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PHARMACOLOGICAL CHARACTERIZATION OF VENOMS OBTAINED FROM MEXICAN TOXOGLOSSATE GASTROPODS ON ISOLATED GUINEA PIG ILEUM

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ABSTRACT: The protein-containing extracts prepared from the venom ducts of *Conus austini*, *Conus spurius* and *Polystira albida* caused a concentration-dependent inhibition of spontaneous contractions in guinea pig ileum. The most potent extract was obtained from *P. albida* venom ducts (IC_{50} = 0.11 ± 0.02 µg protein/mL). The three extracts produced a moderate inhibition of contractions elicited by acetylcholine (ACh 1 µM), suggesting the presence of anticholinergic compounds. The contractile response elicited by nicotine (10 µM) was significantly reduced by the extracts prepared from the ducts of *C. austini* and *P. albida*, which indicates that the venom produced by these species contains toxins that target neuronal nicotinic receptors. All three extracts significantly inhibited contractions evoked by histamine (0.5 µM), particularly those from *C. spurius* and *P. albida*. These findings reveal the presence of antihistaminergic compounds not previously described in any conoidean venom. Finally, we found that only the extract prepared from *C. spurius* ducts decreased KCl (60 mM)-induced contractions, indicating that the venom of this snail contains compounds that block voltage-dependent Ca^{2+} or Na^{+} channels.

KEY WORDS: Conoidea, *Conus austini, Conus spurius*, guinea pig ileum, *Polystira albida*, Turridae.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Marine snails of the superfamily Conoidea produce venom to capture prey and to defend themselves against predators. By far, the most extensively studied toxoglossate gastropods are the cone snails that belong to the Conidae family (20, 28, 29). Modern *Conus* research has primarily focused on purifying and characterizing the toxins from the venom apparatus and on demonstrating that the poison from an individual cone snail contains several toxins, mostly small peptides (conotoxins) that target specific isoforms of receptors or ion channels (3, 6, 20, 25, 27, 35). While Conidae venoms have been widely investigated, there have been only two studies concerning biochemical characterization of toxoglossate venoms other than those of cone snails (17, 21). The results derived from both investigations indicate some differences between conotoxins and venom components found in other toxoglossates.

Although investigations performed during the last two decades have provided significant biochemical, pharmacological, electrophysiological and molecular information regarding cone snail venoms, only a small fraction of the entire conopeptide diversity has been analyzed, mostly peptides obtained from piscivorous *Conus* species venoms found in the Indo-Pacific (9). Therefore, we continue to concentrate our *Conus* venom research on vermivorous and molluscivorous snails that thrive in different marine habitats. Furthermore, given that cone snails comprise only a minority of toxoglossate mollusks, there is a need to study the species that belong to the other eight families of the superfamily Conoidea, since they constitute a potential source of interesting and novel biomolecules.

In this context, as a part of a multidisciplinary research program, designed to obtain bioactive marine natural products potentially valuable for the development of drugs and basic science tools, we have initiated a pharmacological survey of extracts obtained from venom ducts of toxoglossate gastropods collected in the Gulf of Mexico. Guinea pig ileum assay was employed to detect extract effects on intestinal smooth muscle and on the nervous system.

In the present study, results obtained from pharmacological effects of the protein-containing extracts prepared from venom ducts of the vermivorous snails *Conus austini*, *Conus spurius* and *Polystira albida* (Turridae) on guinea pig ileum are described (Figure 1). Although these three species had been previously subjected to

biochemical studies (1, 2, 21-23, 37), neither the extracts prepared from their venom ducts, nor the peptides that were isolated and characterized had been evaluated in a functional model that can provide some insight into the action mechanism that mediates the pharmacological effects of their venoms.

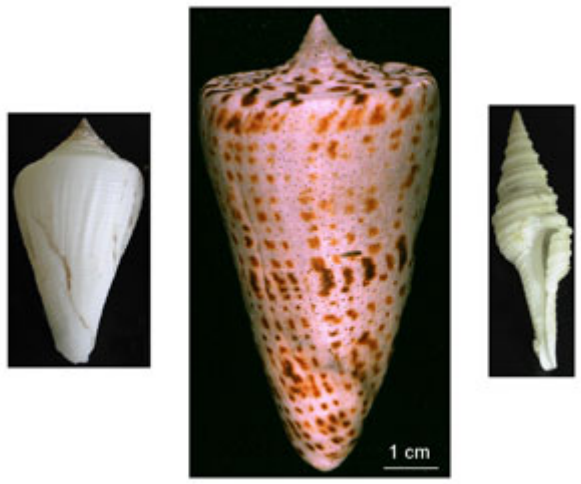

Figure 1. Shells of the three toxoglossate snails studied, left to right: *C. austini*, *C. spurius* and *P. albida*.

