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First egg protein with a neurotoxic effect on mice

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ABSTRACT

While many invertebrates sequester toxic compounds to endow eggs with chemical defences, here we show, for the first time to our knowledge, the identification of a neurotoxin of proteinaceous nature localized inside an egg.

Egg extracts from the freshwater apple snail *Pomacea canaliculata* displayed a neurotoxic effect in mice upon intraperitoneal injection (i.p.) (LD50, 96 h 2.3 mg/kg). Egg protein and total lipids were analysed separately and the only fraction displaying a highly toxic effect (LD50, 96 h 0.25 mg/kg, i.p.) was further purified to homogeneity as an oligomeric glyco-lipoprotein of 400 kDa and two subunits biochemically and immunologically indistinguishable from the previously described perivitellin PV2. The neurotoxin was heat sensitive and there was evidence of circulating antibody response to sublethal i.p. doses on mice.

Clinical signs, histopathological and immunocytochemical studies revealed damage mostly in mice spinal cord. Experiments showed chromatolysis and a decreased response to calbindin D-28K associated with a significant increase of TUNEL-positive cells in the dorsal horn neurons. These results suggest that calcium buffering and apoptosis may play a role in the neurological disorders induced by the toxin in mammalian central nervous system.

This is the first report of a mollusc neurotoxin genetically encoded outside the cone-snail species.

1. Introduction

Pomacea canaliculata (Gastropoda: Ampullariidae) is a freshwater South American species. Commonly referred to as "apple snails" these Arquitaenioglossa are exceptionally well adapted both to temperate regions, where thermal changes during the year are large (Albrecht et al., 1999, 2005) and to subtropical and tropical regions where periods of drought may alternate with periods of excessive rainfall (Pizani et al., 2005). Among their adaptation is the strategy to deposit their eggs above the waterline in a calcareous, colored clutch. This remarkable egg-laying strategy is very unusual for an aquatic invertebrate and exposes the eggs to sunlight, desiccation and terrestrial predators. To cope with these harsh conditions ampullariids have developed some interesting mechanisms, including a protective and nourishing perivitellin fluid surrounding the embryos. *P. canaliculata* eggs are provided with a multifunctional perivitellin called ovorubin which play critical roles for embryo development such as photoprotection, antioxidant, trypsin inhibitor and nutrient provision and also provide the eggs with a pink-reddish

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colour (Heras et al., 1998; Dreon et al., 2004b, 2006; Heras et al., 2007). Ovorubin functions are complemented by perivitellin PV2, which has been described as a source of nutrients during embryogenesis (Heras et al., 1998).

The conspicuous reddish colour of egg clutches suggests a warning coloration (Heras et al., 2007) and, therefore, the presence of egg defences taken for granted. In fact, field evidence of egg unpalatability has been provided in a related species (*Pomacea paludosa*) by the fact that most animals foraging in habitats where the apple snails live ignore these eggs. Moreover, when eggs from *P. paludosa* were offered to captive predators, though they ate them at first, they refused them after repeated feeding (Snyder and Snyder, 1971). The aim of the present work was to study *P. canaliculata* egg composition with a view to identify the unpalatable compound, but unexpectedly it came out with the first toxin of proteinaceous nature in the animal kingdom located inside an egg. As in mouse tests the toxin provoked death showing symptoms suggesting neurological damage, we examined the possible effects of the purified toxin on the central nervous system. In particular, we focused on the Ca^{+2}-binding protein calbindin D-28K and on cell death mediated by apoptosis in spinal cord neurons. We demonstrated the presence of the first mollusc neurotoxin genetically encoded outside the cone-snail family Conidae.

2. Materials and methods

2.1. Eggs

Egg masses from *P. canaliculata* were collected either from females raised in our laboratory or taken from the wild between November and March of consecutive seasons in streams or ponds near La Plata city, Buenos Aires Province, Argentina. All egg masses employed had embryos developed to no more than the morula stage. Embryo development was checked in each egg mass microscopically as described elsewhere (Heras et al., 1998).

