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INHIBITION OF L-GLUTAMATE AND GABA SYNAPTOSOME UPTAKE BY CROTOXIN, THE MAJOR NEUROTOXIN FROM *Crotalus durissus terrificus* VENOM.

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ABSTRACT. This paper describes a brief study on the crotoxin mechanism of action, regarding the transport of GABA and L-glutamate in rats cortico-cerebral synaptosomes and in heterologous systems, such as COS-7 cells expressing gabaergic transporters, and C6 glioma cells and *Xenopus* oocytes expressing glutamatergic transporters. Crotoxin concentrations over 1 μM caused an inhibitory effect of ^{3}H-L-glutamate and ^{3}H-GABA, and reversibly inhibited L-glutamate uptake by C6 glioma cells. When COS-7 cells were assayed, no inhibition of the ^{3}H-GABA transport could be evidenced. Crotoxin kept its inhibitory effect on neurotransmitters uptake even when Ca^{2+} ions were removed from the medium, therefore, independently of its PLA_2 activity. In addition, high concentrations (2 mM) of BPB did not avoid the action of crotoxin on the neurotransmitters uptake. Crotoxin also inhibited ^{3}H-L-glutamate, independently on Na^+ channel blockade by TTX. In addition, an evaluation of the lactic dehydrogenase activity indicated that uptake inhibition does not involve a hydrolytic action of crotoxin upon the membrane. We may also suggest that crotoxin acts, at least partially, altering the electrogenic equilibrium, as evidenced by confocal microscopy, when a fluorescent probe was used to verify cell permeability on C6 glioma cells in presence of crotoxin.

KEY WORDS: Crotoxin, L-glutamate, GABA, *Crotalus durissus terrificus*.

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INTRODUCTION

Animal venoms are complex mixtures of bioactive components, predominantly polypeptides, which play offensive, defensive and digestive roles (15,36,62). Among those components are phospholipases A_2 (PLA_2, E.C. 3.1.1.4), which catalyze the hydrolysis of *sn*-2 ester bond of phospholipids, releasing free fatty acids, initially polyunsaturated, as arachidonic acid. PLA_2s are also found in pancreatic secretions, platelets, neurons, mastocytes, inflammatory exudates, and snake and bee venoms.

Many of these phospholipases, although toxic, do not show detectable catalytic activity (6,53,56). Their M_r is usually about 14,000; they are monomeric, homomultimeric or heterodimeric enzymes, and may be obtained from a single snake, as many as 16 PLA_2s, catalytically active or not (21,24,31).

Some phospholipases A_2 are potent, mainly pre-synaptic, neurotoxins (34). Endogenous PLA_2s are suggested to be involved with brain pathologies, inducing epilepsy, ischemia, trauma, and others (2). They may show neurotoxic, myotoxic, convulsant, inflammatory, and edema inducing activity (16,61). The convulsive activity induced by crotoxin (5) and its basic subunit CB (22) was reported. Crotoxin showed to be convulsive despite its low *in vitro* catalytic activity (34,46). Tzeng *et al*. (58) showed that crotoxin binds to specific sites in the synaptic membranes and that the highly conserved residues Tyr21 and Tyr24 of the basic PLA_2 subunit are determinants of this binding. A 48kDa-membrane protein able to bind to crotoxin was reported by Krizaj *et al*. (33). In brain pathologies, it was already well established that several neurotransmitters (1,28), such as excitatory amino acids (27) are able to increase the release of free fatty acids, especially arachidonic acid.

Little is known about the participation of endogenous PLA_2s in the glutamate uptake. However, it was shown that a toxic PLA_2 paradoxin was able to significantly decrease this process in rat hippocampal mini-slices (9); and that transporter-mediated currents in Purkinje neurons are increased more than threefold by arachidonic acid, a second messenger that is liberated as a result of the activation of phospholipase A_2 by Ca^{2+} (59).