MATERIALS AND METHODS

Specimen Collection and Venom Extraction

C. austini, *C. spurius* and *P. albida* specimens were trawled from deep waters (100 to 200 m for *P. albida*, 50 to 80 m for *C. austini* and *C. spurius*) of Campeche Bay, Campeche State, Mexico. Snails were identified by Dr. Antonio García-Cubas Gutiérrez (Marine Science Institute, National Autonomous University of Mexico), employing classical taxonomical criteria. To dissect the venom ducts from other soft parts, snails were centrifuged (900 g for 2 minutes at 4°C), which detached the columellar muscle from the columella and the complete animals from intact shells. Venom ducts from six animals of each species were dissected, pooled and homogenized (PT 10/35® homogenizer with PTA-7TS® generator, Kinematica, Switzerland) in 10 mL of 0.1% v/v trifluoroacetic acid (sequencing grade, Aldrich, USA) in 40% aqueous acetonitrile (HPLC grade, Fisher, Fair Lawn, USA). The homogenates were centrifuged at 17,000 g for 30 minutes at 4°C; the supernatants containing the peptides (referred to as the protein-containing extracts) were used to

determine pharmacological activity in the isolated guinea pig ileum model. Protein content was measured by the Lowry method.

Guinea Pig Ileum Assay

Male guinea pigs (500 to 800 g) were sacrificed by cervical dislocation and their ileums were removed. Ileum segments (1 cm) were mounted in organ baths containing Krebs-Henseleit solution of the following composition (mM): NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $MgSO_4$, 1.2; and D-(+)-glucose, 11. This solution was gassed with 95% O_2 and 5% CO_2. Contractions were recorded on a Grass polygraph (Astro-Med, Inc. Grass, USA). After a stabilization time of 30 minutes, a 10-minute control period was recorded. Then, the protein-containing extracts (0.001-100 µg/mL) obtained from the venom ducts (*C. austini*, *C. spurius* and *P. albida*) and tetrodotoxin (TTX, 10^{-9} to 10^{-2} µg/mL), dissolved in deionized water, were independently added to the baths at different concentrations, and the responses were recorded for 10 minutes. A concentration-response curve for each test substance was plotted. The effect of the extracts was determined by comparing the areas under the curve inscribed by frequency and amplitude of ileum contractions, before and after the application of the test materials. Areas were calculated from the polygraph tracings using an analog/digital converter, fed into a personal computer, recorded and analyzed with Polyview® software (Astro-Med, Inc. Grass, USA).

Preliminary pharmacological characterization was carried out on the action mechanism involved in smooth muscle relaxant effect elicited by the active extracts. In these experiments, extract effects on contractions of the ileum induced by KCl (60 mM) and submaximal concentrations of acetylcholine (ACh 1 µM), nicotine (10 µM) and histamine (0.5 µM) were studied. The extract was added to the bath at 10 µg protein/mL concentration and permitted to act on the ileum for 5 minutes and afterwards an agonist was administered. The contractions elicited by the agonist were recorded for 5 minutes. The extract effect was determined by comparing the average amplitude of ileum contractions with the amplitude obtained when the agonist alone was added to the bath. Control experiments were carried out with atropine (0.1 µM), the nicotinic antagonist pempidine (1 µM) and the histaminergic

antagonist mepyramine (1 μM), following the same procedure as the one employed for the extracts.

Analysis of Data and Statistics

The results are expressed as the mean of six experiments ± SEM. Concentration-response curves (CRC) for the extracts and TTX were plotted, and experimental data from the CRC were fitted to the non-linear curve-fitting program PRISM, using a Boltzmann sigmoidal function. Statistical significance ($p < 0.05$) of differences between means was assessed by analysis of variance (ANOVA) followed by Dunnett's test.

