta* spider venom and *Conus* marine snail venom, respectively, distinguish some calcium channel subtypes (Olivera *et al*., 1990). The polypeptide PhTx4, isolated from *Phoneutria nigriventer* venom, inhibits L-glutamate uptake in a dose–response manner (Mafra *et al*., 1999).

Parawixia bistriata is a social spider that lives in the South American "*cerrados*". In termites, the injection of this venom produces an irreversible, dose-dependent paralysis (Fontana *et al*., 2000). Intracerebroventricular injection of this venom as well as more purified fractions abolishes convulsive tonic–clonic seizures induced by picrotoxin, bicucuuline and PTZ in rats (Cairrão *et al*., 2002). In this study, we examined the effects of crude and purified fractions from the venom of the spider *P. bistriata* on GABA and L-glutamate uptake and release in synaptosomes from rat cerebral cortex. Here, we demonstrate that a purified component of the venom can enhance the uptake of L-glutamate by a mechanism that appears independent of glutamate receptor activation. Our observation that the active component prevents neuronal death during retinal ischemia implies that transport activity limits the amount of excitotoxicity and suggests the potential for designing novel therapeutic drugs that act by enhancing glutamate clearance.

Methods

Materials

L-[^{14}C]glutamate (272 mCi mmol^{-1}), L-[^{3}H]glutamate (24 Ci mmol^{-1}), D-[^{3}H]aspartate (18 Ci mmol^{-1}), [^{3}H]GABA (40 Ci mmol^{-1}), [^{3}H]AMPA (55 Ci mmol^{-1}), [^{3}H]kainic acid (58 Ci mmol^{-1}) and [^{3}H]MK-801 (24.2 Ci mmol^{-1}) were obtained from New England Nuclear (Boston, MA, U.S.A.).

Methanol, acetonitrile, formic acid and ammonium hydroxide used were of analytical grade. Columns for h.p.l.c. were from Shimadzu Techno-Research, Inc. (Kyoto, Japan) and Asahipack (Asahi Chemical Industry Co., Ltd, Japan). BCA™ Assay kit was from Pierce (Rockford, IL, U.S.A.). Thiopental was from Cristalia (Brazil). Solutions for histological analysis (hematoxylin, eosin, picric acid, acetic acid, formalin, ethanol, xylene and paraffin) were from Reagen (Brazil) and Merck (Germany).

PDC, NBQX, baclofen and SG-209 were purchased from Tocris Cookson Inc. (Ballwin, MO, U.S.A.). Reagents for Tyrode and binding buffers, valinomycin, TTX, ketamine, cadmium chloride and unlabeled L-glutamate, GABA and MK-801 were from Sigma-RBI (St Louis, MO, U.S.A.).

Spider collection and preparation of venom extract

Spiders were collected in the region of Ribeirão Preto, São Paulo, Brazil. Upon arrival in the laboratory, they were frozen and stored at −20°C. The venom glands were removed, crushed in Milli-Q water at 0–4°C, and the extracts boiled for 10 min. The venom gland extract was cleared by centrifugation at 3000 × *g* for 10 min and the supernatant was lyophilized. A standard optical density (OD) of 1.0 at $\lambda_{215\,nm}$ was defined as 1000 units (u). For reference, a single gland in 1.0 ml of water has a concentration of approximately 1500 u.

Fractionation of the venom extract by reverse-phase and ion-exchange chromatography

Methods used were according to Figueiredo *et al*. (1995) and Escoubas *et al*. (2000). A lyophilized extract from 1000 venom glands was dissolved in 1.0 ml of Milli-Q water and applied onto a reverse-phase h.p.l.c. column (PREP-ODS 20 × 250 mm^2, 5 μM). Milli-Q water was degassed (solvent A) and 65% methanol was filtered and degassed (solvent B). Solvent A (95%) 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 was 1.0 ml min^{-1}. Nine fractions were obtained, and all were tested for their effects on L-glutamate and GABA uptake and release in synaptosomes from rat cerebral cortex, as described below.

The fraction referred as to PbTx1 (*P. bistriata* toxin 1) was rechromatographed on an ODS C18 column (4.6 × 150 mm^2, 5 μM) coupled to a precolumn, Shim-pack, C18 (4 × 10 mm^2), equilibrated with the A mobile phase (TFA 0.1% in Milli-Q water). The fraction was chromatographed using an exponential gradient from 0 to 100% of B phase (acetonitrile 60%–TFA 0.1%) in 30 min at a flow rate of 0.5 ml min^{-1}. Two eluted fractions were collected, lyophilized and are referred to as PbTx1.1 and PbTx1.2.

PbTx1.2 was dissolved in water, injected onto a cationic-exchange column, Asahipack, ES-502C (7.5 × 100 mm^2, 9 μM) that had been equilibrated with the mobile phase A (0.1 M ammonium formiate). Solvent A (95%) was maintained for 15 min, and then a linear gradient from 5 to 95% solvent B (0.1 M ammonium formiate containing 50% acetonitrile (1 : 1, v v^{-1}) was applied for 20 min at a flow rate of 1 ml min^{-1}. Three fractions were eluted, referred to as PbTx1.2.1–3, and

lyophilized. The ODs of the elutes were continuously monitored at 215 nm.

Preparation of synaptosomes

All animal experiments were treated according to the guidelines for use of animals in research approved by The Brazilian Society for Neuroscience and Behavior. Cerebral cortex from male Wistar rats (200–250 g) were used to prepare synaptosomes as previously described (Gray & Whittaker, 1962). Synaptosomes were resuspended in Tyrode buffer (136 mM NaCl, 5 mM KCl, 2.5 mM KH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM Tris-HCl, 5 mM glucose, pH 7.4, with equimolar replacement of Na^+ with choline for the Na^+-free conditions) and centrifuged for 20 min at 4°C. Protein content was determined by the BCA protein assay kit.

Uptake assays

Synaptosomes were resuspended in Tyrode buffer and preincubated for 10 min at 37°C in the presence or absence of spider venom or purified toxins. Uptake assays were initiated by adding L-[^{3}H]glutamate (100 nM, final concentration) [^{3}H]GABA (20 nM, final concentration) or D-[^{3}H]-aspartate (100 nM, final concentration) to synaptosomal suspensions (100 μg of protein ml^{-1}), and incubated for 3 min at room temperature in 96-well plates. Nonspecific uptake was determined from samples incubated in the presence of the Na^+-free buffer, and these values were subtracted from the total. All the reactions were stopped by filtration through a Whatman GF/B filter plate followed by three washes with cold buffer using a Brandel M96 Cell Harvester. Radioactivity collected was measured with Wallac M50 microbeta scintillation analyzer.

Calculation of ED_{50} for the effects of the venom

The concentration dependence of the effects of venom (from 3.8×10^{-3} to 3.8×10^{3} u ml^{-1}, final concentration) was measured to verify possible effects on GABA and glutamate uptake. Dose–response curves were fitted to the Hill equation in nonlinear regression analysis using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA, U.S.A.), ED_{50}s and the maximum effects are given as mean ± s.d. of three independent experiments.

To rule out the possibility that the increase in Na^+-dependent L-glutamate uptake might be related to increased intracellular metabolism of L-glutamate, we examined the effects of 2 and 20 u ml^{-1} of PbTx1 on the rate of uptake of D-aspartate, a nonmetabolized analog of L-glutamate. Results were expressed as a percent of the mean ± s.d. of velocity, in pmol min^{-1} mg^{-1} and compared using Student's *t*-test.

Saturation analyses of L-glutamate and GABA uptake performed in the presence of venom extract or PbTx1.2.3

Synaptosomes were preincubated with or without venom or PbTx1.2.3 for 10 min in 0.45 ml volumes in 96-well plates. Assays of L-glutamate or GABA uptake were initiated by the addition of unlabeled L-glutamate or GABA and L-[^{3}H]glutamate or [^{3}H]GABA (in a 50 μl volume, 4.5 nM to 10 μM, final concentration, 90% unlabeled and 10% labeled compound) to synaptosomes (100 μg of protein ml^{-1}). Plates were incubated for 3 min at room temperature (RT), the reactions were stopped and radioactivity counted as described above. For these experiments, the nonspecific uptake was determined from samples incubated in the presence of the L-glutamate uptake specific inhibitor PDC (630 μM, final concentration), or the GABA uptake specific inhibitor nipecotic acid (6 mM, final concentration). The nonspecific uptake was subtracted from the total. The uptake rate measured under these conditions was found to be linear over time and amount of tissue within the range used (not shown).