Several papers have reported that toxins inhibit the uptake of many neurotransmitters, including GABA (γ-aminobutiric acid), noradrenalin, serotonin, and choline (41,42,52,63),

while studies on glutamate uptake are more limited (63). Data on neurotoxicity of PLA_2s are scant and usually directed to β-bungarotoxin (20,23,40). This paper describes a study on the mechanism of action of crotoxin, a heterodimeric PLA_2 from South American rattlesnake venom; regarding the transport of GABA and L-glutamate in rats cortico-cerebral synaptosomes, and in heterologous systems, such as COS-7 cells expressing gabaergic transporters, and C6 glioma cells and *Xenopus* oocytes, expressing glutamatergic transporters.

MATERIALS AND METHODS

^{3}H-GABA, ^{3}H-glutamate, ScintiVerse, sucrose, OptiPhase Supermix, 1900 TR Liquid Scintillation Analyzer, BPB (4-bromophenacyl bromide), DMEM (Dulbecco's Modified Eagle Medium), and protein assay BCA kit were purchased from Perkinhelmer Life Science, Fisher Scientific, Wallac, Packard, Sigma-Aldrich, GIBCO and Pierce, respectively. All other reagents used were purchased from Sigma-Aldrich and Mallinckrodt.

Isolation of synaptosomes

Sprague Dawley male rats (200-250g) from Charles River Laboratory (USA) were sacrificed by decapitation and the brain cortex was separated and dept in 0.32 M sucrose. Brain tissue was homogenized at 130xg with the aid of a Potter-Elvehjen homogenizer and then centrifuged at 1,400xg, 4^{o}C for 10 min. Supernatant was collected and centrifuged at 1,400xg, 4^{o}C for 20 min. The sedimented material (mitochondria, myelin and synaptosomes) was gently dispersed in 0.32 M sucrose, homogenized at 3,200xg and centrifuged under a density gradient of 0.8 M-1,2 M sucrose at 60,000xg, 4^{o}C for 60 min. The upper phase (myelin) was discarded by suction, and the synaptosomal phase was collected and diluted to 0.4 M with Milli Q cold water. After a further centrifugation at 1,600xg, 4^{o}C for 20 min, the supernatant was discarded and the sediment resuspended in Tyrode buffer (136 mM NaCl; 5 mM KCl; 2.5 mM KH_2PO_4; 1 mM $MgSO_4$; 25 mM Tris HCl; 2 mM $CaCl_2$; 5 mM glucose; pH 7.4). For protein determination, the BCA protein assay kit was used. The amount of protein in each reaction well was 30-45 μg (18).

^{3}H-GABA and ^{3}H-L-Glutamate uptake

^{3}H-GABA (36.2 Ci/mmol) and ^{3}H-L-glutamate (22.5 Ci/mM) were used for the uptake experiments. In Rat Cortico-Cerebral Synaptosomes (RCCS), the final concentration of the radioactive component was 100 nM. The different crotoxin concentrations are shown in the corresponding figures. Different incubation times (5, 10, 15 and 20 min.) were assayed at room temperature. For the control, 60 mM of nipecotic acid (for GABA experiments) and 6.3 mM of L-trans-pyrrollidine – 2,4-dicarboxylic acid (PCD) (for L-glutamate experiments) were utilized. Assays were performed on 96 well plates, and after 3 min of incubation with the radioactive material, each well mixture was sucked and filtered through a Brandel System. Radioactivity pulses were counted with the aid of a WALLAC 1450 Microbeta scintillation analyzer using the Optiphase Supermix scintillation liquid. Data were expressed as rate of GABA or L-glutamate uptake in fmol/mg/min (7).

^{3}H-GABA and ^{3}H-L-Glutamate uptake in absence of Ca^{2+}

Synaptosomes were prepared as above described. The Ca^{2+} from Tyrode buffer was now replaced by 7.6 mM of Sr^{2+} and 1 mM of EGTA.

^{3}H-GABA and ^{3}H-L-Glutamate uptake in presence of p-bromophenacyl bromide (BPB)

BPB (0.5, 5.0 and 2000 μM) was incubated with crotoxin for 1 hour at room temperature, using different toxin concentrations.

Tetrodotoxin (TTX)

TTX (1 μM) was incubated for 30 min with RCCS, followed by crotoxin at different concentrations.

