

## EFFECTS OF THE VENOM OF THE BROWN BULLHEAD CATFISH (*AMEIURUS NEBULOSUS*) ON ISOLATED SMOOTH MUSCLES

LORÁND BARTHÓ,<sup>1\*</sup> ZSOLT SÁNDOR<sup>1</sup> and TIMEA BENCSEK<sup>2</sup>

<sup>1</sup>Department of Pharmacology, Faculty of Medicine, University of Pécs,  
Szigeti út 12, H-7624 Pécs, Hungary

<sup>2</sup>Department of Pharmacognosy, Faculty of Pharmacy, University of Pécs,  
Rókus u. 2, H-7624 Pécs, Hungary

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Aqueous extract of the spines of the brown bullhead catfish (*Ameiurus nebulosus* Lesueur, 1819) caused contraction of the guinea-pig small intestine *in vitro*, a widely-used preparation in pharmacology. The action is dependent on extracellular  $Ca^{2+}$ , and probably takes place on the smooth muscle cells. Mouse gastrointestinal preparations were also contracted by the extract. Stings by the spines of this fish species causes a painful sensation in man. We tested the effect of an extract of spines in isolated organ experiments on innervated smooth muscle preparations. In the guinea-pig ileum, the response to the extract was abolished by the  $Ca^{2+}$ -channel blocker nifedipine, but only slightly reduced by atropine (a muscarine receptor antagonist) or tetrodotoxin (TTX; a blocker axonal conduction) or antagonists for  $P_{2X}$  purinoceptors. Blocking of serotonin or histamine  $H_1$  receptors, tachykinin  $NK_1$  receptors, functional impairment of capsaicin-sensitive sensory nerve endings or inhibition of cyclo-oxygenases failed to influence the contractile effect of the extract. No inhibitory action of the extract was detected on the ileum subject to electrical motor nerve stimulation.

**Keywords:** Brown bullhead catfish – venom – smooth muscle effects

### INTRODUCTION

The effects and mechanism of action of the venom of the brown bullhead catfish (*Ameiurus nebulosus*) was studied on innervated smooth muscle preparations. This fish has been introduced to Hungary in the late 19th century. Here, the subspecies *pannonicus* has been formed and is widely distributed both in natural waters and ponds or channels. It is well known that this fish can produce painful stings with its stout stings, spines or horns, located adjacent to the dorsal and pectoral fins. The character of pain caused by the stings is indicative of the presence of venom, not only mechanical injury. The venom of a related fish species (*Ictalurus catus*) has been analyzed [9]. The fraction toxic to mice has been found to be heat-resistant and not destroyed by trypsin, in spite of a high estimated molecular weight (around 10,000) [9]. Catfish stings are usually harmless to human beings, but occasionally complications may occur [6]. Effects of stings of ictalurid catfishes (*Schilbeoides*

\*Corresponding author; e-mail address: lorand.bartho@aok.pte.hu

*exilis* and *S. leptanthus*, *Ictalurus melas* and *I. punctatus*) on test fish have been studied [5], but the tests performed were quite different from those of the current study. The aim of our experiments was to investigate the biological effects of an aqueous extract of spines of *Ameiurus nebulosus* (ssp. *pannonicus*) in isolated organ experiments on the isolated small intestine of the guinea-pig, a tissue containing smooth muscle and nerves and a most widely-used and thoroughly-characterized preparation in experimental pharmacology (see Table 1). Gastrointestinal preparations of the mouse were also used.

## MATERIALS AND METHODS

The experiments have been approved by the Regional Committee for Animal Research, as well as by the respective committee of University of Pécs. These experiments were of the “ex vivo” type and are in accordance with the European Guidelines on Laboratory Animal Care.

One-year old brown bullhead fish were used. They were kept in an aquarium for nearly 1 year (from a size of a few mm) and now weighed 14 g on average. They were fed on standard food for aquarium fish (granulated high-value fish food *plus* dried *Daphnia*). Fish were killed by a strong blow to the head. All three spines were removed, soaked in Krebs solution (50 mg wet tissue/ml solution) for 10 min at room temperature, with intermittent vortexing. The tissue was then removed from the solution (that will hereafter be called extract). Samples of the extract were pooled to make a 10 ml stock solution, then divided into 100  $\mu$ l samples and kept at  $-20^{\circ}\text{C}$ . An extract of the abdominal fins was prepared in the same way.