High-affinity L-glutamate and GABA uptake assays were performed in triplicate for each condition and the mean specific uptake value per concentration was determined. The results were expressed as mean ± s.d. of three independent experiments. Kinetic parameters were determined from a log plot using nonlinear regression analysis. Statistical analysis on the values of V_{max} and K_M, obtained in each experiment in the presence or absence of spider venom, was performed using the Student's *t*-test for paired data. Values were considered to be significantly different when $P < 0.05$.

Effects of agents that alter receptor and ion channel function on the actions of PbTx1.2.3

Synaptosomes were preincubated with or without 10 u ml^{-1} PbTx1.2.3 for 10 min at 37°C and then the following compounds were added: TTX, a sodium channel blocker (1 μM); cadmium chloride, a calcium channel blocker (1 mM, final concentration); baclofen, a GABA(B) receptor agonist (100 μM); ketamine, an NMDA receptor antagonist (100 μM); NBQX, a non-NMDA receptor antagonist (100 μM); valinomycin, a potassium ionophore (4 μM); or SG-209, an ATP-sensitive potassium channel opener (1 mM). L-[^{3}H]glutamate uptake assays were performed as described above. Results for each drug treatment are expressed as mean ± s.d. of the velocity of uptake, in pmol min^{-1} mg^{-1}. Data were analyzed using a Student's *t*-test for comparison between treatments and control, and a one-way ANOVA test for comparison of different treatments, using GraphPad Prism version 3.02.

L-[^{14}C]glutamate and [^{14}C]GABA release

Synaptosomes were homogenized in Tyrode buffer. L-[^{14}C]glutamate (70 nM) or [^{14}C]GABA (84 nM, final concentration) was added and incubated at RT for 20 min. The tubes were centrifuged for 3 min at 7200 × *g* at 4°C and the pellets were washed three times with cold buffer. Six different groups of tubes were prepared in triplicate containing 50 mM KCl, 50 mM KCl plus 20 u ml^{-1} of venom, 1.65 μM tetrodotoxin (TTX), 1.65 μM TTX plus 20 u ml^{-1} of venom, 20 u ml^{-1} of venom or 20 u ml^{-1} of PbTx1.2.3. Preloaded synaptosomes were added to these tubes and incubated for 3 min at 37°C. The reactions were stopped by centrifugation for 3 min at 3000 × *g*, and the amount of radioactivity in the supernatant was determined. The pellets were dissolved in methanol, centrifuged, and the radioactivity in the resulting supernatant was counted by liquid scintillation spectrometry with a counting efficiency of 85% (Beckman, LS-6800).

The statistical analyses used consider the percentage of neurotransmitter released among several treatments as the dependent variable. Two-sample comparison tests were

applied. Statistical tests were performed using baseline, inhibited (1 μM TTX) and stimulated (50 mM KCl) controls. The baseline control is the basal release rate (synaptosomes in Tyrode buffer). Values were considered to be significantly different when $P<0.05$. A one-way ANOVA test was performed to verify differences among different treatments, using GraphPad Prism version 3.02.

Receptor binding assays

The membrane preparation used in all receptor binding experiments was prepared according to Hawkins *et al.* (1995). Protein content was determined using a BCA protein assay kit and membranes were stored at -70°C until used. Prior to the binding assays, membranes were thawed and washed three times in 50 mM Tris-HCl (pH 7.4) by centrifugation at $50{,}000 \times g$ for 10 min. Assays were carried out in a final volume of 100 μl consisting of membrane suspension (100 μg of protein) and binding buffer with or without 100 u PbTx1.2.3. For NMDA receptor binding assays the buffer consisted of 50 mM Tris-HCl (pH 7.4). [^{3}H]MK-801 (20 nM) and coagonists L-glutamate (100 μM) and glycine (30 μM) were added to the reactions. Nonspecific binding was defined in the presence of 100 μM unlabeled MK-801. For AMPA and kainic acid binding assays, the binding buffer consisted of 50 mM Tris-HCl, 100 mM KCl and 2.5 mM $CaCl_2$. [^{3}H]kainic acid (50 nM) or [^{3}H]AMPA (50 nM) was used. Nonspecific binding was obtained in the presence 1 mM L-glutamate. Samples were incubated for 2 h at 25°C until equilibrium is attained. Assays were terminated by vacuum filtration using a Brandel M96 Cell Harvester, through GF/B filters (presoaked in 0.3% polyethyleneimine). Radioactivity collected was counted with Wallac M50 microbeta scintillation analyzer.

Effects of venom and PbTx1.2.3 in an experimental glaucoma model

Male Wistar rats (230–250 g) were anesthetized intraperitoneally with thiopental (50 mg kg^{-1}) and the retinas were made ischemic according to the protocols of Hughes (1991) and Louzada Jr *et al.* (1992).

Intraocular pressure was elevated by cannulating the eye anterior chamber, with a sterile 27-gauge needle attached to a manometer/pump connected to an air reservoir (Hughes, 1991). Elevating the intraocular pressure to 155 mmHg for 60 min induced maximal retinal ischemia (indicated by whitening of the eye fundus as blood flow is interrupted). After the ischemic period, the intraocular pressure was allowed to return to normal levels for 45 min (reperfusion period, during which the fundus color returns to normal). The left retina of each animal was subjected to the experimental condition, ischemia and/or reperfusion, while the right retina served as a nonischemic control. PbTx1.2.3 (10 u) was injected intravenously 15 min before the pressure elevation was induced. After the animals were euthanized, 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, dehydrated, sectioned at 4 μM cut and stained with hematoxylin–eosin. The histology of the retinas was examined to assess the ischemic damage to the cells. Five sections from each of five retinas per treatment condition were imaged, giving a total of 25 measurements per condition. The images of these sections were examined by a light microscope (Axiophot, Zeiss) and digitized with an analog camera (JVC TK1270) connected to a computer. Quantification was made using software KS 400 (Carl Zeiss Vision) and analyzed in a masked fashion. Results were expressed as mean cell number $\pm$ s.e.m. (Lagrèze *et al.*, 1998). Results were statistically analyzed by Student's *t*-test followed by ANOVA on ranks test ($P<0.05$).

Results

Effects of venom on L-glutamate and GABA uptake into synaptosomes are dose dependent

Initial experiments assessing the effects of *P. bistriata* venom on both L-glutamate and GABA uptake into cortical synaptosomes indicated that the venom stimulated L-glutamate uptake, but inhibited GABA uptake. To further explore these observations, we examined the dose dependence of the actions of the venom in this system. Figure 1a shows the dose–response curve of spider venom on L-glutamate uptake in synaptosomes. Spider venom stimulates L-glutamate uptake, the maximum stimulation being 243 ± 28%, in the presence of 3.8×10^2 u ml^{-1}. The ED_{50} is 0.038 ± 0.0092 u ml^{-1}. The dose–response curve for GABA uptake is shown in Figure 1b. In contrast to its actions on L-glutamate transport, spider venom inhibits GABA uptake; the ED_{50} for this effect is 2.45 ± 0.59 u ml^{-1}. The values for nonspecific uptake measured in the absence of sodium were less than 4% for both neurotransmitter substrates (data not shown) and this background was subtracted from the total radioactivity.

As a wide variety of free amino acids have been detected in the venoms of many spiders, particularly GABA, L-glutamate and D-aspartate (Rash & Hodgson, 2002), we verified using h.p.l.c. analysis that the concentration of L-glutamate present in venom is 42.6 pmol gland^{-1} (data not shown). This is equivalent to a concentration of 28 nM for a dose of 1000 u ml^{-1}, for example. This concentration is not sufficiently high to alter the results observed in L-glutamate uptake assays. Indeed, concentrations higher than 3.8×10^2 u of venom produce an inhibition of the dose–response curve (data not shown). The venom does not contain measurable concentrations of GABA, ruling out the possibility that the inhibition observed is due to the presence of GABA in the extract (data not shown).