2.2. Mice

Female BALBcAnN mice, 4 weeks old, weighing 19–21 g from a colony started with a stock provided by NIH USA and bred in a specific pathogen free (SPF) environment were employed in the experiments. All the studies performed with animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

2.3. Preparation of toxin fractions

Fertilized eggs were repeatedly rinsed with ice cold 20 mM Tris–HCl, pH 6.8, containing 0.8 μM aprotinin (Trasylol, Mobay Chemical Co., New York) and homogenised in a Potter type homogeniser (Thomas Sci., Swedesvoro, NJ). Ratio of buffer:sample was kept 5:1 v/w. The crude homogenates were then sonicated for 15 s and centrifuged sequentially at 10,000*g* for 30 min and at 100,000*g* for 60 min. The pellet was discarded and the supernatant was employed for experiments or further fractionated into their proteins and lipids. Lipids were extracted with chlorophorm:methanol (Bligh and Dyer, 1959). Proteins were then further fractionated and each fraction analysed for toxic effect.

2.4. Isolation and purification of toxin

First screening: a 100,000*g* supernatant protein was fractionated using a Merk-Hitachi high performance liquid chromatograph (HPLC) (Hitachi Ltd., Tokyo, Japan) with an L-6200 Intelligent Pump and an L-4200 UV detector set at 280 nm. The sample was analysed in a Mono Q™ 10/100 GL Tricorn column (Amersham Biosciences, Uppsala, Sweden) using a gradient of 0–1 M NaCl in a Tris–HCl 20 mM pH 8.5 buffer. Total protein of each fraction was measured by the method of Lowry et al. (1951), and each fraction assayed for toxicity (see below).

Final purification: an ultracentrifugation step was added (sample was layered over NaBr 1.26 g/ml) and ultracentrifuged at 207,000*g* at 10 °C for 22 h, on a Beckman L8M (Beckman, Palo Alto, CA) and the toxin-containing fraction, which corresponded to the egg perivitellin PV2 was then analysed by ion-exchange chromatography as described above. The toxin peak was then further purified by size-exclusion chromatography and purity of the single peak obtained was checked by native PAGE.

Native and subunit composition of the toxin was determined by PAGE and cross reactivity with PV2 checked by Western blot analysis using an anti-PV2–polyclonal antibody (PAb) prepared in previous studies. Analysis was performed as previously described (Dreon et al., 2002, 2004a). Low and high MW markers for PAGE were purchased from GE Healthcare Bioscience (Uppsala, Sweden). Western blot MW standards were Chemiblot MW markers (Chemicon International, Temecula, CA).

2.5. Toxin assays

2.5.1. Mice toxicity tests

Groups of 10 mice (20 ± 2 g) were injected i.p. with a single dose of 300 ml phosphate buffer saline (PBS) or the same volume of serial dilutions of five toxin concentrations of either cytosol or PV2 toxin and the LD50 determined by a lethality test 96 h after injection. Statistical analysis of data to establish median lethal concentrations (LD50) with 95% confidence limits (CL) were analysed by PROBIT using the program Toxdat (US, EPA). LD50 was calculated for each experiment and averaged.

Lipid extracts obtained from 50 mg egg were dissolved in 20 μl DMSO and 300 μl PBS and injected i.p. to mice. A group receiving only DMSO and PBS was included as control.

2.5.2. Assays for heat susceptibility

Purified PV2 toxin was heated at 100 °C for 15 min and tested for activity by i.p. injection to mice (1 mg/kg, $n = 5$).

2.5.3. Mice immunization analysis

To check if mice were capable of becoming immunized against the toxin, animals were subjected to an intraperitoneal, sublethal injection of 3 μg of purified PV2, with a second injection of 30 μg in PBS 19 days after. No

adjuvants were employed. Serum was harvested 4 days later, and kept at −70 °C until used.

Spots of 1 μg PV2 toxin were deposited onto nitrocellulose membranes (Amersham). After blocking for 1 h at 37 °C with 5% (w/v) non-fat dry milk in PBS–Tween, the membranes were incubated overnight at 4 °C with the anti-sera dilutions in 1% (w/v) non-fat dry milk in PBS–Tween. Specificity of mice sera towards PV2 was detected by anti-mouse IgG horseradish peroxidase conjugate (Bio Rad Laboratories, Inc.). An anti-PV_2 polyclonal antibody was prepared in previous studies and used as positive control (Dreon et al., 2002, 2003). Immunoreactivity was visualized by electro-chemi-luminiscence.