Guinea-pigs (short-haired, coloured) of either sex, weighing 450–580 g were stunned by a blow to the occiput and bled out. Whole segments of the ileum (approximately 2 cm in length) were suspended in organ baths containing 7 ml of oxygenated (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ) Krebs–Henseleit solution at  $37^{\circ}\text{C}$ . Longitudinal movements were recorded auxotonically, using Experimetria (Budapest) force transducers and bridge amplifiers. The transducers were fitted with fine springs for allowing the tissue to contract. The initial load was 6 mN. All experiments commenced after 40 min equilibration period. A maximum of 2 preparations from the same animal were used for a given type of experiment. Some preparations were electrically field stimulated [27]; cholinergic „twitch” responses elicited by single electrical pulses with 20 min intervals, delivered through a pair of ring electrodes. Parameters of the pulses were 30 V/cm amplitude and 0.1 ms pulse width. The spectrum of detection of the guinea-pig ileal preparation is given in Table 1.

Adult female C57 mice were killed by a strong blow to the head. Ileum (whole ileum segments in the longitudinal direction) and gastric fundus preparations were set up as described above, with a resting tone of 5 mN. Each stomach yielded one single fundus preparation, in which the isolated gastric fundus was opened along both the lesser and the greater curvatures. Contractions to 2  $\mu$ l/ml of the extract were reproducible (contact times, 3 min with the ileum and 6–8 min for the fundus), provided that a

Table 1  
Spectrum of detection of the guinea-pig ileum tests

No.	Tests	Activities detected by the test	Selected references
1	Guinea-pig ileum at basal tone: contractors via nerve stimulation	Ganglionic stimulants; serotonin (to a greater extent); cholecystokinin-like drugs, tachykinin NK <sub>3</sub> receptor agonists, P <sub>2X</sub> purinoceptor agonists (like $\alpha,\beta$ -methylene ATP), VIP, PACAP, CGRP, some eicosanoids, endocannabinoids	4, 7, 8, 11, 16, 17, 19–21, 22, 25, 32
2	Guinea-pig ileum at basal tone: contractors via smooth muscle stimulation	Muscarinic receptor agonists, histamine (at H <sub>1</sub> receptors), serotonin (to a lesser extent), tachykinins (substance P, neurokinin A, through NK <sub>1</sub> and NK <sub>2</sub> receptors), some other polypeptides, mast cell activators; spasmogenic effect by unidentified mechanisms	1, 7, 8, 12, 18, 23
3	Guinea-pig ileum, contractions due to electrical field stimulation of cholinergic nerves	Acetylcholine muscarine M <sub>3</sub> receptor antagonists; Na <sup>+</sup> channel blocking (TTX or local anesthetic-like); N-type Ca <sup>2+</sup> -channel blocking effect; negative modulators of intestinal cholinergic nerves (e.g., opioid agonist- or adrenergic $\alpha_2$ agonist-like, nociceptin-, cannabinoid-, adenosine-like) L-type Ca <sup>2+</sup> -channel blocker, K <sup>+</sup> -channel opener or any other smooth-muscle relaxant (e.g., enhancers of intracellular cAMP or cGMP levels)	10, 13, 15, 24, 26, 28, 29, 31

40 min washout period was allowed to the preparations (Table 3). Part of the preparations received TTX (0.5  $\mu$ M) 20 min before the second exposure to the extract.

Drugs used were apamin, atropine sulfate, capsaicin, histamine dihydrochloride, indomethacin, pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), serotonin creatinin sulfate, tetrodotoxin (TTX; Sigma), ATP,  $\alpha,\beta$ -methyleneATP, (2*S*,3*S*)-*N*-(2-methoxyphenyl)methyl-2-diphenylmethyl-1-azabicyclo[2.2.2]octan-3-amine (CP 96345), methysergide, nifedipine, (1-butyl-4-piperidiny)methyl-8-amino-7-chloro-1,4-benzodioxane-5-carboxylate (SB 204 070), suramin, tropisetron, 1,2,3,6-tetrahydro-1,3-dimethyl-N-[4-(1-methylethyl)phenyl]-2,6-dioxo-7H-purine-7-acetamide, 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide (HC 030031, Tocris), chloropyramine hydrochloride (Egis Pharma, Hungary).