Spider venom does not affect apparent transport affinities but does alter the maximum velocity of transport

By measuring the uptake of L-glutamate in synaptosomes over a range of substrate concentrations, the transport activity was shown to be both saturable and of high affinity. Figure 2a shows the kinetic analysis of glutamate uptake in the presence or absence of spider venom, using a new batch of venom. The values obtained were K_M of 3.4 ± 0.8 μM and V_{max} of 42.8 ± 4.6 nmol min^{-1} mg^{-1}. In the presence of 0.038 u ml^{-1} of venom, the K_M was 2.9 ± 0.6 μM and the V_{max} increased to 63.6 ± 5.5 nmol min^{-1} mg^{-1}, or 148 ± 5%. Figure 2b shows a saturation analysis of GABA uptake in the presence or absence of 2.4 u ml^{-1} of spider venom. K_M and V_{max} values were 1.16 ± 0.19 μM and 8.93 ± 0.45 nmol min^{-1} mg^{-1}, respectively. By adding spider venom, V_{max} was reduced to

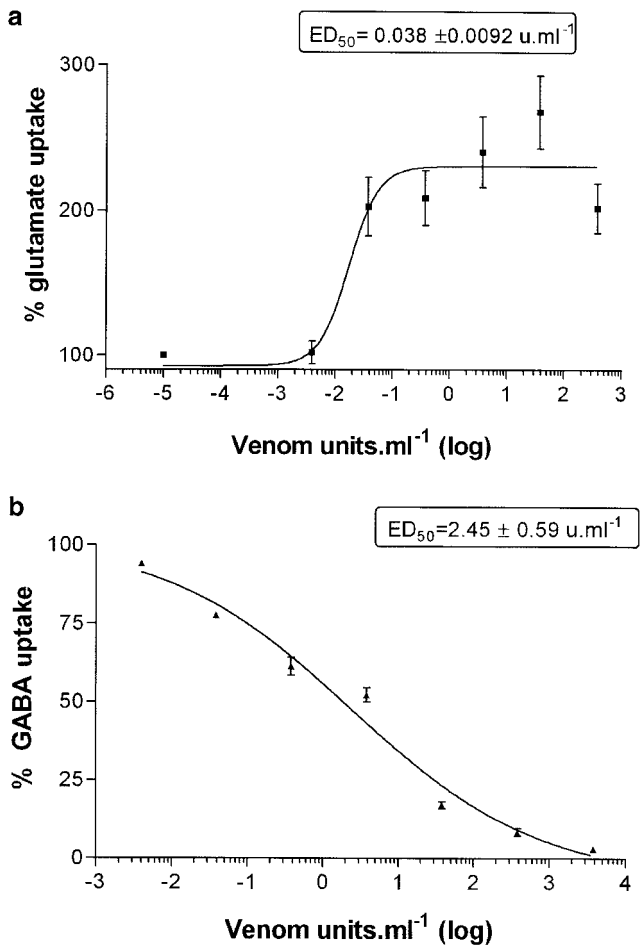

Figure 1 Dose–response curve for the effect of *P. bistriata* venom on L-[^{3}H]glutamate (a) and [^{3}H]GABA uptake (b) in synaptosomes from rat cerebral cortex. Synaptosomes were preincubated in the presence or absence of venom (at concentrations from 3.8×10^{-2} to 3.8×10^{3} u ml^{-1}) for 10 min at 37°C. Higher concentrations than those shown inhibit L-glutamate uptake and were not included in the graph. Concentrations of venom are plotted on a logarithmic scale. Uptake assays were initiated by adding L-[^{3}H]glutamate (100 nM, final concentration) or [^{3}H]GABA (20 nM, final concentration). Data from four independent experiments generated an $ED_{50}=0.038\pm0.0092$ u ml^{-1} in the L-glutamate transport assay (a) and an $ED_{50}=2.45\pm0.59$ u ml^{-1} in the GABA transport assay (b).

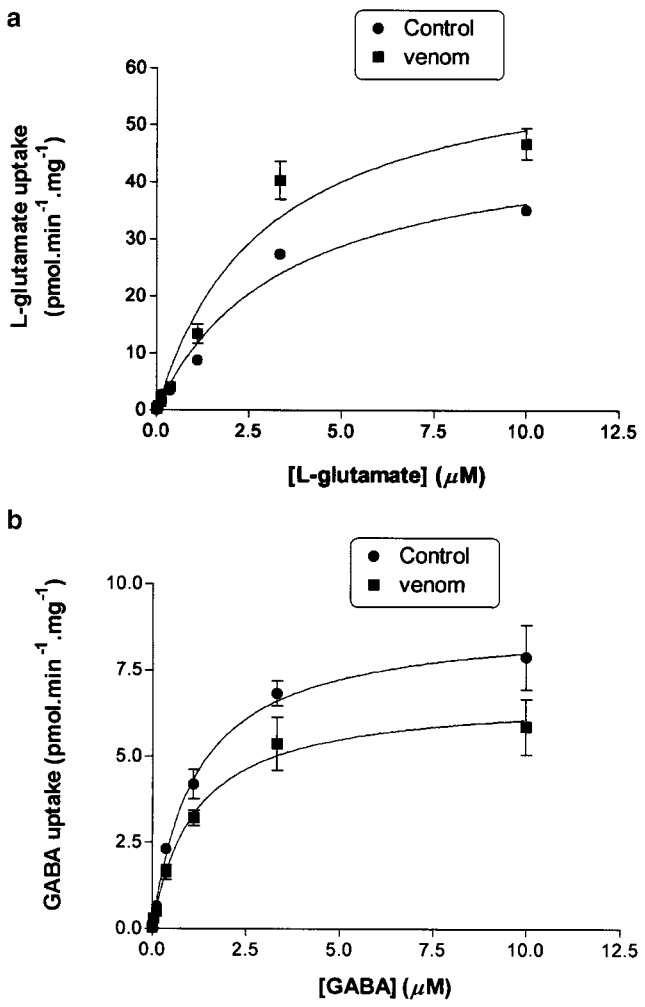

Figure 2 Kinetic analysis of high-affinity L-glutamate uptake (a) and GABA uptake (b) by synaptosomes preincubated in the absence (circles) or presence (squares) of *P. bistriata* venom. Venom was added at the concentration that produced a half maximal effect on L-glutamate (a) or GABA (b) uptake. (a) Data from three independent experiments using eight substrate concentrations generated values of $3.4\pm0.8\,\mu$M for the K_M and 42.8 ± 2.8 pmol min^{-1} mg^{-1} for the V_{max} under control conditions and $2.9\pm0.6\,\mu$M for K_M and 63.6 ± 5.5 pmol min^{-1} mg^{-1} for the V_{max} in the presence of venom. (b) Data from three independent experiments using eight substrate concentrations generated values of $1.16\pm0.19\,\mu$M for the K_M and 8.93 ± 0.45 pmol min^{-1} mg^{-1} for the V_{max} under control conditions and $1.14\pm0.22\,\mu$M for the K_M and 6.48 ± 0.38 pmol min^{-1} mg^{-1} for the V_{max} in the presence of venom.

6.48 ± 0.38 nmol min^{-1} mg^{-1}, that is $73\pm0.4\%$, and K_M was unaltered at $1.14\pm0.22\,\mu$M. Thus, spider venom noncompetitively inhibits high-affinity GABA uptake.

Purification of active fraction from P. bistriata venom by h.p.l.c.

Figure 3 shows the chromatographic profiles of the three steps of fractionation utilized in this work. The profile of the spider venom extract injected on a PREP ODS C18 column is illustrated in Figure 3a. The fractions were detected at $\lambda_{215\,nm}$, a wavelength that primarily detects carbon–nitrogen bonds. The eluted fractions, which were collected and lyophilized, are referred to as PbTx1–9 (*P. bistriata* toxin), as indicated above the figure. The sum of the ODs ($\lambda_{215\,nm}$) across all fractions gave a recovery of 70% compared to the crude venom. The compounds eluted include both highly hydrophilic and hydrophobic substances.