Reagents

TTX, ACh, nicotine, histamine, atropine, pempidine and mepyramine were obtained from Sigma (USA). Salts and other reagents were provided by J. T. Baker (USA) or Sigma.

RESULTS

Effect of the Extracts Obtained from the Venom Ducts of *C. austini*, *C. spurius* and *P. albida* on the Spontaneous Contractions of Isolated Guinea Pig Ileum

The protein-containing extracts prepared from the venom ducts of *C. austini*, *C. spurius* and *P. albida* (tested from 0.001 to 100 μg protein/mL) inhibited the spontaneous contractions of guinea pig ileum. This smooth-muscle-relaxant effect was characterized by a decrease in basal tone and amplitude of spontaneous ileal contractions. In all cases the response was immediate, within 6 to 7 minutes after addition of the extracts to the bath (Figure 2A). Ileum spontaneous contractions were completely restored after the tissues were washed. Similar behavior was obtained for TTX, which was used as a positive control.

Figure 3 shows the CRC for the extracts and TTX. The values of the mean inhibitory concentrations (IC_{50}), maximum inhibitory effects (E_{max}) and potencies of the extracts are presented in Table 1. The most potent extract was obtained from *P. albida* venom ducts and the least active was from *C. austini* ducts. All the extracts were considerably less potent than the positive control.

The extracts lost their smooth-muscle-relaxant effect when incubated at 98°C for 30 minutes (Figure 2B).

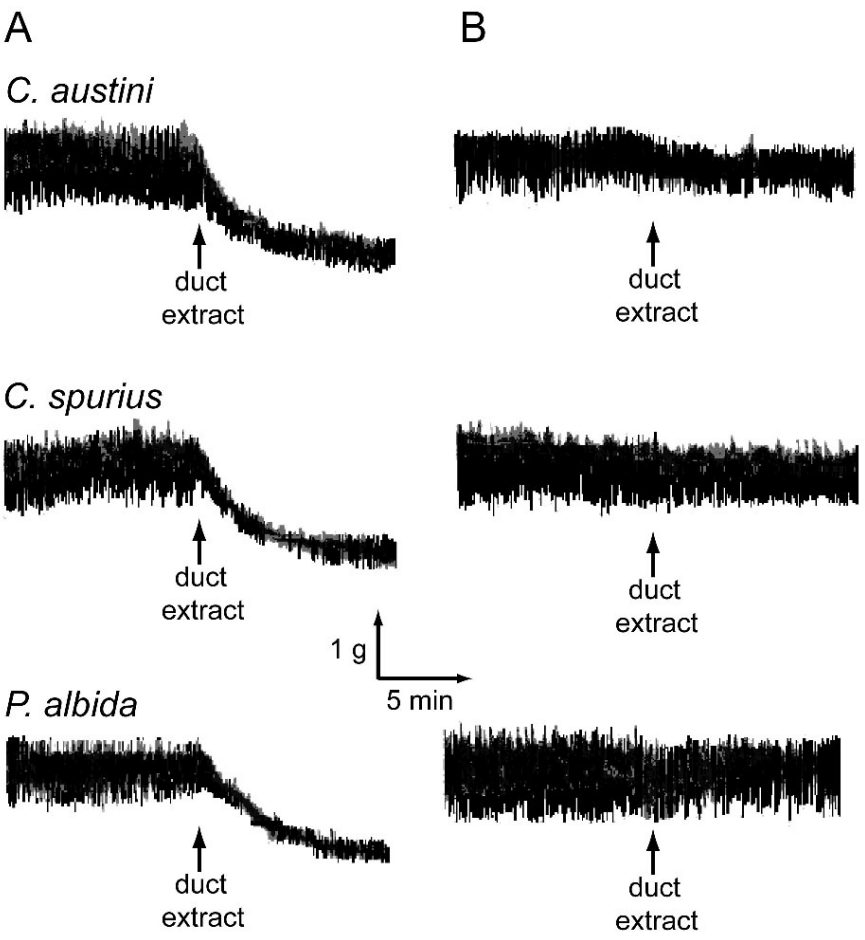

Figure 2. Tracings of guinea pig ileal contractions showing the effect of venom duct extracts from *C. austini*, *C. spurius* and *P. albida* (10 µg protein/mL), before (**A**) and after (**B**) the extracts were preincubated at 98°C for 30 minutes.

