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Neuroprotective effect on brain injury by neurotoxins from the spider *Phoneutria nigriventer*

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Abstract

The role of calcium channels blockers in ischemic condition has been well documented. The PhTx3 neurotoxic fraction of the spider *Phoneutria nigriventer* venom is a broad-spectrum calcium channel blocker that inhibits glutamate release, calcium uptake and also glutamate uptake in synaptosomes. In the present study we describe the effect of PhTx3 (1.0 μg/mL), ω-conotoxin GVIA (1.0 μmol/L) and ω-conotoxin MVIIC (100 nmol/L) on neuroprotection of hippocampal slices and SN56 cells subjected to ischemia by oxygen deprivation and low glucose insult (ODLG). After the insult, cell viability in the slices and SN56 cells was assessed by confocal microscopy and epifluorescence, using live/dead kit containing calcein-AM and ethidium homodimer. Confocal images of CA1 region of the rat hippocampal slices subjected to ischemia insult and treated with ω-conotoxin GVIA, ω-conotoxin MVIIC and PhTx3 showed a percentage of dead cells of 68%, 54% and 18%, respectively. The SN56 cells subjected to ischemia were almost completely protected from damage by PhTx3 while with ω-conotoxin GVIA or ω-conotoxin MVIIC the cell protection was only partial. Thus, PhTx3 provided robust ischemic neuroprotection showing potential as a novel class of agents that targets multiple components and exerts neuroprotection in *in vitro* model of brain ischemia.

Keywords: Brain ischemia; Glutamate; Hippocampus slices; Neuroprotection; SN56 cells

1. Introduction

The high energy demand of the brain results from its requirement to maintain the ionic gradients and the resting membrane potential. Energy deprivation exceeding few minutes may cause irreversible damage to brain tissue. During cerebral ischemia, the lack of energy in the brain may depolarize neurons causing a large increase in release of neurotransmitters such as glutamate, aspartate, dopamine and serotonin (Siesjo, 1992a,b; Milusheva et al., 1996).

Cell calcium plays a key role in mediating ischemic neuronal damage and thus it is crucial to understand how calcium is regulated in ischemic conditions. The main entry of calcium in neurons is through voltage-dependent calcium channels (VDCC), which open in response to cell depolarization (Siesjo, 1992a,b). Glutamate, through an action on *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-metil-4isoxiazole propionate (AMPA) receptors, also allows calcium entry into the cell (Choi, 1992; McCulloch, 1992).

Glutamate action on metabotropic receptors leads to the formation of diacylglycerol and inositol tri-phosphate, which upon activation of enzymes causes the release of calcium from intracellular stores (Choi, 1992). The net result of the above mechanisms is an increase in cytoplasmic calcium concentration that activates proteases, nucleases, phospholipases, nitric oxide synthase and other degradative enzymes. This leads to an increase in free radical production and subsequent cell death (Siesjo, 1992a,b). Thus, calcium ions play an important role in

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the pathogenesis of ischemic brain injury (Siesjo, 1981). Consistent with this model, a growing body of experimental evidence shows that calcium blocker agents that reduce calcium entry through either neurotransmitter receptor-regulated ionotropic channels or voltage-dependent calcium channels (VDCC) have the potential to diminish brain injury (Choi, 1995).

At least six classes of neuronal VDCC, designated L, N, T, P, Q and R have been described in mammalian systems (Dunlop et al., 1995). However there are few available pharmacological agents that modulate these channels in a selective manner. Moreover, the role of individual VDCC subtypes in ischemia-induced neuronal injury remains largely unknown. Nevertheless, accumulating evidence suggests that certain neuronal VDCC subtypes may be valuable targets for therapeutic intervention in ischemic injury. For example, selective L-type channel blockers are useful to treat a variety of cardiovascular disorders through their actions on VDCC in cardiac and smooth muscle as well as decreasing the incidence and severity of ischemic deficits in patients with subarachnoidal hemorrhage. However, these blockers do not protect the neurons from the consequences of ischemia (Wauquier et al., 1988).

There is a strong relationship between excessive Ca^{2+} influx and glutamate release on neuronal injury (Choi, 1988). Therefore, the initial focus for cytoprotective approaches was to use antagonists of glutamate receptors to block glutamate-induced raise in $[Ca^{2+}]_i$. However, it is now apparent that blocking glutamate receptors may not the best approach, mainly due to the adverse side effects produced by these antagonists on normal brain (Muir and Lees, 1995).