Each fraction was tested for its ability to modulate L-glutamate and GABA uptake into synaptosomes. The fraction PbTx1, which showed the greatest ability to enhance L-glutamate transport, was subjected to an analytical reverse-phase chromatographic step, the profile of which is shown in Figure 3b. The total recovered percentage relative to PbTx1 was 42.5%. Eluted fractions are referred to as PbTx1.1 and PbTx1.2, as indicated in Figure 3b. The active fraction, PbTx1.2, was chromatographed on an ion-exchange column (Figure 3c). The percentage of activity recovered relative to PbTx1 was 61.3%. The fractions are denoted as PbTx1.2.1–PbTx.1.2.3.

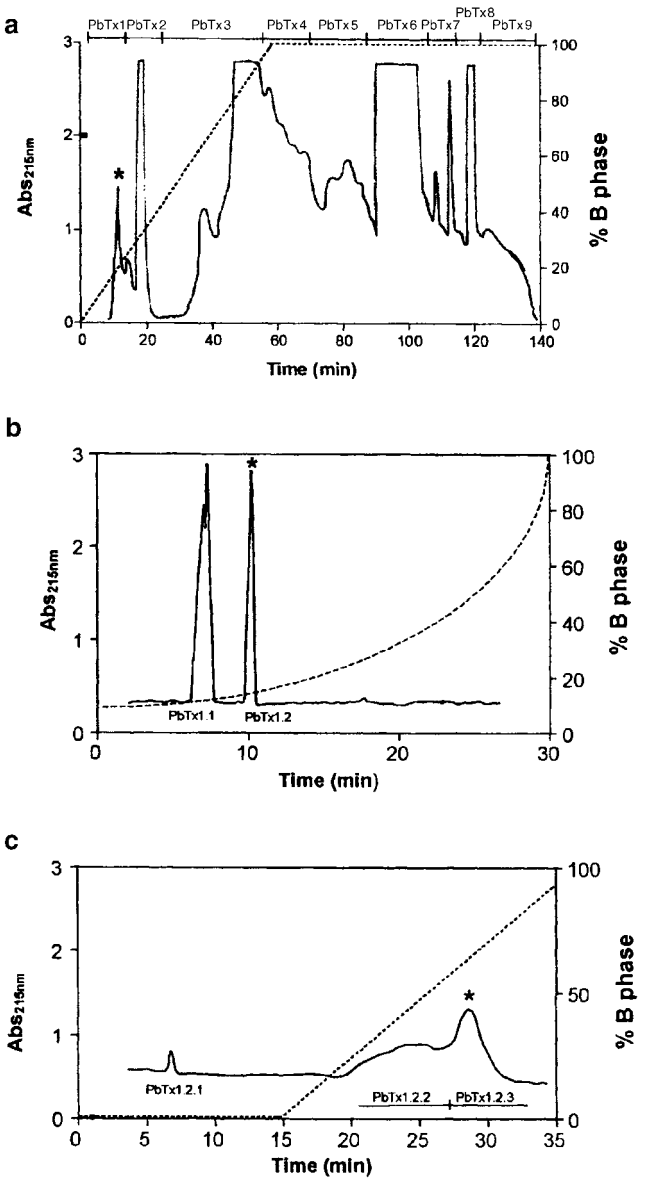

Figure 3 Chromatographic profiles showing the purification of the biologically active fraction from *P. bistriata* spider venom. (a) Reverse-phase chromatography of boiled extract of *P. bistriata* venom. The extract from 1000 venom glands was applied to a PREP-ODS C18 column ($20 \times 250\,mm^2$, $5\,\mu M$), equilibrated with water (A phase) and eluted using a linear gradient of 65% methanol (B phase, dotted line) over a 60 min period at a flow rate of $1\,ml\,min^{-1}$. The fraction PbTx1 was lyophilized for further purification. (b) Reverse-phase fractionation of PbTx1 from panel a on an ODS C18 column ($4.6 \times 150\,mm^2$) equilibrated with 0.1% TFA (A phase) and eluted using an exponential gradient (5–95%) of 65% acetonitrile/0.1% TFA (dotted line) over a 30 min period at a flow rate of $0.5\,ml\,min^{-1}$. Fraction PbTx1.2 was collected and lyophilized for further purification. (c) Ion-exchange chromatography of PbTx1.2 obtained from panel b on an ES-502C column ($7.5 \times 100\,mm^2$), Asahipack, equilibrated with 0.1 M ammonium formiate pH 8.25 (A phase) and eluted with a linear gradient of 0.1 M ammonium formiate containing 50% acetonitrile pH 8.25 (B phase, dotted line) over a 35 min period at a flow rate of $1\,ml\,min^{-1}$. Fraction referred as to PbTx1.2.3 was active in synaptosomes from rat cerebral cortex. Eluates were monitored at $\lambda = 215\,nm$. PbTx = *P. bistriata* toxin. *Denotes the active fractions.

Effect of the purified fraction, PbTx1, on L-*glutamate, GABA and* D-*aspartate uptake*

Figure 4 shows the effects of PbTx1 on L-glutamate GABA and D-aspartate uptake. We observed a significant increase in the rate of L-[^{3}H]glutamate uptake in the presence of PbTx1. The maximum enhancement observed was $316 \pm 11\%$ of control, in the presence of $27.6\,u\,ml^{-1}$ of PbTx1. No significant

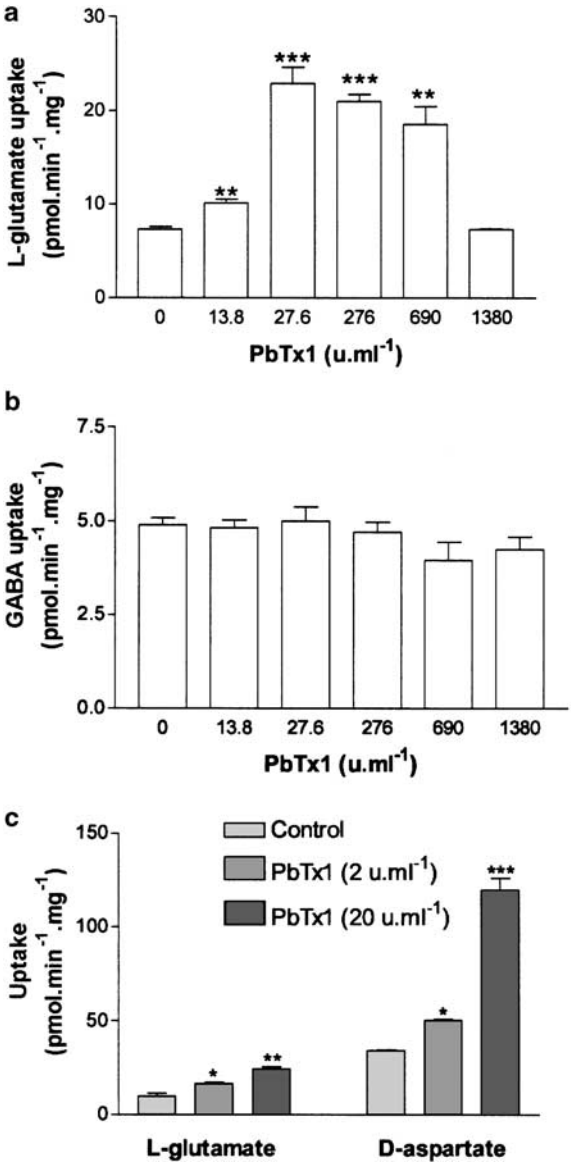

Figure 4 Effect of increasing concentrations of PbTx1 on L-glutamate (a), GABA (b) and D-aspartate (c) uptake in synaptosomes from rat cerebral cortex. (a) The maximum enhancement of L-glutamate uptake activity observed was $316 \pm 11\%$ of control in the presence of $27.6\,u\,ml^{-1}$ of PbTx1. (b) No effects on GABA uptake are observed at any concentration of PbTx1. Data are means $\pm$ s.d. of three independent experiments for both L-glutamate and GABA uptake. Fractions PbTx2-9 did not alter the rate of glutamate uptake in synaptosomes (not shown). (c) Effect of PbTx1 (2 and $20\,u\,ml^{-1}$) on L-glutamate and D-aspartate uptake in synaptosomes from rat cerebral cortex. Data are means $\pm$ s.d. of three and two independent experiments for L-glutamate and D-aspartate, respectively. Determinations of significance were made using Student's *t*-test (*$P<0.05$, **$P<0.001$, ***$P<0.001$).

effect in glutamate uptake was observed upon addition of PbTx2-9 (data not shown). In contrast to the effects of PbTx1 on L-glutamate uptake, GABA uptake was not altered by any of the concentrations of PbTx1 assayed. We concluded that the component(s) responsible for GABA uptake inhibition (Figures 1b and 2b) are different from those responsible for the activation of L-glutamate uptake. The component that inhibits GABA uptake elutes in fraction PbTx2, and efforts to purify and characterize the active component are ongoing. However, in the subsequent studies presented here, we have focused on the enhancement of glutamate uptake by PbTx1 and the more highly purified fractions PbTx1.2 and PbTx1.2.3.