2.5.4. Histopathology analysis

Groups of six mice were injected i.p. with a single dose of PBS (control group) or with 1 mg/kg PV2 (19 μg per mouse). After 40 h animals were anesthetized by an injection of Ketamine hydrochloride (40 mg/kg i.p.) plus Xylazine (8 mg/kg, intramuscular) and then perfused transcardially with 50 ml (PBS) followed by 50 ml of 4% paraformaldehyde in PBS.

2.5.5. Tissue samples

Samples of the main organs namely heart, liver, kidney, spleen, uterus, stomach and intestine were post-fixed in formaldehyde and then embedded in paraffin wax. Spinal cord was carefully removed, kept in the same fixative for 3 h and then in Bouin for 3 days.

Representative 5–7 μm sections were stained with haematoxylin and eosin for histological examination of general morphology. Besides, gallocyanin staining was performed in nervous tissue samples to highlight nuclear morphology and Nissl substance.

2.5.6. Calbindin immunohistochemistry

Eight sections representative of lumbar, dorsal and cervical regions of spinal cord of each animal were assayed by immunohistochemistry (IHC) to evaluate calbindin levels. Sections were incubated with a primary monoclonal mouse anti-calbindin 1:100 (Novocastra, USA) overnight at 4 °C. The Envision plus kit (Dako, Carpinteria, CA, USA) based on a dextran polymer coupled to horseradish peroxidase (HRP) and secondary antibodies was employed as IHC detection system. Positively stained cells showed a golden dark brown colour using 3,3′-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 reaction product. All sections were then counterstained with Mayer's haematoxylin before analysis. Primary antibody was replaced by normal mouse anti-serum in control sections.

2.5.7. TUNEL staining of DNA fragmentation

Apoptosis was revealed by *in situ* terminal deoxynucleotidyl transferase (TdT)–UTP–biotin nick end labelling (TUNEL) using the Apoptag Plus Peroxidase *in situ* Apoptosis Detection kit (Chemicon International, USA). In short, DNA fragmentation on apoptotic cells generates many free 3′-OH DNA ends of double-stranded and single-stranded DNA which are *in situ* revealed by adding chemically labelled and unlabelled nucleotides using TdT. The incorporated nucleotides form an oligomer composed of digoxigenin-conjugated nucleotide and unlabelled nucleotide in a random sequence. DNA fragments labelled with the digoxigenin–nucleotide are then allowed to bind an anti-digoxigenin antibody conjugated to a peroxidase reporter molecule. The bound peroxidase antibody conjugate generated a localized stain from chromogenic substrates. The slides were then counterstained with Mayer haematoxylin. Negative controls were always included in each series of assayed sections.

3. Results

3.1. Isolation and identification of the toxin

The crude-egg extract (100,000*g* supernatant) was first loaded into an anion-exchange column and three proteins fractions eluted (Fig. 1A). Only the fraction containing the PV2 perivitellin was identified as toxic, while ovorubin and the other apple snail egg proteins showed no toxicity to mice upon i.p. injection of 6 mg/kg. Lipid extracts were also non-toxic, discarding the possibility of a lipidic or lipophilic toxin.

To further purify and identify the toxin, an ultracentrifugation step was added and the fraction enriched in PV2 ($\delta = 1.26$ g/ml) was collected and injected into an ion-exchange column. The fraction, now very much enriched in PV2 was checked on mice and conserved toxicity. A final purification by size-exclusion chromatography was performed (Fig. 1B) and the purity and homogeneity of toxin were checked by native PAGE electrophoresis giving a single protein band (Fig. 1B, inset). The purified protein has a mass of 400 kDa and two subunits of 31 and 67 kDa further indicating a similarity with PV2 perivitellin (Fig. 2A and B). The strong cross reactivity observed by Western blot analysis using anti-PV2 antibodies gave the final evidence that the toxin indeed corresponds to the perivitellin PV2 (Fig. 2C). Toxicity was completely lost by heating the toxin. Interestingly, toxicity of either the extracts or the purified toxin did not seem to diminish by freezing. Moreover, mice receiving sublethal doses of toxin became immunized towards the toxin and doses up to LD100 did not kill them. Sera of immunized mouse were positive in a Dot blot performed using purified PV2 as antigen (Fig. 3).

The LD50, 96-h values determined were 2.3 mg/kg for whole egg extract and 0.25 mg/kg for purified PV2 neurotoxin.