C6 Glioma cells

These cells were originally cloned from a rat glioma (3), and then grown in 24 well plates supplemented with Dulbecco's Modified Eagle Medium (DMEM) and 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). They were then kept at 37^{o}C under 5% (v/v) CO_2. In experiments involving pre-incubation, the toxin was added in the cell medium kept at room temperature.

^{3}H-L-Glutamate uptake in C6 glioma cells

C6 glioma cells are undifferentiated cells from kidney tumour, able to express the glutamatergic transporters EAAC1 (EAAT-3) endogenously (8,14,44). The cells were kept in Corning culture flasks containing DMEM, supplemented with FCS (10% final) plus penicillin (100 U/ml) and streptomycin (100 μg/ml), and plated every two days. For glutamate uptake assay, the following buffer solutions were used: 5 mM Tris base; 10 Mm HEPES; 2.5 mM KCl; 1.2 mM $CaCl_2$; 1.2 mM $MgCl_2$; 12 mM KH_2PO_4; 10 mM Dextrose; 140 mM NaCl; pH 7.2. As control, 140 mM choline chloride was used to substitute 140 mM NaCl. The treated cells were pre-incubated for 20 min with 0.015, 1.0 and 500 μM of crotoxin, and then ^{3}H-glutamate (22.5 Ci/mM) was added up to 1 μM. After 5 min of incubation, the reaction was stopped by washing the cells twice with cold buffer containing Na^{+} or not, and then the cells were disrupted by 450 μl of 0.1% SDS/0.1 N NaOH. The plates were placed on an electronic shaker for 20 min, in order that all cells could get loose from the plate, and 400 μl was transferred into a vessel containing 4.4 ml of ScintiVerse scintillation liquid. Radioactive pulses were then counted in a 1900 TR Scintillation Analyzer.

C6 Glioma cells recovery

The cells were incubated with 500 μM crotoxin for 20 min and then washed with PBS four times. They were then incubated at 37ºC, and L-glutamate uptake assay was carried out 10, 20, 30, 45 and 60 min after incubation, in order to evaluate the cell recovery ability. In regard to the controls, Milli-Q water and incubation of cells with crotoxin for 20 min was followed by the uptake assay.

COS-7 cells

These cells were used for expression of GAT-1 and GAT-3 GABA transporter DNAs. They were kept in DMEM, in Corning culture flasks, transferred every 3 days using transference protocol, and washed with PBS buffer, which was then sucked out. 12 ml of DMEM plus penicillin (100 U/ml) and streptomycin (100 μg/ml) were then added as before, and 2 ml of the suspension was transferred to a new flask and kept at 37^{o}C for an additional 3-days period before the next transference, when cells were also transferred to 24 well plates. On the next

day, transfixion of cells and gabaergic transporter DNA was carried out using Fugene protocol for experiment on GABA uptake.

Transformation of E. coli DH5α cells

GAT-1 and GAT-3 transporter DNAs were obtained from *E. coli* DH5α cells, and for the expressed DNA extraction, Maxi-Prep protocol was used (48).

^{3}H-GABA uptake in COS-7 cells

These assays were carried out in 24 well plates. Two days before, the cells were transfected with GABA, GAT-1 and GAT-3 transporter DNAs using CMV vector. ^{3}H-GABA was added, and ^{3}H-GABA uptake data were obtained from a β counter. The treated cells were pre-incubated for 20 min with 0.015, 1 and 500 μM crotoxin and then, the radioactive material was added up to 1 μM. After 10 min of incubation, the reaction was stopped, the cells were washed twice with cold PBS, and then disrupted with 450 μl of 0.1% SDS/0.1 N NaOH. The plates were placed in a shaker for 20 min as above, and 400 μl were transferred to a vessel containing 4.5 ml of scintillation liquid. Counting was then performed during 1 min/vessel.

Statistics Analysis

Results are presented as the means ± S.E.M of values obtained. Statistical significance of differences between groups was evaluated using Student's unpaired *t*-Test. *P* values <0.05 were considered significant.

RESULTS

Figure 1 shows the action of crotoxin on the uptake of ^{3}H-GABA and ^{3}H-L-glutamate by RCCS, depending on pre-incubation time. Both 0.1 mM and 0.5 mM of crotoxin were able to significantly inhibit neurotransmitters uptake. Inhibition was time-dependent, since the uptake decreased as the incubation time of RCCS and crotoxin increased. The assay was repeated at least 3 times following the same conditions of temperature, incubation time, and dilutions, calculated by the crotoxin ε.