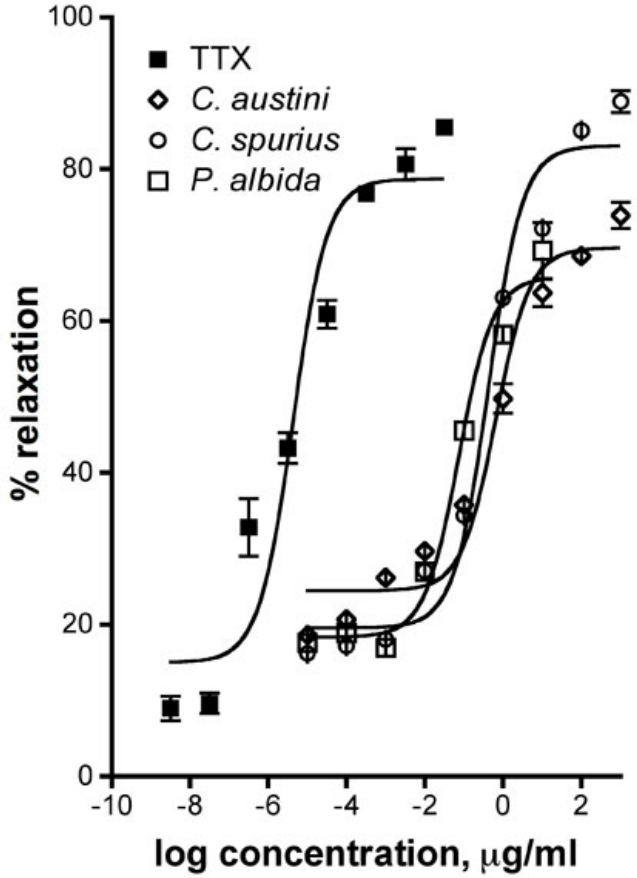

Figure 3. Concentration-response curves for the inhibitory effect of TTX and the venom duct extracts from *C. austini*, *C. spurius* and *P. albida* on the spontaneous contractions of isolated guinea pig ileum. Values are expressed as percent inhibition of contractile response calculated as the mean from six measurements ± SEM.

Table 1. Spontaneous contraction inhibition of isolated guinea pig ileum induced by venom duct extracts from *C. austini*, *C. spurius* and *P. albida*

Duct extracts	**EC_{50} (µg/mL)**	**E_{max}**	**Potency**
Tetrodotoxin	$4.3 \times 10^{-6} \pm 2.1 \times 10^{-6}$	78.71 ± 4.978	1
Conus austini	0.85 ± 0.21	65.92 ± 3.84	0.003
Conus spurius	0.48 ± 0.09	83.72 ± 4.52	0.006
Polystira albida	0.11 ± 0.02	67.59 ± 2.93	0.026

Values are means ± SEM; n = 6
Potency was obtained by the formula: EC_{50} tetrodotoxin/EC_{50} extract, assuming a value of 1.00 for tetrodotoxin

Preliminary Characterization of the Action Mechanism of the Extracts

In order to identify the target of the extracts on the ileum, we investigated their influence on cholinergic, histaminergic and ion-induced smooth-muscle contraction. In these experiments, all extracts were evaluated at a 10 µg protein/mL concentration. As summarized in Table 2, the extracts inhibited to various degrees the contractions provoked by ACh; however, in all cases the inhibition was less than 31% (Figures 4A and 5A). Atropine completely inhibited ACh-induced contractions (Figure 5A).

In the experiments with nicotine, the extracts elicited different responses. Thus, the extract prepared from *P. albida* venom ducts blocked the nicotine-induced contractions (Figure 4B), while the one prepared from ducts of *C. austini* displayed a less potent inhibition and the extract from *C. spurius* ducts had no effect (Table 2). Pempidine reduced, but did not abolish the nicotine-induced contractions (Figure 5B). The data presented in Figure 5C and in Table 2 show that all extracts inhibited the contractions evoked by histamine. The greatest inhibitory effects were those from *P. albida* (Figure 4C) and *C. spurius* ducts, which significantly reduced the histamine response. As expected, mepyramine blocked the contractions caused by the agonist (Figure 5C).