Figure 4c shows the effects of PbTx1 on L-[^{3}H]glutamate or D-[^{3}H]aspartate uptake. Addition of the active fraction produced significant changes in the rate of L-glutamate and D-aspartate uptake, when compared to the control. PbTx1 (2 and 20 u ml^{-1}) increased the uptake of both L-glutamate and D-aspartate. Interestingly, with D-aspartate, a nonmetabolized substrate, the increase in transport produced by PbTx1 was more pronounced. Maximum increases were observed in the presence of 20 u ml^{-1} of PbTx1, by 140±2 and 248±5% for L-glutamate and D-aspartate uptake, respectively.

Purified venom fractions stimulate L-*glutamate uptake*

Figure 5a,b shows the effects of PbTx1.2 and PbTx1.2.3 on L-glutamate uptake. PbTx1.2 at 28 u ml^{-1} stimulated L-glutamate uptake by 48±7% (Figure 5a). Figure 5b shows that 10 u ml^{-1} and 100 u ml^{-1} PbTx1.2.3 enhanced L-glutamate uptake by 76±2 and 161±5%, respectively. Other fractions obtained in the first round of h.p.l.c. purification (PbTx1.1) or the second round of h.p.l.c. purification (PbTx1.2.1 and PbTx1.2.2) did not affect glutamate uptake in cortical synaptosomes (data not shown). The pH of the stock solutions of PbTx1.2.3 is ~6.5 and does not affect the pH of the assay solution. Figure 5c shows the kinetic analysis of L-glutamate uptake in the presence or absence of 10 u ml^{-1} of PbTx1.2.3. Under control conditions, the K_M for L-glutamate uptake was 2.74±0.73 μM, with a V_{max} of 42.28±4.26 pmol min^{-1} mg^{-1}. When PbTx1.2.3 was added, the K_M remained the same (3.06±0.57 μM) while the V_{max} increased ~60% to 67.88±4.9 pmol min^{-1} mg^{-1}. This observation parallels the effects seen with the venom, which also produces an increase in the V_{max} without altering the apparent affinity for L-glutamate transport.

Enhancement of uptake is not caused by alterations in L-*glutamate release*

Synaptosomes can also mediate glutamate release. If the venom and purified fractions were preventing basal release, they could act to reduce the concentrations of unlabeled glutamate in the assay buffer producing an apparent enhancement of glutamate transport. Our finding that the venom and PbTx1.2.3 have no effect on the transport K_M would argue against this interpretation. However, to confirm that glutamate release was not directly affected, we measured the release of glutamate in the presence or absence of venom or PbTx1.2.3. Figure 6 shows the effects of KCl, TTX, spider venom and PbTx1.2.3 on L-glutamate release. Spontaneous release of glutamate was 25±2% of the total internal pool and this was not altered by addition of 20 u ml^{-1} of venom or

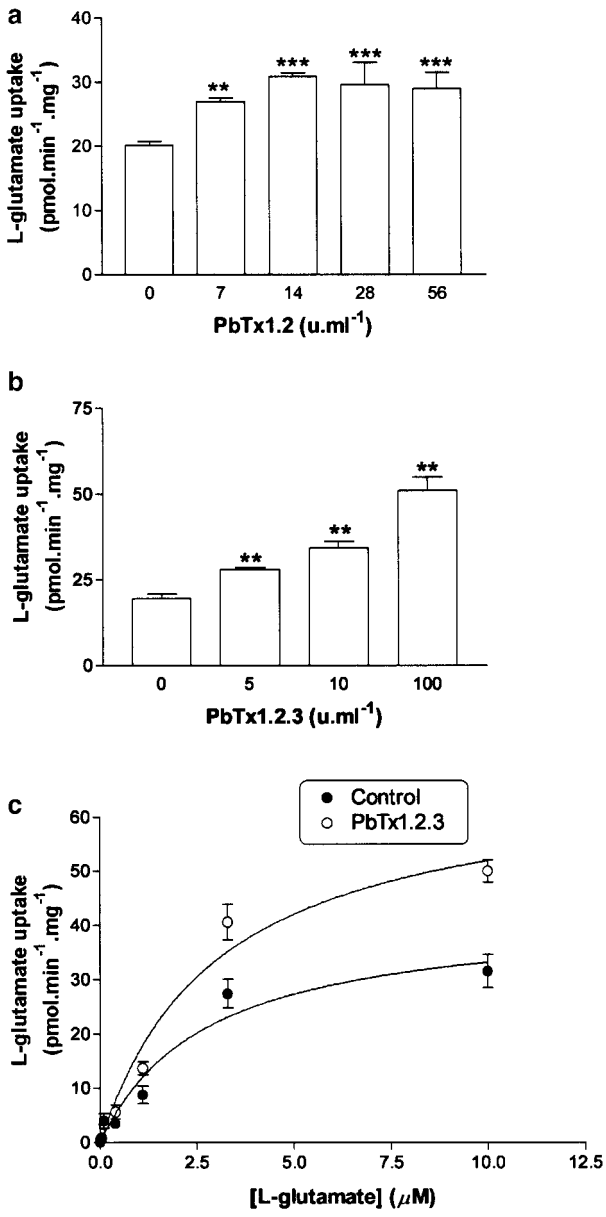

Figure 5 Effects of PbTx1.2 (a) and 1.2.3 (b, c) fractions purified from PbTx1 and PbTx1.2, respectively, on L-[^{3}H]glutamate uptake in synaptosomes from rat cerebral cortex. Concentrations of the fractions are indicated. Data are means±s.d. from three experiments (**$P<0.01$; ***$P<0.001$). PbTx1.1, PbTx1.2.1 and PbTx1.2.2 did not alter the rate of glutamate uptake in synaptosomes (not shown). (c) Kinetic analysis of high-affinity L-glutamate uptake by synaptosomes in the absence (closed circles) or presence (open circles) of 10 u ml^{-1} PbTx1.2.3. Uptake was measured in the presence of L-glutamate (4.5 nM to 10 μM, final concentrations). The amount of radiotracer was always 10% of the total L-glutamate added and the uptake velocities are expressed as pmol min^{-1} mg^{-1}. Data from three independent experiments generated values of 2.74 ±0.73 μM for the K_M and 42.28±4.26 pmol min^{-1} mg^{-1} for the V_{max} under control conditions, and 3.06±0.57 μM for the K_M and 67.88±4.9 pmol min^{-1} mg^{-1} for the V_{max} in the presence of PbTx1.2.3.