The PhTx3 fraction purified from the venom of the spider *Phoneutria nigriventer* inhibits glutamate uptake (Reis et al., 1999) and also acts on P/Q types of calcium channels abolishing both the calcium-dependent glutamate release and the increase on $[Ca^{2+}]_i$ induced by K^+ depolarization from synaptosomes (Prado et al., 1996). Spider and snail toxins have shown to be fundamental to the identification and pharmacological definition of distinct channels (Prado et al., 1996; Rajendra et al., 2004). In the present study, we investigated the cytoprotection induced by spider neurotoxin fraction PhTx3 and snail toxins ω-conotoxin GVIA and ω-conotoxin MVIIC, on neuronal damage induced by ODLG insult on hippocampal slices.

2. Materials and methods

2.1. Drugs

The PhTx3 was purified according to Cordeiro et al. (1993) and contains six toxin isoforms, Tx3-1 to Tx3-6. The snail toxins ω-conotoxin MVIIC and ω-conotoxin GVIA were obtained from Peptides, Japan. All other chemical reagents were of analytical grade.

2.2. Preparation of hippocampal slices

All animal procedures were approved by a local Ethics Committee and followed the guidelines for the Use and Care of Animals for Research issued by the NIH. Wistar rats (180–200 g) were killed by decapitation and their brains rapidly (<1 min) removed and submerged in cold (4 °C) artificial cerebrospinal fluid (ACSF) containing (in mmol/L): 127 NaCl, 2 KCl, 10 glucose, 1.2 KH_2PO_4, 26 $NaHCO_3$, 2 $MgSO_4$, 2 $CaCl_2$, 10 HEPES bubbled with 95% O_2/5% CO_2. Both hippocampi were dissected and 7/8 transverse slices (400 μm) were obtained with a McIlwain Tissue Chopper (Brinkman Instruments Inc., UK). The first three (septal) slices of each hippocampus were discarded and the other slices were placed on nylon mesh platforms in a covered incubation chamber. The slices were submerged on ACSF bubbled with 95% O_2/5% CO_2 for 60–90 min at 36.5°. The slices that were to be used during ODLG insult were transferred to small chambers (25 mL). After 30 min the slices on their floating nylon platforms were transferred to insult chambers (25 mL) 95% N_2/5% CO_2 bubbled ACSF with 4 mmol/L glucose for 10 min at 36.5 °C in the presence or absence of the test toxins (Monette et al., 1998). Treatments (test toxins) were present for 30 min prior to, and during 10 min of ODLG insult on hippocampus. Thus the slices obtained from the same animal were incubated in ischemic and control conditions. For each experiment ($n = 3$), there were 3–4 ischemic slices untreated/treated toxins that were incubated together on the perfusion chamber. Thereafter, the slices were washed (with ACSF) during the 4 h reperfusion period prior to assessing neuronal viability. Under the above conditions, hippocampal slices can be maintained in a viable state for up to 12 h (Monette et al., 1998).

2.3. Confocal microscopy

To analyze cell viability the slices were stained with 6 μmol/L ethidium homodimer-AM and 4 μmol/L calcein-AM (live/dead assay, Molecular Probes, Eugene, OR) for 30 min and then washed for 15 min in 2 mL of 95% 02/5%$C0_2$ ACSF at room temperature. During the staining procedure the slices were protected from light. Viable cells show green fluorescence of calcein and dead cells were indicated by the red fluorescence of the ethidium homodimer. Slices were imaged in a Bio-Rad MRC 1024 UV confocal system equipped with a krypton–argon laser. Confocal images showing a specific red-fluorescent nucleic acid staining of dead cells were collected and used to quantify the ischemia-induced neurotoxicity. We used the Confocal Assistant© software to combine consecutive optical sections (1024 × 1024) from a Z-series to create image reconstructions. To improve images for quantitative analysis, they were processed using the median filter. Quantification of dead cells required identification of their nuclei fluorescently stained with ethidium homodimer. In the current approach, we define nucleus as connected pixels that were above a threshold. This threshold was calculated using the image histogram, and pixels below the threshold were set to 0. Regions in the CA1 area of hippocampus were selected from the threshold images and analyzed using The MetaMorph® Imaging System to calculate the percentage of threshold area in the image which reflects the number of dead cells.