Crotoxin concentrations higher than 1 μM caused an inhibitory effect of ^{3}H-L-glutamate and ^{3}H-GABA (Figure 2) for a chosen 20 min incubation period. Within this longer time, we could detect the uptake inhibition even at lower concentrations. This assay shows that crotoxin reversibly inhibited L-glutamate uptake by C6 glioma cells and did not disrupt cell membrane as cause of inhibition of the neurotransmitter uptake.

The word "*crotoxin*" in Figure 3 represents the assay in presence of this toxin after 20 min of incubation; and zero represents the assay in absence of crotoxin, which was replaced by water, washed twice with PBS, and incubated at 37^{o}C. We can see that cells were recovered with crotoxin after incubation, and that the toxin did not irreversibly injure them. When the heterologous system constituted by COS-7 cells was assayed, no inhibition of ^{3}H-GABA transport by crotoxin could be evidenced (Figure 4).

Crotoxin keeps its inhibitory effect on neurotransmitters uptake even when Ca^{2+} ions are removed by EGTA and replaced by Sr^{2+} ions (Figure 5), therefore, independently of its PLA_2 activity. In addition, high concentrations (2 mM) of BPB, which inhibits PLA_2 activity, did not avoid the action of crotoxin on the neurotransmitters uptake, even after the incubation of BPB with crotoxin (Figure 6), showing that there is no heterodimer dissociation to evoke its activity. Figure 6 also shows that crotoxin inhibits ^{3}H-L-glutamate, independently of Na^{+} channel blockade by TTX, demonstrating that this toxin does not potentiate crotoxin activity, and that its activity is not dependent on Na^{+} channel.

DISCUSSION

Crotoxin, the major neurotoxic component of *Crotalus durissus terrificus* venom (51) is a complex of two proteins: crotapotin, acidic component, catalytically inactive, ~9kDa, and PLA_2, basic component, catalytically active, ~14kDa. Little is known about the crotoxin mode of action in the central nervous system. It was already shown that it acts initially on the peripheral nervous system blocking the Ach release on nerve terminals (29).

Ng and Howgood (42) showed that crotoxin (7 nM) inhibits 50% GABA uptake, and that isolated basic subunit presents half of this activity. Despite the available data regarding the action of crotoxin on the central nervous system are scant, mainly in experimental models as synaptosomes, it was already established by these authors that this toxin causes some alterations, inhibiting GABA transport (38), as well as the L-glutamate excitatory activity potentiation by some PLA_2s (12,32,37).

Our results showed that increasing concentrations of crotoxin inhibit ^{3}H-L-glutamate and ^{3}H-GABA uptake (Figure 2). This inhibition is time-dependent, but independent on extracellular Ca^{2+} (Figures 5 and 6), since lower crotoxin concentrations (0.1 mM) result in a significant decrease of neurotransmitter uptake, mainly from 5 to 15 min (Figure 1), both in presence and absence of Ca^{2+}. Neurons and glia accumulate neurotransmitters dependent on Na^+ cotransport, through which the energy stored along transmembrane electrochemical transport may be used to move solute into the cell (17, 26, 57). This transporter is located in the neurons synaptic membrane and uses the same transmitter. This is probably the main mechanism to end synaptic transmission. When COS-7 heterologous cells or Xenopus oocytes (60) were used to express GABA or L-glutamate transporters, respectively, we could not reproduce the results obtained with synaptosomes (Figure 3). A suggested explanation for this fact is that crotoxin does not act directly upon transporters, but rather in their neighborhoods.

These cells, which express neuron components are not neurons and hence do not possess all components of a central nervous system cell. However, we cannot discard the possibility that the cloned transporters utilized are not able to express the binding site for crotoxin, as it was clearly evidenced in K^+ channels, which were cloned and expressed in mammal cells (13) C6 glioma cells originated from a rat adrenal tumour, which display L-Glutamate transporters similar to those from neuron cells. Our assays showed that 1.2 mM of crotoxin inhibits up to 60% the uptake (Figure 3). After 45 min, the cells were already recovered with full ability to uptake ^{3}H-L-glutamate.