3.2. Symptomatology and toxin assays

Mice injected i.p. (19 μg/20 g) with purified PV2 showed first behavioural changes after 16–20 h. These included weakness and lethargy, low head and bent down position (ortopneic), half-closed eyes, taquipnea, and hirsute hair. Besides, they presented extreme abduction of the rear limbs, paresia and were not able to support their own body weight (tetraplejic). The anal, perineal, abdominal, flexor and palpebral reflexes were absent while the plantar reflex was partially conserved. When mice tried to raise their tail, muscles showed spastic movements (tremors). As the toxin continued to take effect, alterations of deep sensitivity were observed with abnormal postures,

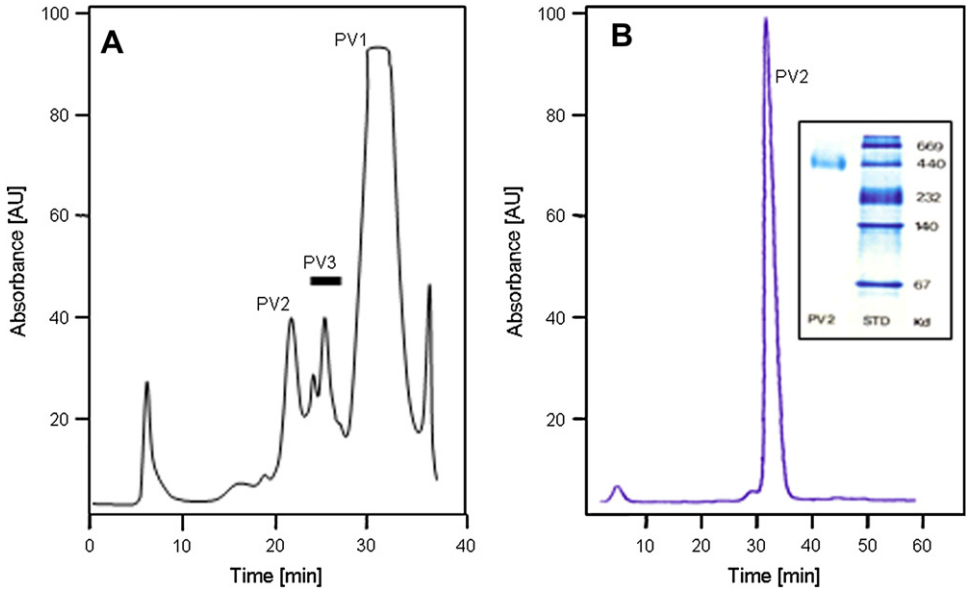

Fig. 1. Purification of toxin. (A) Crude-egg extract fractionated by ion-exchange chromatography. Three groups of proteins were assayed for toxicity (PV1, PV2 and PV3 brackets). (B) PV2 fraction from panel (A) was further purified by gel permeation, and purity checked by native PAGE electrophoresis (inset) and Western blot (see Fig. 2).

weakness, staggering standing, uncontrolled movements, oscillations and lack of muscle tone. After 30 h post-inoculation mice showed flaccid paraplegia. The rear limbs were unable to support their own body weight whereas the forelimbs remained functional.

Death in the majority of mice came after 40 h of injection with flaccid paralysis of the outstretched rear limbs. At higher doses (near LD100) death occurred in less than 30 h.

3.3. Histopathology and immunohistochemistry

Histopathological studies performed on dying mice after intraperitoneal injection did not reveal any morphological changes in most of the main internal organs examined, the only noticeable fact was a slight hyperhemia without the presence of inflammatory cells in uterus, intestine and nervous system. The only target organ for the toxin from the histopathologic screening was the spinal cord, so further analyses were performed in this organ.

Haematoxylin–eosin and gallocyanin stainings of spinal cord section indicated chromatolysis in the big motoneurons of the grey matter (not shown).

The immunohistochemical reaction for the Ca^{+2}-binding protein calbindin in spinal cord showed a decreased intensity in the dorsal horn of the grey matter of the toxin-treated animals. This was particularly evident in laminae II and III (following the laminar model proposed by Rexed (1952) and Paxinos and Watson (1986)) regardless of the medullar regions analysed (cervical, thoracic or