In order to determine wether the smooth-muscle-relaxant effect produced by the extracts involved an interference with sodium and calcium influx into the enteric neurons or smooth muscle cells, the effect of the extracts on the KCl-induced contractions was investigated. In these experiments, the *C. austini* and *P. albida* extracts presented no effect on the KCl-induced contractions whereas the *C. spurius* extract had a moderate effect (Table 2).

Table 2. Extract effects of the venom ducts obtained from *C. austini*, *C. spurius* and *P. albida* on contractions elicited by ACh (1 µM), nicotine (10 µM), histamine (0.5 µM) and KCl (60 mM)

	Duct extracts (percentage of inhibition)		
	Conus austini	***Conus spurius***	***Polystira albida***
ACh	17.0 ± 1.56	13.2 ± 1.53	30.3 ± 3.78
Nicotine	22.3 ± 1.69	3.4 ± 0.94	97.9 ± 1.52
Histamine	28.2 ± 2.13	76.9 ± 3.13	90.1 ± 1.15
KCl	1.3 ± 0.63	32.5 ± 2.14	4 ± 0.79

Values are means ± SEM; n = 6

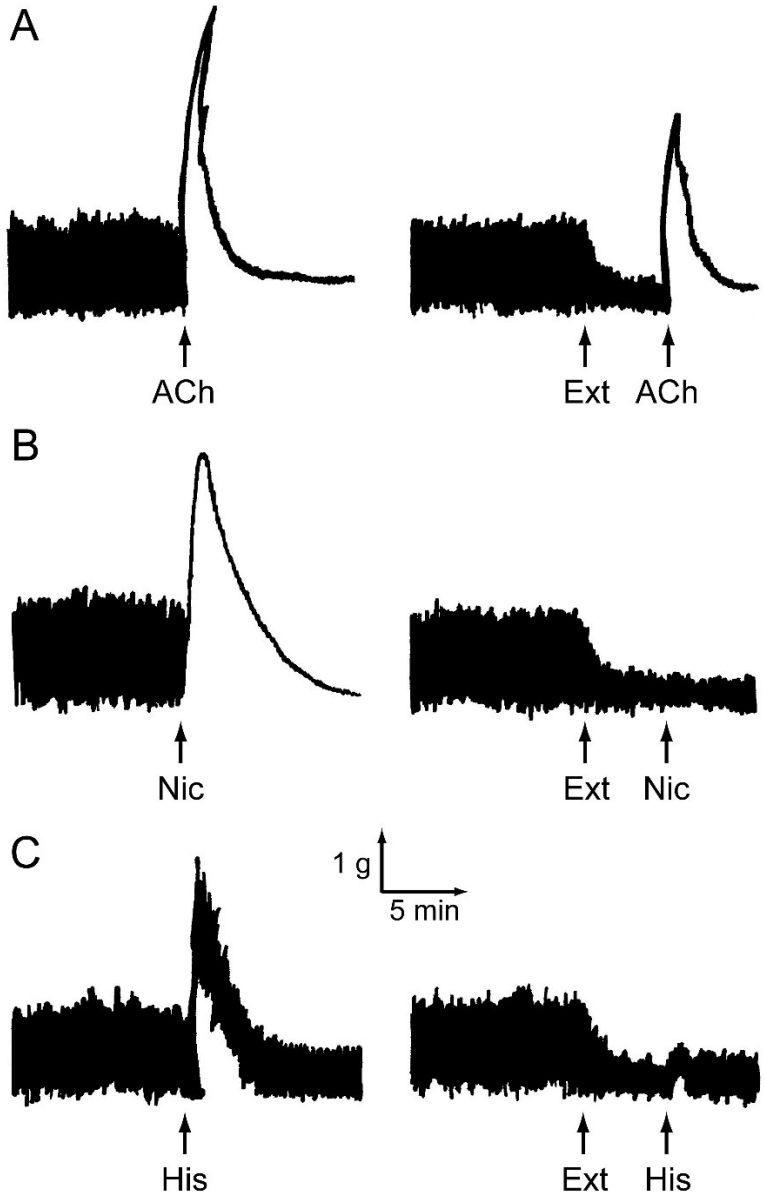

Figure 4. Effect of the protein-containing extract prepared from the venom ducts of *P. albida* (10 µg protein/mL) on the contractions induced by (**A**) 1 µM ACh, (**B**) 10 µM nicotine and (**C**) 0.5 µM histamine (right side). The tracings on the left side show the response induced by the agonists alone.