Crotoxin induces convulsion when injected intracerebroventricularly. Recently, Dorandeu *et al*. (10) showed that intracerebralventricular (i.c.v.) injection of low doses (7 pmol) of crotoxin caused a prominent tonic-clonic convulsion. Increased L-Glu in the synaptic cleft has

been one of the explanations for convulsive diseases (39). Our results show that extracellular Ca^{2+} does not significantly interfere in L-Glu (Figure 5) or GABA uptake (data not shown), in disagreement with Zhu *et al*. (64) and Mafra *et al*. data (35). We have to point out that L-Glu or GABA uptake indeed slightly decreases in the absence of external Ca^{2+}, which was not significant. GABA is the main inhibitory neurotransmitter of mammal brain and is efficiently removed from the synaptic cleft and extracellular space by high affinity transporters of neurons and glial cells. At least three cDNAs of GABA transporters have already been isolated from rat brain, namely GAT-1 (19), GAT-2 (4) and GAT-3 (4,7). High levels of GAT-1 mRNA have been found along brain cortex and hippocampus, hence the choice of brain cortex for the synaptosomal preparations in this work.

An important aspect of neurotransmitter transport is that they are potentially electrogenic. For GABA and L-Glu, both Na^+-dependent, a charge translocation through the membrane and the process itself, generate the current. When TTX (Figure 6), a Na^+ channel blocking toxin, is added, no alteration in the neurotransmitter uptake is observed, since the blocked channel does not belong to the system that promotes the transport electrogenic equilibrium. Note, however, that crotoxin does not potentiate this blockade to compete with TTX since the inhibitory action of crotoxin on L-Glu uptake was kept. Norepinefrine (57), GABA (49,50), and L-glutamate (55) carriers can reversibly act, transporting substrates to the outside of cells when external K^+ concentration increases and cell depolarization occurs. This reverse uptake may explain the non-vesicular Ca^{2+} independent release of GABA and L-Glu (43).

Irreversible inactivation of PLA_2s by BPB is usually used to abolish catalytic, toxic, and consequently, epileptogenic activity (10). Addition of BPB, in order to inhibit any PLA_2 activity resulting from the dissociation of the crotoxin complex, showed that crotoxin does not dissociate before exerting its activity (Figure 6). Consequently, no alteration of the crotoxin inhibitory activity on ^{3}H-L-Glutamate could be detected, since the crotoxin complex is insensitive to the inhibitor (45,54). However, we cannot discard the possibility that crotoxin produces its effects indirectly. Our results show that crotoxin does not directly interfere in the neurotransmitters uptake mechanism inhibiting its carriers, but in neighboring targets, also present in the membranes of C6 glioma cells, where it causes GABA and L-glutamate uptake

inhibition. Crotoxin does not dissociate into crotapotin and PLA_2 before interacting with synaptosome membranes.

In addition, an evaluation of lactic dehydrogenase activity (data not shown) evidenced no statistical differences of values for synaptosomes in presence and absence of crotoxin, indicating that uptake inhibition does not involve a hydrolytic action of crotoxin upon the membrane. We may also suggest that crotoxin acts, at least partially, altering the electrogenic equilibrium, as evidenced by confocal microscopy, when a fluorescent probe $DIBAC_4$ (36) {bis [1,3-dibutylbarbituric acid-(56)] trimethin-eoxolnol} was used to verify permeability on C6 glioma cells in presence of crotoxin (data not shown). While the precise mechanism of action of many venom components is still unknown, it is already established that it involves interactions with specific membrane components (25,30,47,48). Although it unclearly remains which role transporters play in presence of neurotoxins (in this case, crotoxin), the activation of a proton current could serve as another mechanism, maybe involving local pH changes, through which synaptic excitability could be modulated by substrate binding and transport (11). These interactions make these venom components attractive tools for development of therapeutic agents in the study of molecular targets on cell membranes.