20 u ml^{-1} of PbTx1.2.3. In the presence of 50 mM KCl, glutamate release was increased only modestly (by 31±1%) and this amount of KCl-evoked efflux was not altered in the presence of 10 u ml^{-1} of venom. This release evoked by 50 mM

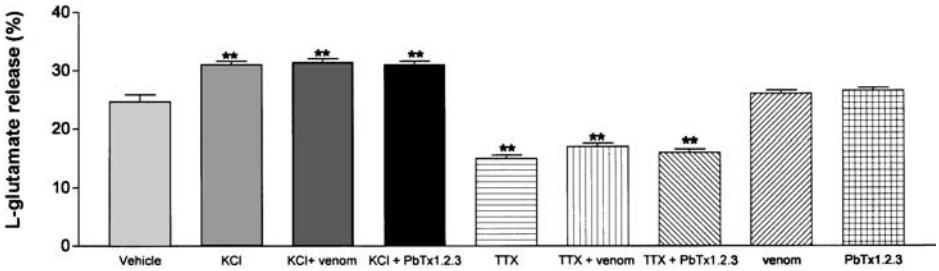

Figure 6 Percentage of L-glutamate released by synaptosomes preloaded for 20 min with L-[^{14}C]glutamate (70 nM final concentration). Concentrations of the different reagents used were: 50 mM KCl, 1.65 μM TTX, 20 u ml^{-1} venom and 20 u ml^{-1} PbTx1.2.3. Statistical analyses were based on the assumption that the percentage of neurotransmitter released during the various treatments is the dependent variable (**$P < 0.01$). One-way ANOVA test showed no difference among the percentage of glutamate release in KCl or TTX-treated synaptosomes in the presence and absence of venom or PbTx1.2.3.

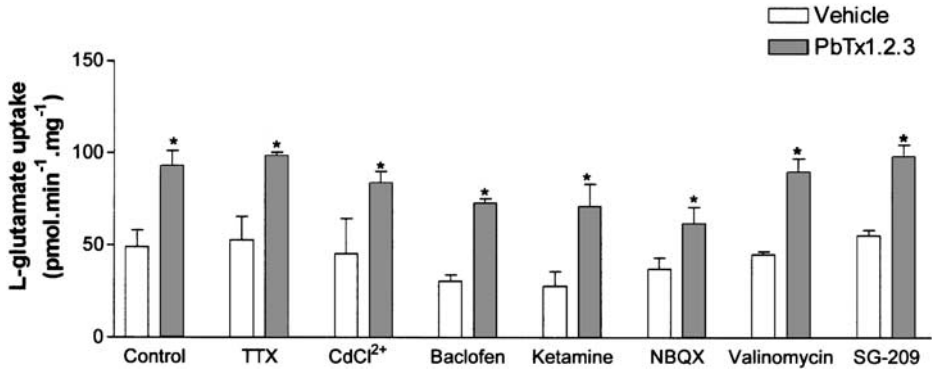

Figure 7 Effect of pretreatment with PbTx1.2.3 (10 u ml^{-1}) on L-glutamate uptake into synaptosomes in the presence of receptor and ion channel agonist or antagonists. Final concentrations were: TTX (1 μM), cadmium chloride (1 mM), baclofen (100 μM), ketamine (100 μM), NBQX (100 μM), valinomycin (4 μM) or SG-209 (1 mM). Data are means $\pm$ s.d. from four experiments (Student's *t*-test, *$P < 0.05$). Analysis using a one-way ANOVA indicated no difference in uptake between different treatments either in the absence or presence of PbTx1.2.3.

KCl would be comprised of both Ca^{+2}-dependent release and a small portion of reverse transport (Dawson *et al*., 2000). We suggest that reverse transport was not affected by PbTx1.2.3 and does not have a prominent effect on stimulated release. In contrast, the release was decreased by 1.65 μM TTX (to 15 $\pm$ 1%). However, the release observed in the presence of TTX was also not altered by the addition of venom and PbTx1.2.3. Similarly, no effects were observed when the venom and PbTx1.2.3 were tested for their effects on GABA release under all these conditions (data not shown).

Drug treatment of synaptosomes in the presence of PbTx1.2.3

Figure 7 shows the effects of receptor antagonists and channel blockers and openers on L-glutamate uptake in synaptosomes preincubated with PbTx1.2.3. TTX, cadmium chloride, baclofen, ketamine, NBQX, valinomycin and SG-209, at the concentrations indicated, did not alter the glutamate uptake. Preincubation with PbTx1.2.3 stimulated L-glutamate uptake in all treatments (Figure 7a, *$P < 0.05$). Thus, the effects of PbTx1.2.3 are not dependent on the activation of sodium, calcium or potassium channels, NMDA and AMPA receptors or GABA(B) receptors.

PbTx1.2.3 does not affect ionotropic glutamate receptors binding

To establish whether PbTx1.2.3 might also block or interact with ionotropic glutamate receptors, binding assays were performed on brain membranes in the presence or absence of 100 u ml^{-1} PbTx1.2.3, the maximal concentration used in glutamate uptake experiments (Figure 5b). The results are shown in Table 1. Even at this high concentration, the compound did not alter the binding of [^{3}H]MK-801, [^{3}H]kainic acid or [^{3}H]AMPA to their respective receptors, indicating that the actions of PbTx1.2.3 are unlikely to be mediated through blockade of glutamate receptors. However, we cannot exclude the possibility that PbTx1.2.3 may noncompetitively block receptor activation at sites on glutamate receptors that would not be detected with the ligands used.

Neuroprotective effects of PbTx1.2.3 in an experimental glaucoma model

To investigate the effect of PbTx1.2.3 *in vivo,* rat retinas were subjected to experimental glaucoma. In these experiments, the rats received i.v. injections of 10 u PbTx1.2.3 or saline 15 min prior to ischemic treatments. Figure 8 shows micrograph images of retinas subjected to control conditions, to ischemia

Table 1 Effect of PbTx1.2.3 on binding of [^{3}H]MK-801, [^{3}H]kainate and [^{3}H]AMPA to synaptic membranes and on uptake of L-[^{3}H]glutamate into cortical synaptosomes

	Synaptic membranes *Specific binding* (pmol mg^{-1})			*Synaptosomes* *Uptake* (pmol mg^{-1} min^{-1})
	[^{3}H]MK-801	[^{3}H]kainic acid	[^{3}H]AMPA	L-[^{3}H]glutamate
Vehicle	0.59±0.14	0.42±0.02	0.10±0.03	19.5±2.2
PbTx1.2.3 (100 u ml^{-1})	0.52±0.05	0.53±0.13	0.07±0.01	51±7***

The values represent the mean±s.d. of specific binding or uptake from three experiments carried out in triplicate. Incubation conditions are described in the Methods section. ***$P<0.001$, different from vehicle.

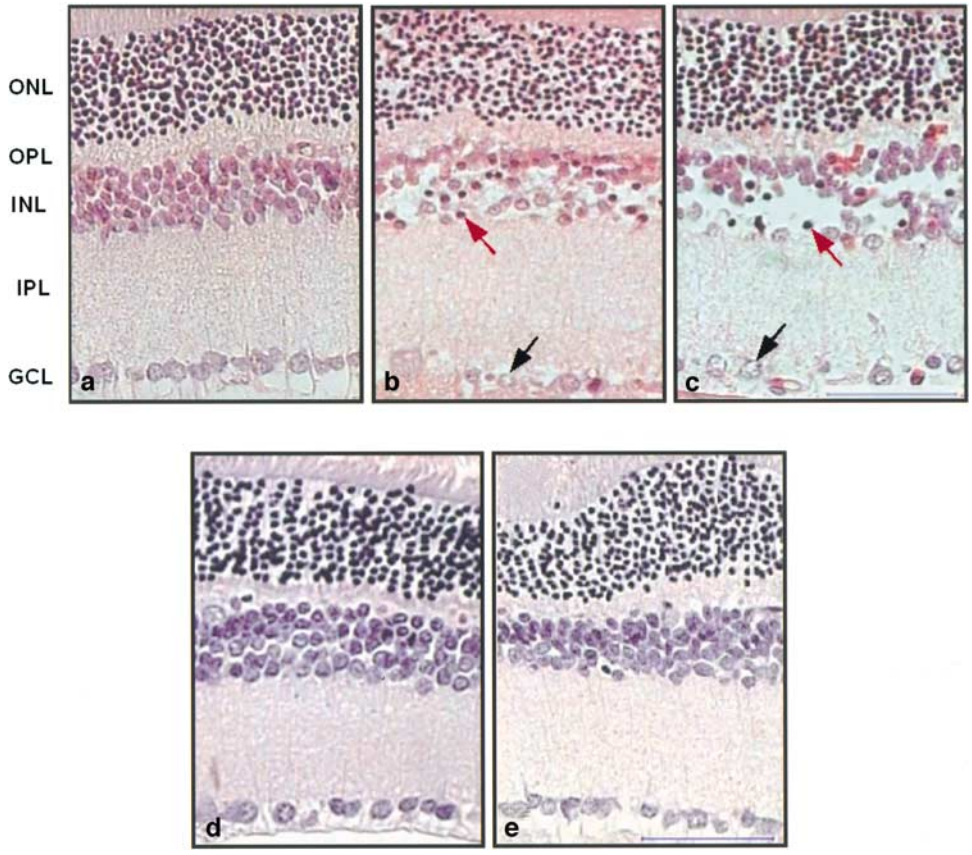

Figure 8 Effects of intravenous injection of PbTx1.2.3 (10 u) in ischemic and ischemic/reperfused retinas. Retinal sections were stained with hematoxilin–eosin. Bars = 50 μM. Black arrows denote areas of vacuolization and red arrows denote pyknotic nuclei. (a) Control; (b) ischemic; (c) ischemic/reperfused; (d) ischemic pretreated with 10 u of PbTx1.2.3; (e) ischemic/reperfused retinas pretreated with 10 u of PbTx1.2.3. ONL, outer nuclear layer; OPL, outer plexiform layer, INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