2.4. Cell culture

SN56 cells were a generous gift of Prof. Bruce Wainer (Department of Pathology, Emory University School of Medicine, Atlanta, GA) and were maintained as previously described (Santos et al., 2001). Medium was changed every four days and they were differentiated in serum-free medium supplemented with 1 mmol/L dibutyryl-cyclic AMP (Sigma Chemical Co., St. Louis, MO) for 4 days. The SN56 cells were subjected to ischemia during 10 min in the presence/absence of the toxins. After 4 h reperfusion they were stained with 4.0 mmol/L calcein and 2.0 mmol/L ethidium homodimer (Molecular Probes, Eugene, OR). The images were obtained with an Axioskop Zeiss microscope coupled to a Micromax CCD camera. Image analysis and processing were performed with the software Lasersharp (BioRad)®, Confocal Assistant®, and Metamorph® (Universal Imaging, Wester Chester, PA).

2.5. Statistical

Statistical analysis was performed by analysis of ANOVA with a significance of $P < 0.05$.

3. Results

The neuroprotective effect of PhTx3 and two different Ca^{2+}-channel blockers ω-conotoxin MVIIC and ω-conotoxin GVIA on slices of hippocampus subjected to ischemia was tested against the cell death due to the ODLG. Fig. 1A shows ischemic slices treated/untreated by the toxins. The hippocampal slices subjected to a period of 10 min ODLG insult in the presence of 1.0 μmol/L of ω-conotoxin GVIA, a blocker of N-type Ca^{2+}-channel, showed a partial cell protection with a reduction of red cells (dead cells) and a slight increase of live cells (green cells) of CA1 hippocampal slices. Quantitative analysis of dead cells showed a value of $68 \pm 2.3\%$ ($P < 0.05$) (Fig. 1B). The confocal images of CA1 hippocampal slices subjected to ischemia in the presence of 100 nmol/L ω-conotoxin MVIIC, a blocker of N-, P- and Q-type Ca^{2+}-channels, showed a higher cell protection than that induced by ω-conotoxin GVIA. Thus it was observed an increase of live cells (green cells). Quantitative analysis of dead cells showed a value of $54 \pm 2.9\%$ ($P < 0.05$) (Fig. 1B). There was a statistical difference between the protection induced by ω-conotoxin GVIA and that of ω-conotoxin MVIIC ($P < 0.05$).

The neuroprotection of the *P. nigriventer* toxin fraction, PhTx-3, a wide-spectrum blocker of Ca^{2+}-channels on hippocampal slices subjected to 10 min ODLG insult (Fig. 1A) shows that in the presence of 1 μg/mL PhTx-3, the green live cells were abundant with a virtual exclusion of red dead cells, indicating that deprived slices treated with PhTx-3 were much healthier than those subjected to other treatments. Confocal images of treated-deprived slices with PhTx3 were almost identical to non-deprived slices of control experiments. Quantitative analysis of dead cells showed a value of $18 \pm 4.1\%$ (Fig. 1B).

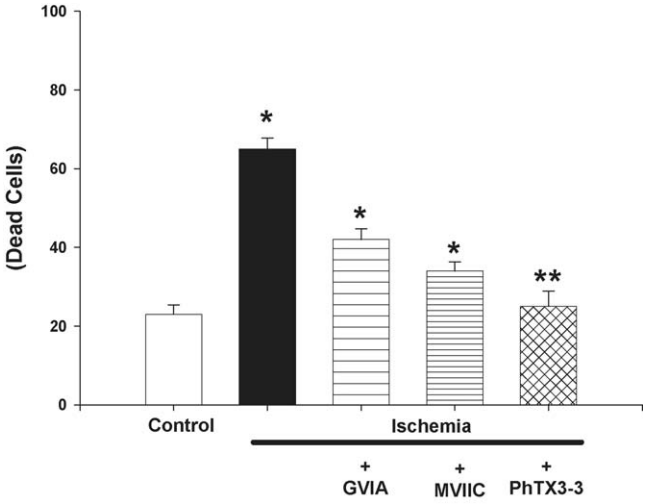

Fig. 2. Effect of 1.0 μmol/L of ω-conotoxin GVIA, 100 nmol/L of ω-conotoxin MVIIC and 1.0 μg/mL of PhTx3 on SN56 cells subjected to ODLG insult. The results express the mean ± S.E.M. of dead cells per field obtained by the analysis of 8 fields in 3 different experiments (100 cells were counted/field). Control cells were not subjected to ODLG insult. *Different from control, $P < 0.05$; **no difference from control, $P > 0.05$.