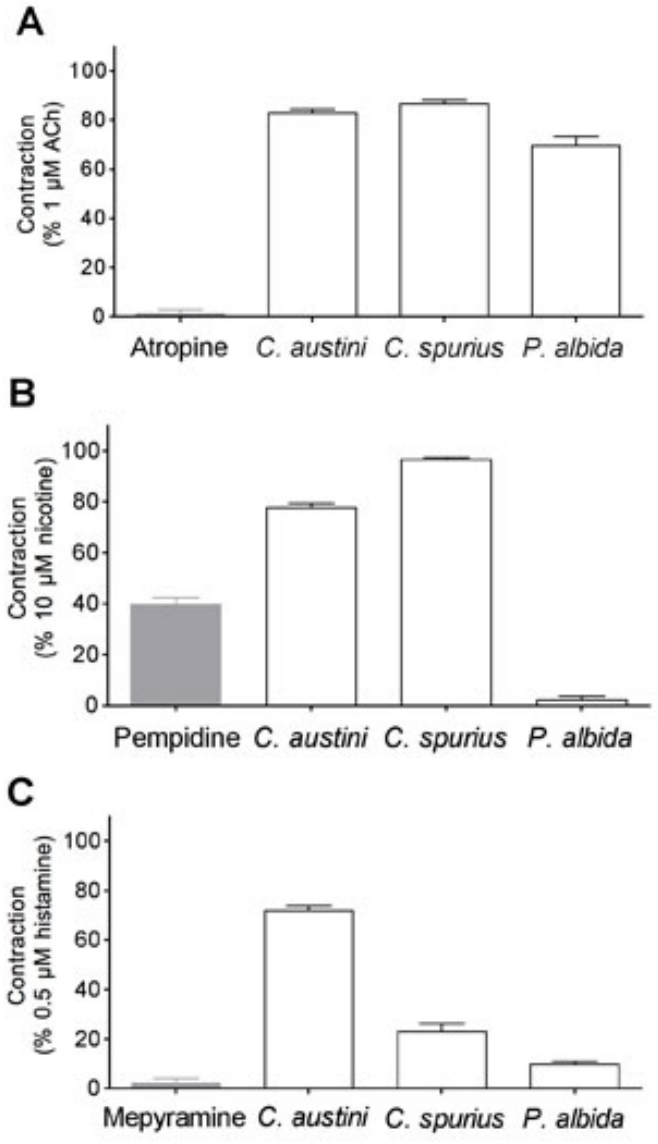

Figure 5. Effect of the extracts prepared from *C. austini*, *C. spurius* and *P. albida* venom ducts (10 µg protein/mL) on contractions elicited by (**A**) 1 µM ACh, (**B**) 10 µM nicotine and (**C**) 0.5 µM histamine. Values are expressed as percentages of the mean responses when the agonist alone was added to the bath, calculated as the mean from six measurements ± SEM.

DISCUSSION

The pharmacological results of the current study employing the guinea pig ileum assay, clearly indicated that the extracts prepared from the venom ducts of *C. austini*, *C. spurius* and *P. albida* contain compounds capable of inducing a significant relaxation of intestinal smooth muscle. The loss of this effect, when the extracts were incubated at 98°C for 30 minutes, suggests that active substances are peptides and/or proteins.

In guinea pig ileum, smooth muscle contraction depends upon axonal transmission, neurotransmitter release and receptor binding at the end-plate, as well as activation of the contractile mechanism. At each of these levels, there is a number of molecular sites at which a compound may act. Therefore, a series of experiments to identify the level at which the active extracts might be exerting their smooth-muscle inhibitory effect was performed.

First, we investigated whether relaxation induced by the extracts in guinea pig ileum could result from an interference in the transduction mechanism initiated by the binding of ACh to its receptors in the enteric nervous system (ENS) or in smooth muscle cells. ACh is considered the main excitatory neurotransmitter in the ENS, where it regulates both the state of mucosa and the gut motor function. Neuronal nicotinic receptors contribute to rapid synaptic transmission in the enteric plexuses (4, 11-13, 18, 26, 33), whereas muscarinic receptors, located mainly in smooth-muscle-cell membranes, mediate the contraction elicited by ACh on smooth muscle layers (8, 36). All extracts inhibited the contractions evoked by ACh, suggesting the presence of anticholinergic compounds in the venom obtained from the three species under investigation.