and to ischemia followed by reperfusion. Histological alterations could be observed when comparing control (Figure 8a) and ischemic (Figure 8b) and ischemic/reperfused retinas (Figure 8c). Relative to control retinas, the ischemic retinas (Figure 8b) showed a decrease in cell number, and increases in cytoplasmic vacuolization and the number of pyknotic nuclei in the ganglion cell layer (GCL). The inner nuclear layer (INL) displays more edema, pyknotic nuclei and cellular disorganization. The outer nuclear layer (ONL) exhibits a decreased cell number, and greater edema and cell disorganization compared with control retinas. Edema and matrix disorganization is also observed in inner plexiforme layer (IPL).

In the ischemia/reperfusion retinas (Figure 8c), the GCL showed lower cell density, and increases in vacuolization, number of pyknotic nuclei, and cellular disorganization. The INL also had fewer cells and those remaining had more pyknotic nuclei and cytoplasmic vacuoles as well as enhanced edema and cellular disorganization. There were also fewer cells in the ONL.

Retinas treated with 10 u PbTx1.2.3 (Figure 8d and e) displayed normal cellular morphology and a dramatic reduction in cell death in all layers when compared to ischemia- and ischemia/reperfusion-induced retinas (Figure 8b and c). Figure 8d shows ischemia-induced retinas treated with PbTx1.2.3. We observed no degeneration, pyknotic nuclei or disorganization of the cells, although some cell loss is also observed. Figure 8e shows ischemic/reperfused retinas treated with PbTx1.2.3, where protection is observed in all layers and there is a decrease in cellular loss.

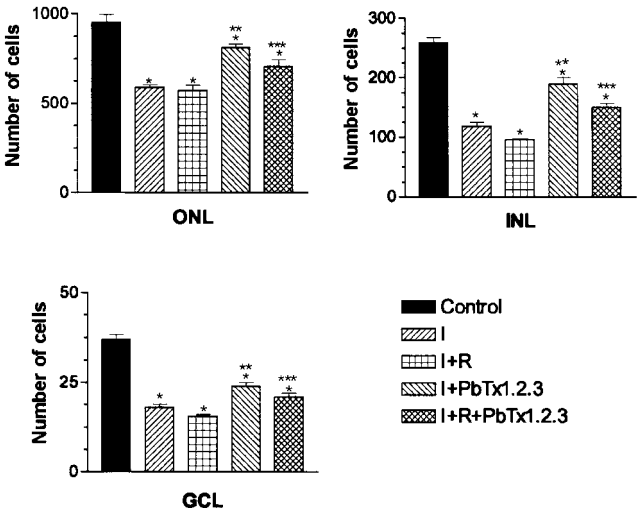

Figure 9 Number of cells in retinal layers: ONL (outer nuclear layer), INL (inner nuclear layer) and GCL (ganglion cell layer). I = ischemic. I + R = ischemic/reperfused. ($n = 5$, 5 images/retina). *Significantly different from the control retinas ($P < 0.05$, using a Student's *t*-test followed by ANOVA on ranks (Student–Newman–Keuls method). ***Significantly different from ischemic/reperfused retinas ($P < 0.05$, Student's *t*-test). In all, 10 u of PbTx1.2.3 was i.v. injected 15 min before induction of ischemia (ischemic and ischemic/reperfused) in rat retina *in vivo*.

The number of cells in control, ischemic and ischemic/reperfused retinas with or without treatment with PbTx1.2.3 is presented in Figure 9. There was a significant decrease in the number of cells in all layers following ischemia and those that were reperfused following ischemia. The cell numbers were 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, 63% in the INL and 57% in the GCL in the retinas subjected to ischemia/reperfusion. Smaller decreases in the number of cells were observed in all retinal layers in either of the two conditions when the animals were pretreated with 10 u of PbTx1.2.3. In contrast to the retinas not treated with PbTx1.2.3, the cell number in the treated retina was only decreased by 14% in ONL, 27% in INL and 42% in GCL after ischemia and by 25% in ONL, 42% in INL and 43% in GCL in ischemic/reperfused retinas (Figure 9).

Discussion

In this study, we evaluated the effects of crude venom and purified toxins from the spider *P. bistriata*. The paralyzing properties of this venom have been demonstrated previously in a novel bioassay using termites as an insect model (Fontana *et al*., 2000). These studies indicated that this venom might mediate its action through effects on glutamatergic or perhaps GABAergic systems, as L-glutamate serves as the major neurotransmitter at the insect neuromuscular junction (Usherwood, 1994). Synaptosomes served as a useful model in our studies, as many of the functional properties of transporters, receptors and ion channels are maintained and can be rapidly assayed. Morphological examination by electron microscopy of the synaptosomal preparation demonstrated that the membrane remained intact and the levels of lactic acid dehydrogenase, which rise when the plasma membrane is disrupted (Phillis *et al*., 1994), were not changed when synaptosomes were incubated with either spider venom or any toxins purified from it (results not shown).

We found that addition of the venom and the purified venom fractions to synaptosomal uptake assays results in a saturable and concentration-dependent stimulation of L-glutamate transport (Figure 1a). The observation that a component or components present in the venom stimulate L-glutamate uptake is a novel result not observed to date for a spider venom. This enhancement of uptake by the venom is a consequence of an increase in the V_{max} for L-glutamate transport, and does not appear to involve a change in the apparent affinity for uptake (Figure 2a).

Although multiple glutamate transporter subtypes probably contribute to the activity measured in synaptosomes, many studies indicate that much of the uptake measured in cortical synaptosome preparations is mediated by a single predominantly glial carrier, GLT-1/EAAT2 (Danbolt, 2001). Indeed, the pharmacology of the glutamate uptake in synaptosomes aligns most closely with glutamate uptake mediated by GLT-1/EAAT2, including sensitivity to inhibition by kainate, dihydrokainate and selectivity for different transporter substrates (Bridges *et al*., 1999). Thus, it seems likely that the effects we observe reflect the action of PbTx1.2.3 on GLT-1. Preliminary studies of the actions of PbTx1.2.3 on the cloned human subtype EAAT2 expressed in COS cells are consistent with this idea. Although uptake by GLT-1/EAAT2 into glia accounts for much of the glutamate transport activity in the CNS, nerve terminals may also display a transport activity with similar pharmacological properties (Suchak *et al*., 2003). Several recent studies demonstrate the expression of alternatively spliced C-terminal variant of GLT1/EAAT2 in neurons (Chen *et al*., 2002; Schmitt *et al*., 2002). This variant isoform, which is pharmacologically indistinguishable from GLT1/EAAT2 (Chen *et al*., 2002), may be the major site for presynaptic uptake of glutamate. Ultimately, it will be important to know whether PbTx1.2.3 has similar effects on uptake in both neuronal and glial cells.