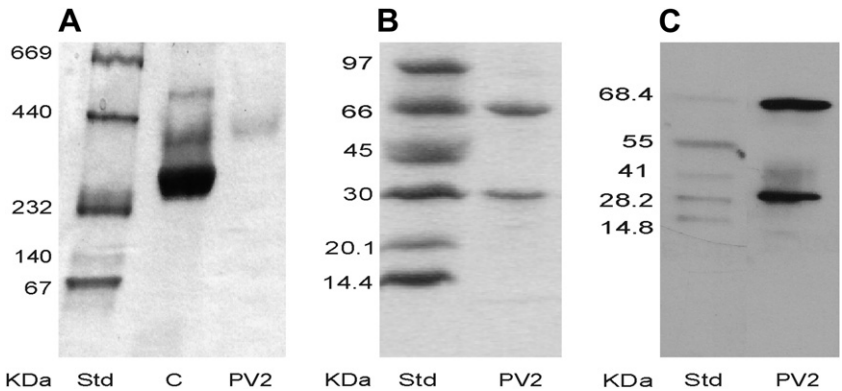

Fig. 2. Native (A) and dissociating gel electrophoresis (B) and Western blot analysis of toxic protein (C). Panel A: Native PAGE was carried out using 4–20% w/v polyacrylamide gradient. Lane 1: High molecular weight standards; Lane 2: egg cytosol (20 μg); Lane 3: purified toxin (3 μg) Panel B: SDS-PAGE was performed in 4–20% w/v polyacrylamide gradient. Lane 1: Low molecular weight standards; Lane 2: toxic protein (10 μg). Panel C: Immunoblotting of toxic protein using PV2–PAbs. A strong cross reactivity can be seen. Lane 1: Molecular weight standards; Lane 2: toxic protein subunits. PV2–PAb diluted 1:1000. Proteins were blotted onto nitrocellulose from 4 to 20% SDS-PAGE.

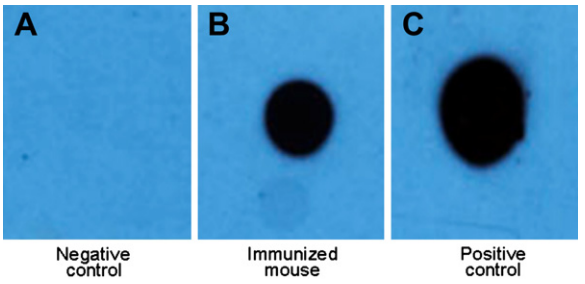

Fig. 3. Dot blot analysis of sera from immunized mice. Strong positive immunoreactivity can be seen towards PV2 antigen. Dots A and B: Sera from control and immunized mice, respectively, diluted 1:1000, Dot C: anti-PV2 polyclonal antibody diluted 1:2000. PV2 was blotted onto nitrocellulose (1 μg diluted in 5 μl PBS).

lumbar). Inoculated mice were either negative or showed minimum immunostaining for calbindin as compared to control animals, the reaction being more evident in the cervical region (Table 1 and Fig. 4).

As regards the white matter, no significant differences were observed between control and toxin-treated animals (results not shown).

The use of TUNEL stain revealed positive immunostaining of toxin-inoculated animals, at the dorsal horn of the grey matter, but only at the level of laminae II and III (Fig. 5). Labelled neurons were most notoriously observed at cervical level in a fashion similar to calbindin expression. This localization was coincident with the lack of immunostaining for calbindin on laminae II and III in the same group of animals. On the other hand, there were no TUNEL-positive neurons in spinal cord of control mice.

4. Discussion

4.1. Purification and identification of toxin and toxicity to mice

We have described the partial characterization of a novel toxin isolated from the eggs of the freshwater mollusc *P. canaliculata* which, upon intraperitoneal injection, produces a strong neurotoxicity in mice. After assaying egg extracts, total lipids and several protein fractions, we found that the toxicity was associated with only one of the proteins.

Table 1
Quali-quantitative analysis of calbindin levels in dorsal horn of the grey matter at three spinal cord levels

	Cervical 4	Thoracic 7	Lumbar 2
Control			
1	4+	3+	3+
2	4+	3+	3+
3	4+	2+	3+
4	3+	3+	2+
Toxin-treated			
1	3+	(−) 1+	−1+
2	(−) 1+	(−) 1+	2+
3	(−) 1+	(−)	(−)
4	3+	(−) 1+	(−)
5	(−)	(−)	(−)
6	(−)	(−)	(−)

(−) to 4 denote ascendant immunostaining gradient.