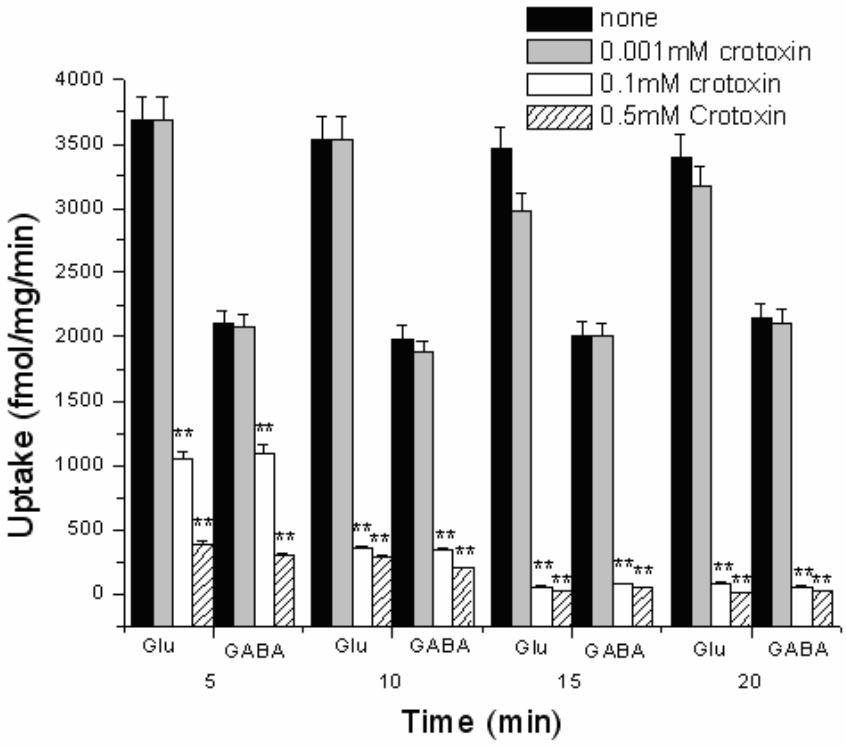

Figure 1. Inhibition of ^{3}H-L-glutamate and ^{3}H-GABA uptake by different crotoxin concentrations and incubation time in RCCS. Concentrations of 0.1 and 0.5 mM were effective (**$p<0.01$) in all periods assayed.

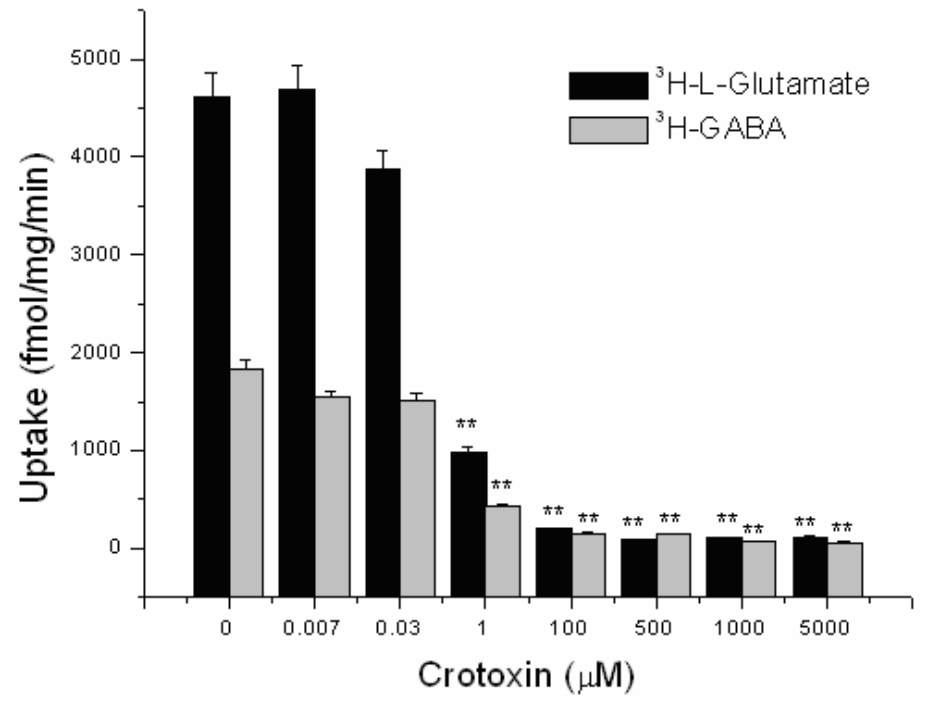

Figure 2. Inhibition of ^{3}H-L-glutamate and ^{3}H-GABA uptake by different crotoxin concentrations in RCCS for a fixed time of 20 min. Concentrations from 1 to 5000 μM were effective (***p<0.01*).