The venom also contains a component capable of regulating GABA uptake. Figure 1b shows that increasing concentrations of spider venom dramatically inhibited GABA uptake. As shown in Figure 2b, the venom inhibits the maximal velocity of GABA uptake, while the K_M remains unchanged indicating a noncompetitive inhibition by the venom. This ability of a toxin to mediate the inhibition of GABA uptake in synaptosomes was also observed with extracts from venom of the wasp *Agelaia vicina* (Pizzo *et al*., 2000). Drugs that inhibit GABA uptake may provide an effective means for protecting the brain against neuronal injury (Fisher & Bogousslavsky, 1998) and for treating epilepsy (Meldrum, 1997), as illustrated by the clinically used drug tiagabine.

Venom was fractionated in search of the component(s) responsible for enhancing L-glutamate uptake. Three chromatographic purification steps, reverse-phase on preparative ODS-C18, on analytical ODS-C18 and ion-exchange columns, provided fractions that were surveyed for the glutamatergic activity in synaptosomes. We found that a single fraction, referred to as PbTx1, enhanced L-glutamate uptake in synaptosomes (Figure 4a). PbTx1 increases both L-glutamate and D-aspartate uptake, but does not alter GABA uptake in synaptosomes (Figure 4b and c), indicating that the active

component of this fraction is specific for glutamate transport. Both L-glutamate and D-aspartate are substrates for the same transporters (Arriza *et al*., 1994), but D-aspartate is less subject to cellular metabolism. These data provide strong evidence that increase in L-glutamate transport produced by the venom and PbTx1 cannot be attributed to an increase in L-glutamate metabolism.

PbTx1 was resolved into two fractions: PbTx1.1 and PbTx1.2 (Figure 3b). PbTx1.2 showed activity in enhancing L-glutamate uptake in synaptosomes (Figure 5a), and this activity was further purified by ion-exchange chromatography to yield PbTx1.2.3 (Figure 3c). PbTx1.2.3 stimulates glutamate uptake in a dose-dependent manner and appears to mediate its effect by increasing the V_{max} for L-glutamate uptake without changing the K_M (Figure 5c). This observation suggests that the active component could either act directly or indirectly to increase the turnover rate of the carrier or, alternatively, it could act to increase the number of functional transporter molecules at the cell surface. The latter hypothesis is unlikely since a greater number of transporters at the plasma membrane would also result in a greater efflux of labeled glutamate, which we did not observe (Figure 6). We speculate that PbTx1.2.3 is not a protein since its activity was maintained after boiling and is highly hydrophilic. In addition, liquid chromatography coupled to FAB/MS analysis revealed a molecular weight of 437 D (data not shown). The molecular structure is currently under investigation.

We also examined the effect of PbTx1.2.3 on the amount of glutamate efflux. The synaptosomal release of neurotransmitters was stimulated by K^+ and blocked by TTX, confirming the integrity of the preparation (Figure 6). PbTx1.2.3 did not affect the levels of basal L-glutamate and GABA release, nor did it alter the percentage of K^+-stimulated efflux or TTX-blocked release from preloaded synaptosomes. These results indicate that PbTx1.2.3 does not act indirectly by altering tonic or depolarization-dependent glutamate release.

In order to determine whether the enhancement of glutamate uptake by PbTx1.2.3 is mediated by an indirect action through receptors or channels, we investigated whether various channel and receptor agonists and antagonists could interfere with the effects of PbTx1.2.3 on glutamate uptake. These agents included TTX, a sodium channel blocker; cadmium chloride, a calcium channel blocker; baclofen, a GABA-B receptor agonist; ketamine, an NMDA receptor antagonist; and NBQX, a non-NMDA receptor antagonist (Figure 7). Neither basal nor PbTx1.2.3-stimulated glutamate uptake was altered by blockade of voltage-dependent sodium channels, or calcium channels, using blockers at concentrations known to produce effective inhibition of channel activity.

GABA type B receptors are present on excitatory terminals throughout the CNS. Baclofen, a GABA-B receptor agonist, appears to decrease excitatory transmission by inhibition of glutamate release and has been shown to increase the amount of glutamate inside nerve terminals (Martire *et al*., 2000). Despite its potential effects on neuronal glutamate concentrations, baclofen did not alter glutamate uptake or the ability of PbTx1.2.3 to enhance uptake into synaptosomes, suggesting that the effects of PbTx1.2.3 are not dependent on GABA receptor activation. Similarly, glutamate receptor antagonists ketamine and NBQX did not alter basal glutamate uptake or the enhancing effect of PbTx1.2.3 (Figure 7).

To address the possible influence of membrane potential changes resulting from potassium flux, we used the potassium ionophore valinomycin and the potassium channel opener SG-209. Valinomycin has been shown to elevate the level of glutamate uptake level in membrane vesicles from rat brain and glial plasmalemmal vesicles from rat hippocampus, suggesting that the inside-negative membrane potential induced by K^+ diffusion enhances the uptake activity (Kanner & Sharon, 1978; Nakamura & Kataoka, 1993). However, neither facilitating potassium diffusion by valinomycin nor opening potassium channels by SG-209 affected glutamate uptake in synaptosomes. Most likely, this is because synaptosomes already have a negative membrane potential. Since PbTx1.2.3 exerted its stimulating effects in the presence of both valinomycin and SG-209, we strongly believe that it does not act by facilitating potassium channel opening and membrane hyperpolarization, but instead acts directly on glutamate transporters.

To investigate whether PbTx1.2.3 could have neuroprotective actions as a consequence of enhancing glutamate uptake, we examined its effects during retinal ischemia and ischemia followed by reperfusion using a rat model of experimental glaucoma. The organization and accessibility of the retina has made it the best-characterized model system for examining the physiology and function of amino-acid transporters (Eliasof *et al*., 1998) and it is an excellent model to study the effects of drugs in ischemia/reperfusion in the CNS (Louzada Jr *et al*., 1992). In our experiments, PbTx1.2.3 was found to protect neurons from injury in all retinal layers, but the INL had the greatest response. The INL contains the cell bodies of the retina interneurons, which includes horizontal cells, bipolar cells and amacrine cells. In the retina, GLT-1 is preferentially expressed by the different types of bipolar cells; however, it was also found in amacrine cells and cones (Rauen *et al*., 1996). Hence, the GLT-1 expression pattern correlates with the INL, where we find the highest protection, consistent with the idea that the compound protects neurons by increasing glutamate uptake through GLT-1 transporters.

The neuroprotective effects of PbTx1.2.3 are observed in both ischemic and ischemic/reperfused retinas. Louzada Jr *et al*. (1992) have shown that there is a higher percentage of glutamate release and neuronal death during the reperfusion period than during ischemia; yet we still observe protection when PbTx1.2.3 is administered in the reperfusion model. Surprisingly, although PbTx1.2.3 is hydrophilic, it protects retinas against neuronal damage when administered intravenously, suggesting that the active agent is able to cross the retina blood barrier.

Many previous studies have shown that glutamate receptor blockers can be effective neuroprotective agents (reviewed in Choi, 1994). To eliminate the possibility that PbTx1.2.3 prevents neuronal death by blocking glutamate receptors, we examined whether it could inhibit binding to NMDA, AMPA or kainate receptors. As shown in Table 1, PbTx1.2.3 at high concentrations does not alter the binding of radiolabeled ligands to NMDA, kainate or AMPA receptors. We conclude that PbTx1.2.3 is not a competitive inhibitor of these receptors, although we cannot exclude the possibility that the compound may be a noncompetitive inhibitor. These results suggest that the retina neuroprotection of PbTx1.2.3 is mediated through glutamate transporters rather than antagonizing ionotropic glutamate receptors.

Our study provides unique insights into a newly identified compound from spider venom that modulates glutamate and GABA transport. A few drugs have been shown to stimulate glutamate uptake, such as bromocriptine (Yamashita *et al*., 1998) and arachidonic acid (Zerangue *et al*., 1995). Although the chemical nature of the active component(s) is still unknown, the significant enhancement of L-glutamate uptake mediated by *P. bistriata* spider venom and PbTx1.2.3 suggests their potential utility in studies of transport mechanisms and the role of uptake during glutamatergic neurotransmission. Moreover, an understanding of the structure and activity of the active compounds may provide 'proof of principle' for a new class of neuroprotective drugs that act by increasing glutamate clearance.

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