## RESULTS

The extract showed contractile activity on the guinea-pig ileum (Fig. 1A). Concentration-dependence was found with 0.5 or 5  $\mu$ l extract/ml bath fluid, which resulted in contractions of  $38.3 \pm 2.9$  and  $74.1 \pm 5.0\%$  ( $n = 4$ ), respectively, of the maximal spasm evoked by histamine (10  $\mu$ M). One  $\mu$ l/ml of the extract was used for pharmacological analysis. Contractions were quick in onset, reached their maximum

within 0.5 min and showed only a moderate tendency to decline during the 3 min observation period. If an interval of 40 min was allowed to the preparations as wash-out time, the responses to 1 µl/ml extract were reproducible (Table 2). Pretreatments that partially diminished responses to exposure to the extract were the Na<sup>+</sup> channel blocker TTX, the muscarinic acetylcholine receptor antagonist atropine, or a combination of the two P<sub>2</sub> purinoceptor antagonists PPADS and suramin. The Ca<sup>2+</sup> channel blocker nifedipine abolished the response (Table 2). The effect of the extract was not inhibited by the histamine H<sub>1</sub> receptor antagonist chloropyramine, a combination of serotonin receptor antagonists, the tachykinin NK<sub>1</sub> receptor antagonist CP 96345, the cyclooxygenase inhibitor indomethacin, and capsaicin desensitization (Table 2). The TRPA<sub>1</sub> receptor/channel inhibitor HC 030031 slightly, while the Ca<sup>2+</sup> sensitive, small conductance K<sup>+</sup> channel blocker apamin moderately enhanced the response to the extract (Table 2).

Table 2  
Effect of the fish spine extract (1 µl/ml bath fluid) on the guinea-pig isolated small intestine

Pretreatment	Contraction (% of maximum)	Contact time of pretreatment	
1st administration: no pretreatment 2nd administration: no pretreatment	49.8±2.7 53.6±2.9		n = 14
1st: control (solvent: saline) 2nd: tetrodotoxin (0.5 µM)	53.9±2.4 42.6±2.6*	20 min	n = 10
1st: control (solvent: saline) 2nd: atropine (0.5 µM)	46.9±5.0 41.2±5.7*	20 min	n = 8
1st: control (solvent: saline) 2nd: chloropyramine (0.3 µM)	51.3±4.9 53.0±5.4	20 min	n = 7
1st: control (solvent: saline) 2nd: PPADS (50 µM) + suramin (100 µM)	54.7±4.8 41.8±5.01*	30 min	n = 8
1st: control (solvents: DMSO) 2nd: serotonin receptor antagonists <sup>#</sup>	52.1±7.8 55.9±4.2	20 min	n = 7
1st: control (solvent: alcohol) 2nd: nifedipine (1 µM)	50.3±5.2 5.1±2.1*	20 min	n = 7
1st: control (solvent: DMSO) 2nd: CP 96345 (1.5 µM)	52.2±2.4 40.9±3.9	45 min	n = 8
1st: control (solvent: saline) 2nd: apamin (0.1 µM)	46.3±3.0 65.9±4.7*	20 min	n = 6
1st: control: (solvent: alcohol) 2nd: indomethacin (5 µM)	44.1±5.5 38.8±4.5	45 min	n = 7
1st: control (solvent: alcohol) 2nd: capsaicin desensitization <sup>##</sup>	45.1±3.1 54.0±6.5	(see below)	n = 6
1st: control (solvent: DMSO) 2nd: HC 030031 (30 µM)	47.9±2.4 54.8±1.7*	30 min	n = 8

<sup>#</sup>Methysergide (0.3 µM, predominantly inhibiting 5-HT<sub>2</sub> receptors, dissolved in saline), tropisetron (3 µM, for 5-HT<sub>3</sub> receptors, dissolved in saline) and SB 204 070 (3 µM, for 5-HT<sub>4</sub> receptors, dissolved in DMSO).

<sup>##</sup>Capsaicin, 10 µM for 10 min, followed by 60 min washout period.

Main±s.e.m. are shown. \*denotes statistically significant differences from the solvent-treated responses (Wilcoxon test).