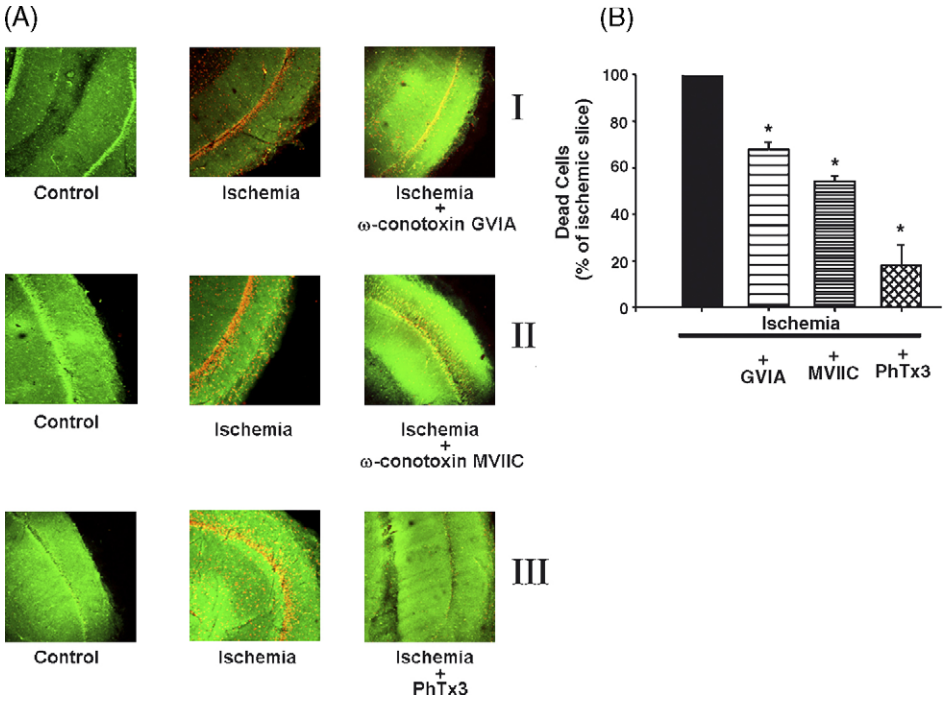

Fig. 1. (A) Confocal images (10× magnification) of hippocampal slices subjected to ischemia by oxygen deprivation and low glucose (ODLG) insult in treated/untreated slices with 1.0 μmol/L of ω-conotoxin GVIA (I), 100 nmol/L ω-conotoxin MVIIC (II) and 1.0 μg/mL of PhTx3 (III). Control slices were not subjected to ischemia induced by ODLG insult. The red color indicates cell dead and green color live cells (see Section 2 for details). (B) Percentage of dead cells on hippocampal CA1 region of the slices submitted to ischemia insult and treated with 1.0 μmol/L of ω-conotoxin GVIA, 100 nmol/L ω-conotoxin MVIIC and 1.0 μg/mL of PhTx3. Dead cells in the slices is given as percentage of the respective ischemic untreated slices, as shown in (A). The result represents the mean ± S.E.M. of three separated experiments. *Different from ischemic slice without treatment, $P < 0.05$.

Fig. 2 shows the effect of ω-conotoxin GVIA, ω-conotoxin MVIIC and PhTx3 on SN56 cells subjected to oxygen deprivation and low glucose (ODLG) insult. In the presence of 1.0 μmol/L ω-conotoxin GVIA or 100 nmol/L ω-conotoxin MVIIC the percentage of dead cells were $44 \pm 3.8\%$ and $36 \pm 2.8\%$, respectively. The percentage of dead cells in the presence of 1.0 μg/mL PhTx3 was $27 \pm 4.7\%$. This value of dead cells was not statistically different from that found in cells incubated in non-ischemic condition (control) (25 ± 3.5), suggesting total protection.

4. Discussion

The *in vitro* model of ischemia induced by ODLG insult on hippocampal slices has proven to be useful to test the neuroprotector efficacy of novel therapeutic agents. It is widely accepted that neurons and oligodendrocytes seem to be more vulnerable to ischemia than astroglial and endothelial cells. Among neurons, specific populations appear to be particularly susceptible to energy deprivation, such as CA1 hippocampal pyramidal striatal neurons and cerebellar Purkinje cells (Pulsinelli, 1985; Centonze et al., 2001). The fluorescence procedure used in our experiments using confocal microscopy by staining with calcein-AM and ethidium homodimer (Small et al., 1997) to determine the viability of cells or slices subjected to ischemia, facilitates reliable and quantitative assessment of cell damage.