The biochemical, biophysical and immunological characterization of this toxic protein indicated that it was indistinguishable from PV2, one of the major proteins of the egg perivitellin fluid. This protein was first described more than 10 years ago and a considerable body of information is available. PV2 is moderately lipidated and glycosylated and composed of two subunits of 67 and 31 kDa. (Garín et al., 1996; Heras et al., 1998; Dreon et al., 2004a). Interestingly, the N-terminal amino acid sequences of PV2 subunits showed no homology with other related or non-related proteins (Dreon et al., 2002). Moreover, immunological studies have shown that PV2 does not cross react with perivitellins from *P. scalaris*, a related snail species (Ituarte et al., 2008), further highlighting the singularity of this protein.

When ascribing the toxic activity to PV2, we relied on their identical ultracentrifugation, ion-exchange HPLC, gel filtration, FPLC and electrophoretic properties (Figs. 1 and 2), which use different physicochemical separation principles. Both particles have the same electrophoretic pattern under native and dissociating conditions (see Garín et al., 1996 for comparison). Besides, polyclonal antibodies against PV2 strongly cross-reacted with the neurotoxin. This was further supported by the fact that mice receiving sublethal doses of the toxin became immunized and their sera also immunoreacted with purified PV2 (Fig. 3). This comprehensive purification scheme and the observation that the toxin was heat inactivated, reinforce its proteinaceous nature. In addition, the fact that lipid extracts were not toxic to mice discards the possibility that the lipid moiety of PV2 would be involved.

Compared to other toxins, PV2 can be classified as extremely toxic, considering that the LD50 96 h with a single dose was around 0.25 mg/kg. Although it is not as potent as marine snail conotoxins, it is in the same order as many snake neurotoxins (Gawade, 2004).

The highly specific action of neurotoxins has led neuroscientists to study key components of both vertebrate and invertebrate nervous systems, and many neurotoxins or their derivatives are being used as therapeutic agents, the snail toxins among them (Adams and Olivera, 1994). Snails possessing toxic compounds are usually divided into two groups: (a) venomous snails (such as conids) that are equipped with a specific apparatus for the synthesis and delivery of their venom and (b) poisonous snails (i.e., Opistobranchs, Neogastropoda and Neotaenioglossa) that lack a specialized venom apparatus but still have toxic compounds in their tissues (West et al., 1996). *P. canaliculata*, therefore, can be included in the latter group, their eggs being the first ones described in gastropod molluscs containing a neurotoxin.

4.2. Symptomatology and histopathology

We do not know if the intoxicated mice perceive pain (nociceptive), though no retraction of paralyzed limbs was observed when stimulated with a stubbing element.

Toxin produces acute toxicity in mice, the first clinical signs appearing 16 h after inoculation. The acute treatment of mice with PV2 produced morphofunctional modifications in neurons of the dorsal horn, with cytological