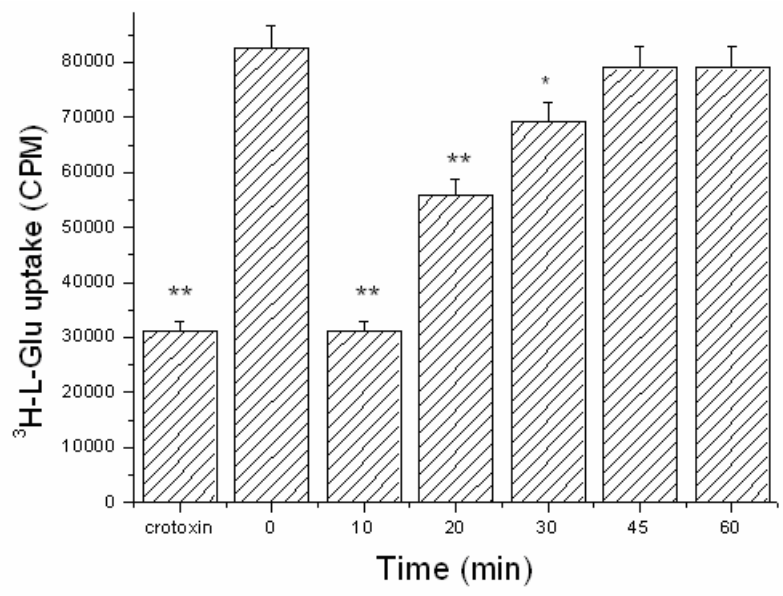

Figure 3. Recovery of C6 glioma cells after incubation with 500 μM of crotoxin. Cells were incubated for 20 min with the toxin, washed twice with PBS buffer and then incubated again at 37^{o}C. After 10, 20, 30, 45 and 60 min, the cells were removed and assayed for ^{3}H-L-glutamate uptake. The word "*crotoxin*" represents inhibition of the neurotransmitter uptake by crotoxin after 20 min of incubation with the toxin (***p<0.01*; * *p< 0.05*).

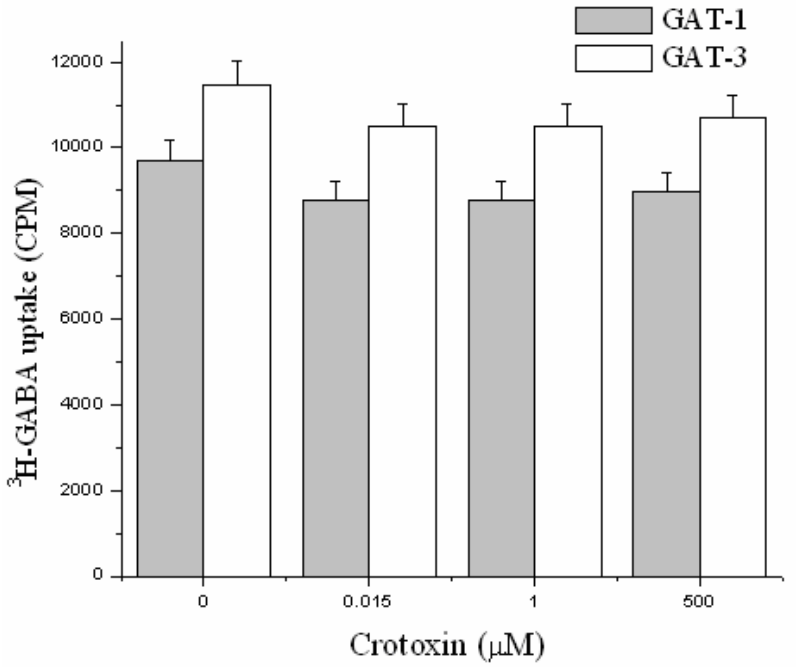

Figure 4. ^{3}H-GABA uptake by COS-7 cells expressing the gabaergic transporters GAT-1 and GAT-3. After incubation of crotoxin at different concentrations for 20 min, the neurotransmitter uptake was assayed. Both for GAT-1 and GAT-3, there was no significant inhibition of ^{3}H-GABA uptake (n=3).