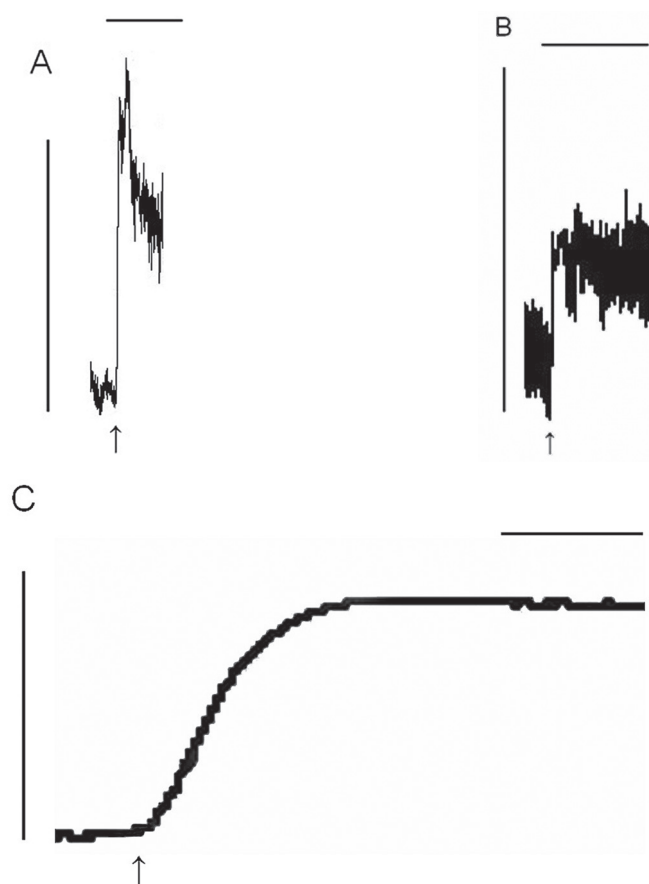


Fig. 1. Original tracings showing the contractile effects of the catfish spine extract (added at the arrow) on the guinea-pig ileum (A; 1  $\mu$ l/ml extract), mouse ileum (B; 2  $\mu$ l/ml extract) and mouse gastric fundus strip (C; 2  $\mu$ l/ml extract). Calibrations, vertical: 50% of maximal contraction to histamine (10  $\mu$ M, A) or acetylcholine (10  $\mu$ M, B, C); horizontal: 3 min

Two concentrations of the extract (0.25 and 1  $\mu$ l/ml) were added while the preparations were field stimulated. The smaller concentration enhanced the amplitude of the cholinergic “twitch” response, with only a small and transient elevation of the baseline, while the larger one elevated the baseline without modifying the “twitch” response throughout the 15 min observation period ( $n = 4$  each). No inhibitory effect was seen whatsoever, which indicates that mechanisms listed in Table 1, row 3 are not activated by the extract.

An extract of the abdominal fins (2  $\mu$ l/ml) had practically no effect on the ileum (0–6% contraction, 1.2% on average,  $n = 5$ ).

Positive controls from previous and the current experiments in the ileum for the inhibitors (in the concentrations used) were as follows. TTX and atropine practically

abolish half-maximal, cholinergic „twitch” responses to electrical stimulation; chlorpyramine blocks near-maximal contractions to histamine (0.1  $\mu\text{M}$ ), without inhibiting the “twitch” contraction; PPADS+suramin suppresses contractions to ATP (300  $\mu\text{M}$ ) or  $\alpha,\beta$ -methyleneATP (10  $\mu\text{M}$ ); nifedipine (1  $\mu\text{M}$ ) blocks half-maximal contractions to exogenous acetylcholine or histamine (also the cholinergic „twitch”); the combination of serotonin receptor antagonists blocks near-maximal ileum contractions to serotonin (0.3–1  $\mu\text{M}$ ); the tachykinin NK<sub>1</sub> receptor antagonist CP 96345 (1.5  $\mu\text{M}$ ) causes an approximately 30-fold shift to the right of concentration-response curves for substance P (0.3–6 nM). Capsaicin desensitization blocks all effects of acute capsaicin administration (0.3–1  $\mu\text{M}$ ). These results were obtained on 3–10 preparations and partly served as confirmation of data of literature [see 1, 3]. To date, we have no positive control for the TRPA1 receptor/channel inhibitor HC 030031 in the guinea-pig ileum, since the putative agonists show conflicting results (unpublished observations).

The extract (2  $\mu\text{l/ml}$ ) also contracted the mouse gastric fundus and mouse ileum preparations. The effect of TTX on these responses was similar to that seen in the guinea-pig ileum (Fig. 1B, C; Table 3).

Table 3  
Effect of the fish spine extract (2  $\mu\text{l/ml}$  bath fluid) on the mouse ileum and stomach fundus strip

Pretreatment	Contraction (% of maximum)	Contact time of pretreatment	
Mouse ileum			
1st administration: no pretreatment 2nd administration: no pretreatment	26.2 $\pm$ 2.5 25.0 $\pm$ 2.4		n = 8
1st: control (solvent: saline) 2nd: tetrodotoxin (0.5 $\mu\text{M}$ )	24.4 $\pm$ 2.2 19.3 $\pm$ 2.1	20 min	n = 7
Mouse gastric fundus			
1st administration: no pretreatment 2nd administration: no pretreatment	47.0 $\pm$ 6.3 50.3 $\pm$ 7.0		n = 5
1st: control (solvent: saline) 2nd: tetrodotoxin (0.5 $\mu\text{M}$ )	45.8 $\pm$ 5.2 49.9 $\pm$ 5.5	20 min	n = 4

Main  $\pm$ s.e.m. are shown.