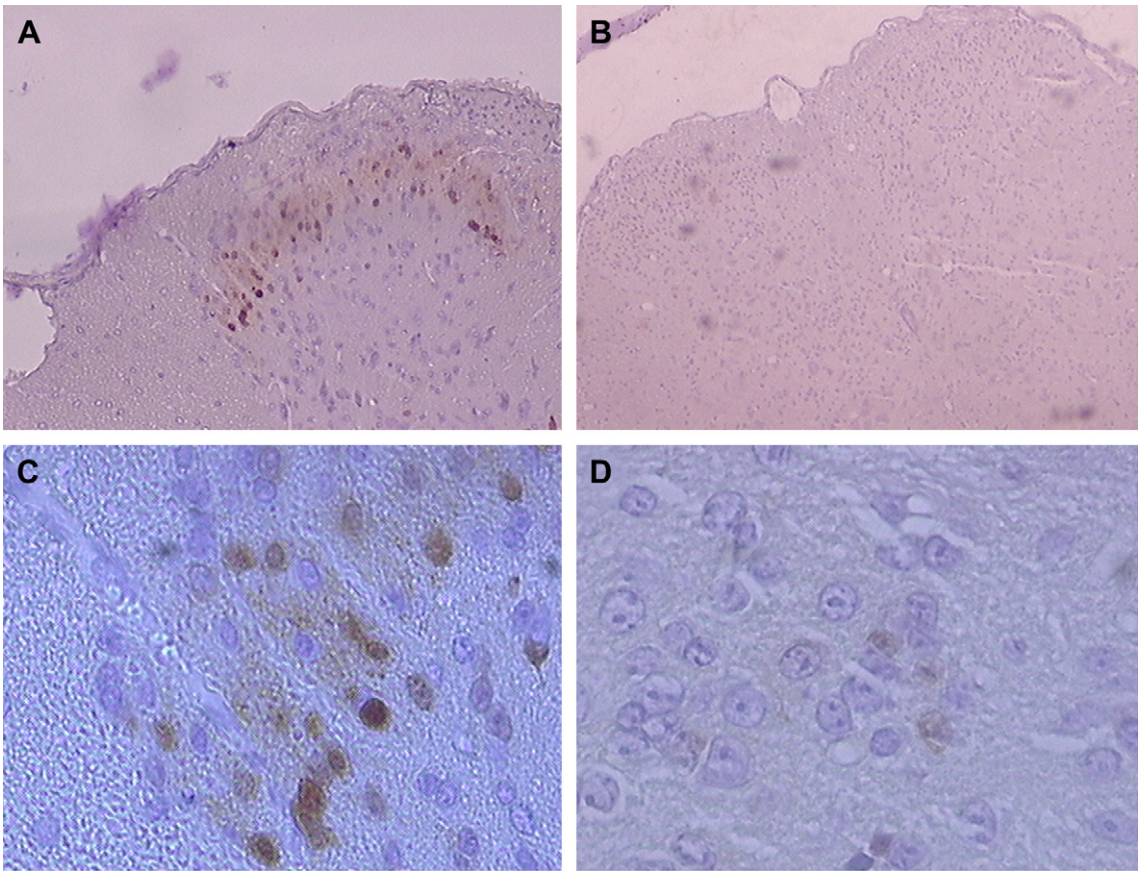

Fig. 4. Immunolocalization of calbindin at the dorsal horn (cervical spinal cord) from control (A, C) and PV2-injected mice (B, D). In treated samples, anti-calbindin decreased. The nuclei were counterstained with haematoxylin. A, B: 4×; C, D: 40×.

damage in laminae II and III at cervical, dorsal and lumbar regions.

Neuronal calcium plays a key role in neuron survival as well as programmed cell death (apoptosis) and pathological neuronal degeneration, and it has been shown that disturbances in calcium regulation can have potentially lethal effects (Bastianelli, 2003). The molecular basis of neurodegeneration in the central nervous system is related to an increased concentration of cytosolic free calcium (Ramirez-Exposito and Martinez-Martos, 1998). Thus, the regulation of calcium-binding protein expression is a critical factor to agents that disrupt calcium homeostasis in neurons (Lema Tome et al., 2006). The absence of calcium buffering proteins would lead to intracellular calcium accumulation rapidly reaching toxic amounts (Iacopino et al., 1990; Iacopino and Christakos, 1990; Mattson et al., 1991; Bastianelli, 2003). In fact, neurons rich in calcium-binding proteins, especially calbindin and parvalbumin, are supposed to be relatively resistant to degeneration in a variety of acute and chronic

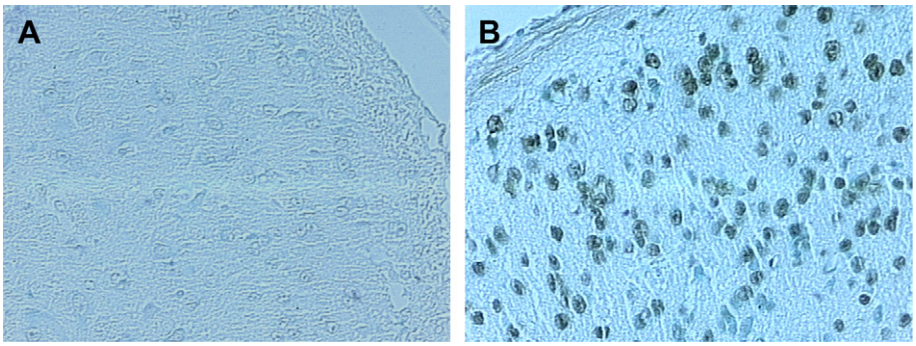

Fig. 5. TUNEL staining in the dorsal horn after PV2 intoxication. (A) Control, (B) 40 h post-injection (40×).