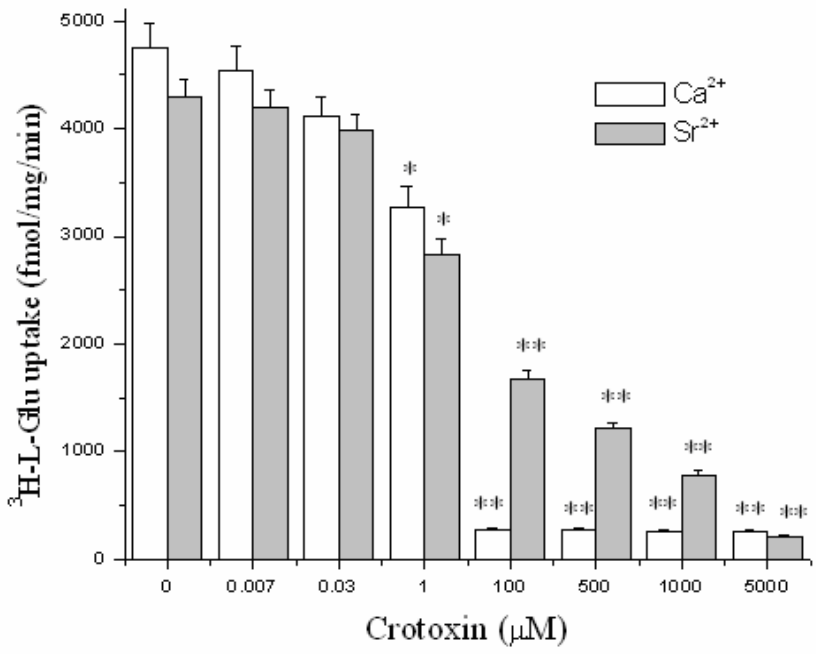

Figure 5. ^{3}H-L-glutamate uptake by RCCS. 2 mM of Ca^{2+} or 7.6 mM of Sr^{2+} and 1 mM of EGTA were added. Different crotoxin concentrations were incubated at room temperature with RCCS for 20 min followed by the uptake assay. Asterisks indicate significant uptake inhibition (**$p<0.01$; *$p<0.05$).

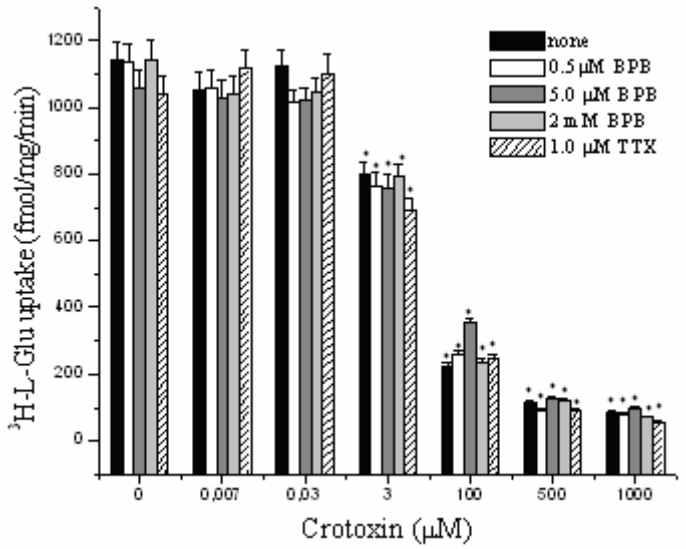

Figure 6. ^{3}H-L-glutamate uptake by RCCS. BPB was incubated with the toxin at different concentrations for 1h at room temperature. 1 µM of TTX was previously incubated with RCCS for 30 min before the uptake assay. (** *p<0.01*).

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