The inhibition of nicotine-induced contractions elicited by the extract prepared from *C. austini* ducts indicates that the venom produced by this species contains toxins that target neuronal nicotinic receptors. It is probable that these toxins are α-conotoxins, which inhibit neuronal homomeric and heteromeric nicotinic receptors (31, 32, 35). This toxin type has not been previously isolated from *C. austini*, since the only reported study performed on this snail venom allowed the purification of a γ-conotoxin-like peptide that belongs to the O-conotoxin superfamily (37).

The strong inhibition of nicotine-induced contractions produced by the extract prepared from *P. albida* venom ducts indicates that this snail synthesizes toxins that

either block neuronal nicotinic receptors or interfere in the transduction mechanisms activated by the binding of ACh to these receptors. It has been determined that phylogenetic differences between conoidean families, and the diversity of biotic interactions between an individual species of these families and other species in the environment are determinant factors that influence the nature of the snail-expressed toxins (28). Therefore, it is very likely that nicotinic antagonists contained in *P. albida* venom are structurally different from α-conotoxins.

Although the extract prepared from *C. spurius* ducts inhibited ACh-induced contractions, it did not modify ileum response to nicotine. This lack of antagonism against the nicotine effect supports the idea that this snail venom may contain antimuscarinic toxins that directly target M_2 or M_3 muscarinic receptors or act at any level of either cAMP/PKA or PLC pathways, involved in receptor activation (8, 36).

The extracts obtained from all three species inhibited histamine-evoked contractions. To our knowledge, this is the first time that an antihistaminergic activity has been reported for a toxoglossate snail venom. The guinea pig ENS bears the three subtypes of histamine receptors H_1, H_2 and H_3 (5, 15, 34), while smooth muscle cells mainly possess H_1 receptors (16). In the ileum, the contractile response to histamine is primarily stimulated through H_1 receptor activation (7, 19). Thus, it is expected that venoms from *C. austini*, *C. spurius* and *P. albida* contain toxins that are antagonists of H_1 histamine receptors or that may block one of the steps in the signaling pathway that mediates the contractile effect of histamine in smooth muscle cells.

Finally, we found that only the extract prepared from *C. spurius* ducts inhibited KCl-induced contractions. In the isolated guinea pig ileum assay, high extracellular potassium is employed to provoke membrane depolarization in neurons and smooth muscle cells, thereby activating voltage-dependent Na^+ and Ca^{2+} channels. Consequently, KCl-induced ileum contractions are primarily the result of an increased Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels located on the membranes of the smooth muscle cells (30). Previous studies revealed that *C. spurius* venom contains an I-conotoxin (peptide sr11a), an O-conotoxin (peptide, sr7a) and a T-1-conotoxin (peptide sr5a). Given that the O-superfamily includes ω-conotoxins that selectively block voltage-gated Ca^{2+} channels (10, 24) and δ and μO-conotoxins – which interfere with the voltage-sensor elements of the voltage-gated Na^+ channels

(14) – it is likely that toxins from the O-superfamily are responsible for the inhibition displayed by the *C. spurius* extract on KCl-induced contractions

The present study revealed that *C. austini, C. spurius* and *P. albida* produce peptides that inhibit contractility of smooth muscle. Venom ducts of all three species produce anticholinergic toxins. Those present in *C. austini* and *P. albida* venoms apparently block neuronal nicotinic receptors or interfere in the transduction mechanisms activated by the binding of ACh to these receptors. On the other hand, *C. spurius* venom probably contains antimuscarinic compounds. The venoms of *C. austini*, *C. spurius* and *P. albida* may contain toxins with unusual antihistaminergic properties, not previously described in any conoidean toxin. Finally, *C. spurius* produces toxins that block either voltage-gated Ca^{2+} or Na^{+} channels located on membranes of neurons or smooth muscle cells. The pharmacological activity found in the extracts prepared from venom ducts of the three snail species clearly indicates that these animals represent a potential source of interesting bioactive compounds that deserve further investigation. At present, biochemical studies are being performed in order to characterize the toxins responsible for pharmacological activities displayed by the crude extracts.

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