disorders (McMahon et al., 1998). In particular, calbindin D-28K is normally expressed in restricted neuronal populations of the mammalian brain where it plays a crucial role in protecting neurons against excitotoxic insults (Lee et al., 2004). Neurons containing calbindin, were immunostained in the superficial dorsal horn of the spinal cord, in laminae II, III in control rodents, similarly to what has previously been described for rats. In these rodents it has been suggested they may be important in primary afferent processing (Yamamoto et al., 1989; Yoshida et al., 1990). We observed in intoxicated animals that these regions displayed a significantly decreased immunostaining for calbindin. Moreover, our data showed that apoptotic cell death was restricted to the same localization in selected portions of the dorsal horn as the neuronal population which depicted the reduction in calbindin expression. This is coincident with many investigations reporting that the participation of calbindin as calcium buffer can avoid apoptosis in susceptible cells from the central nervous system (Wernyj et al., 1999). We can, therefore, suggest that reduced calbindin expression may proceed to marked degeneration of neurons at the laminae II and III or may accelerate the mechanism of neuronal degeneration by further facilitating the calcium-mediated cytotoxic events. The decrease in calbindin levels may be considered an early indicator of neurological disorders induced by acute PV2 toxicity. Moreover, a lower expression of calbindin has also been reported in the aged-brain and in several neurodegenerative disorders such as Parkinson disease, Alzheimer disease, Huntington's corea, brain isquemia and epilepsy (Iacopino and Christakos, 1990; Krzywkowski et al., 1995).

We have shown here that the PV2 toxin administration to mice may induce neuronal apoptosis within the dorsal horn of the spinal cord, and that a depletion of calbindin expression is involved. However, the mechanism of toxin-induced apoptosis in these areas needs further investigation.

The fact that PV2 is a large protein excludes any direct action on the spinal cord due to low permeability of the blood–nerve barrier. Nevertheless, its two subunits are small enough to be transported across the blood–nerve or blood–brain barriers and it is, therefore, reasonable to hypothesize that they could penetrate by any of the mechanisms postulated for proteins such as fluid-phase endocytosis (Poduslo et al., 1994). In addition, it has been reported that protein glycosilation, as it is the case of PV2, increases its permeability across the barrier (Poduslo and Curran, 1994). At present we do not know whether one or both subunits or their fragments reach the CNS, but the effect in spinal cord is manifest.

4.3. Possible function of the toxin in eggs

Animals may acquire toxicity by absorbing toxic compounds from their food, e.g., from plants or other animals. Sequestration and accumulation of toxins may provide protection from predators, which learn to avoid this defended prey because of unpleasant experiences such as bitter taste. This is a common phenomenon in marine as well as in terrestrial ecosystems (Mebs, 1998). These toxins may be transferred into eggs for embryo protection, where they accumulate reaching high, often lethal concentrations some of them being potent neurotoxins (Miyazawa et al., 1987). Unlike all neurotoxins from eggs thus far purified, we report here the presence of a neurotoxin of protein nature.

The metabolism of this protein has been previously studied, reporting its expression and storage restricted to an accessory gland of the female reproductive tract, to later become part of the egg perivitellin fluid and finally incorporated and degraded by the embryos during development (Heras et al., 1998; Dreon et al., 2002; Catalán et al., 2006). These early studies were made assuming PV2 was a classical reserve perivitellin, and not a multifunctional protein.

If we consider that the eggs of *P. canaliculata* are poisonous, and we recall that usually poisons consist of a complex mixture of toxic components that include protein and peptide toxins, enzymes and other active agents, we could expect other components of the egg apart from PV2 to be involved. In fact, eggs of *P. canaliculata* are equipped with an interesting assortment of chemical defences, that includes the carotenoprotein ovorubin with protease inhibitor activity and photoprotective actions, agglutinins and strong antioxidants such as astaxanthin (Norden, 1972; Uhlenbruck et al., 1973; Dreon et al., 2004b, 2006). Eggs collected in the wild were toxic if orally administered to mice (not shown), indicating that the potent neurotoxin PV2 probably complements these defences, in conformity with the defensive strategy of displaying a warning coloration (usual in chemically defended organisms), that allow snail embryos to develop outside the water protected from harsh conditions and from predators.

The observed effect of the toxin suggests a specific action in the vertebrate central nervous system.

Further biophysical, molecular biology and pharmacological analyses of the toxin are currently in progress. The field of snail toxinology outside the *Conus* species is still in its infancy, but its potential yield should attract more interest in the coming years.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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