## DISCUSSION

The extract of the spines (but not of the caudal fins) of the brown bullhead catfish caused smooth contraction on the guinea-pig small intestine; no inhibitory action has been detected (e.g., on the field-stimulated ileum). We interpret the results obtained from a pharmacological analysis as follows.

The strongest (practically full) inhibition of the contractile response to the extract was found with the Ca<sup>2+</sup> channel blocker nifedipine, which indicates an essential role of voltage-dependent, L-type Ca<sup>2+</sup> channels in this response. For comparison, acetyl-

choline- or histamine-evoked responses of similar size are also strongly inhibited by nifedipine, which points to the involvement of voltage-dependent  $\text{Ca}^{2+}$  channels in the course of the Gq-protein-mediated response to these agonists. Whether or not a release of intracellular  $\text{Ca}^{2+}$  is also involved in the effect of the extract cannot be decided on the basis of the current study.

Tetrodotoxin and atropine caused a rather slight reduction in the effect of the extract. Given that both pretreatments are able to fully inhibit the “twitch” responses (tetrodotoxin pre- and atropine postjunctionally), the modest effect of these drugs on the response to the extract might be attributed to an overall reduction of the excitability of the preparations, rather than a specific inhibition of the effect of the extract. This indicates that mechanisms, listed in Table 1, 1st row are unlikely to mediate the contraction caused by the extract. The moderate enhancement of the effect of the extract by the  $\text{K}^+$  channel inhibitor apamin [see 1] may also be a result of an overall increase of the excitability of the preparations.

CP 96345 is a tachykinin  $\text{NK}_1$  receptor antagonist with an  $\text{IC}_{50}$  below 100 nM [14]. On the longitudinal muscle of the guinea-pig small intestine the main tachykinin receptor is the  $\text{NK}_1$  type and the effectiveness of CP 96345 (1.5  $\mu\text{M}$ ) against exogenous substance P was confirmed in the current study. Its failure to inhibit the effect of the extract indicates that a substance P-like peptide is not involved in the contractile effect of the extract. Cyclo-oxygenase products seem not to be involved, as shown by the lack of influence of indomethacin. A mediating role of histamine, serotonin or an ATP-like purinoceptor agonist is improbable, given the lack of influence of chloropyramine (histamine  $\text{H}_1$  receptor antagonist), or serotonin receptor antagonists, and a modest inhibition by PPADS+suramin, respectively. The lack of inhibitory effect of the extract on the electrically-induced “twitch” contractions of the guinea-pig ileum indicates that no major involvement of smooth muscle-relaxant mediators (e.g., the cGMP enhancer nitric oxide, NO) takes place.

Taken together, these data indicate that the extract acts on the ileum directly on the smooth muscle, in a  $\text{Ca}^{2+}$ -dependent manner. Yet, a tetrodotoxin-insensitive release of some (unidentified) neurotransmitter from intestinal nerves cannot be fully excluded as mechanism. Although the pharmacological sensitivity of the effect of the extract does not match the excitatory effect of the sensory stimulant drug capsaicin in this preparation [see 3], we found advisable to study the influence of capsaicin desensitization, a procedure that renders capsaicin-sensitive sensory nerve endings functionally inactive, i.e. insensitive not only against capsaicin, but also any type of stimulus, including antidromic electrical stimulation [2, 3, 30]. In spite of the apparent pain-producing effect of *Ameiurus* stings, no involvement of capsaicin-sensitive sensory nerves in the gut-contracting effect of the extract could be verified. This, however, does not exclude other ways of sensory stimulation by the venom. Further studies will be needed for the clarification of this point.

Experiments on mouse preparations yielded rather similar results to those obtained on the guinea-pig gut, also as far as the modest (statistically insignificant) decrease of the response in the presence of TTX.

In conclusion, the venom of the brown bullhead catfish causes Ca<sup>2+</sup>-dependent smooth muscle contraction. This effect, as well as the contractile action on mouse preparations seem not to involve Na<sup>+</sup>-dependent nerve action potentials. As far as the mechanism of this effect is concerned, the roles of a number of important mediators have been excluded. The stimulatory mechanism(s) encountered might contribute to the toxicity and pain produced by the venom.

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