Toxins that interfere with discrete populations of calcium channels are proving to be useful to manipulate neurotransmitter release in pathological conditions, such as ischemia injuries and pain (Miljallich and Ramachandran, 1995). Therefore, toxins from *P. nigriventer* venom that block VDCC (Prado et al., 1996; Miranda et al., 1998, 2001) may provide a new source of drugs for therapeutic intervention on neuronal calcium channels. The venom of the Brazilian spider *P. nigriventer* possesses several toxic polypeptides fractions, some of which are neurotoxic (Cordeiro et al., 1993). Over the past decade there has been a series of *in vitro* studies examining the effect of *Phoneutria* neurotoxins on channel activity. One of these components, designated PhTx3, suppressed both the Ca^{2+}-dependent glutamate release and the increase in cytosolic free calcium that occurred in response to membrane depolarization by KCl (Prado et al., 1996). The purification of PhTx3 yielded six homogeneous isotoxins, Tx3-1 to Tx3-6 (Cordeiro et al., 1993). The isoforms Tx3-3 and Tx3-4 inhibit glutamate release through blockade of P/Q calcium channels (Prado et al., 1996) and they also are very effective to inhibit tityustoxin-induced $^{45}Ca^{2+}$ uptake in synaptosomes, IC_{50} of 0.32 and 7.9 nmol/L, respectively (Miranda et al., 1998, 2001). Recently, we also demonstrated that Tx3-6, another isoform of PhTx3, causes inhibition of N-type calcium channel expressed in HEK cells (Vieira et al., 2005). Therefore, the PhTx3 fraction purified from the venom of the spider *P. nigriventer* is a broad-spectrum neuronal calcium channel blocker that also presents a specific action to inhibit glutamate uptake (Reis et al., 1999). To our knowledge, there are no other polypeptide fraction described that present such effects. It has been shown that glutamate release that occurs during severe brain ischemia is mainly caused by reversed operation of glutamate uptake (Rossi et al., 2000).

This manuscript is the first to examine spider neurotoxin in a model of ischemia, and as such brings us a step closer to utilizing this compound or components from this compound *in vivo* systems. The data also provides further evidence to support the concept that multidrug or multicomponent action is more beneficial than a compound that interferes with only one pathway. However, the use of neurotoxins as therapeutic agents must be extensively investigated before it undergoes clinical studies. Therefore, it is essential to test all these toxins *in vivo*, in various mammals models.

In addition the release of the excitatory neurotransmitter glutamate is regulated by more than one calcium channel blockers (Olivera et al., 1994) and it has been suggested that broad-spectrum neuronal Ca^{2+} channels blockers might be better cytoprotective agents than more specific neuronal Ca^{2+} channels blockers (Maroto et al., 1996). PhTx3 fraction, derived from spider *P. nigriventer* contains several isoforms of toxins that blocks N and P/Q calcium channels is more effective to attenuate cell damage than the snail derived toxins used in our experiments. Notably, the comparison between the effects of PhTx3, ω-conotoxin GVIA and ω-conotoxin MVIIC showed that the spider toxin-fraction PhTx3 provides better protection of hippocampal slices and SN56 cells subjected to ischemia than both snail toxins.

Our results showing neuroprotection induced by ω-conotoxin GVIA agrees with the relative contribution of N-type calcium channel to synaptic transmission in the hippocampus, known to control 40% of glutamate release (Wu and Saggau, 1994). On the other side, in our hands, the cytoprotection induced by ω-conotoxin MVIIC was close to half of that reported by Small et al. (1997). The lower cytoprotective effect of ω-conotoxin MVIIC demonstrated in our present experiments agrees with our previously published data whereby Tx3-3 and Tx3-4 isoforms contained in the PhTx3 fraction were more efficient than ω-conotoxin MVIIC to inhibit the increase in $^{45}Ca^{2+}$ uptake induced by synaptosomes depolarization (Miranda et al., 2001).

In conclusion, the spider fraction of PhTx3, which blocks a broad-spectrum of VDCC and also attenuates calcium-independent glutamate release due to the reversal of glutamate transporters was more neuroprotective than the more specific calcium channel blockers, ω-conotoxin GVIA and ω-conotoxin MVIIC in hippocampal slices and SN56 cells subjected to ODLG insult. Future experiments aimed to develop therapies to prevent neuronal damage could use some of the individual components present in the PhTx3 fraction that represent a new class of toxins to be tested for *in vivo* cell protection.

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