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PhKv a toxin isolated from the spider venom induces antinociception by inhibition of cholinesterase activating cholinergic system

Flavia Karine Rigo[a,1], Mateus Fortes Rossato[b,1], Gabriela Trevisan[a], Samira Dal-Toé De Prá[a], Rafael Porto Ineu[c], Mariane Bernardo Duarte[a], Célio José de Castro Junior[d], Juliano Ferreira[e], Marcus Vinicius Gomez[d,*]

[a] *Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense (UNESC), Criciúma, SC, Brazil*
[b] *Faculdade de Medicina de Ribeirão Preto USP, Departamento de Farmacologia, Ribeirão Preto, SP, Brazil*
[c] *Programa de Pós-graduação em Ciências Biológicas, Bioquímica Toxicológica, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil*
[d] *Programa de Pós-graduação em Ciências da Saúde, Biomedicina e Medicina, Instituto de Ensino e Pesquisa da Santa Casa de Belo Horizonte, Grupo Santa Casa de Belo Horizonte, Belo Horizonte, MG, Brazil*
[e] *Programa de Pós graduação em Farmacologia, Universidade Federal de Santa Catarina, Florianopolis, SC, Brazil*

HIGHLIGHTS

- PhKv, a peptide from *P. nigriventer* spider venom, does not affect thermal or mechanical sensitivity in mice.
- PhKv caused antinociception in the chronic constriction injury model and after intraplantar injection of capsaicin.
- Pretreatment of mice with atropine or mecamylamine inhibited neostigmine and PhKv-induced antinociception.
- The antinociceptive activity of PhKv may be mediated by inhibition of AChE.
- The inhibition of AChE activity by PhkV is competitive.

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ABSTRACT

Background and aims: Cholinergic agents cause antinociception by mimicking the release of acetylcholine (ACh) from spinal cholinergic nerves. PhKv is a peptide isolated from the venom of the armed spider *Phoneutria nigriventer*. It has an antiarrythmogenic activity that involves the enhanced release of acetylcholine. The aim of this study was to investigate whether PhKv had an antinociceptive action in mice.

Methods: Male albino Swiss mice (25–35 g) were used in this study. The PhKv toxin was purified from a PhTx3 fraction of the *Phoneutria nigriventer* spider's venom. Because of its peptide nature, PhKv is not orally available and it was delivered directly into the central nervous system by an intrathecal (i.t.) route. PhKV on the thermal and mechanical sensitivity was evaluated using plantar test apparatus and the up-and-down method. The analgesic effects of PhKv were studied in neuropathic pain (CCI) and in the peripheral capsicin test. In order to test whether PhKv interfered with the cholinergic system, the mice were pre-treated with atropine (5 mg/kg, i.p.) or mecamylamine (0.001 mg/kg, i.p.) and the PhKv toxin (30 pmol/site i.t.) or neostigmine (100 pmol/site) were applied 15 min before the intraplantar capsaicin (1 nmol/paw) administrations. To investigate PhKv action on the AChE activities, was performed *in vitro* and *ex vivo* assay for AChE. For the *in vitro* experiments, mice spinal cord supernatants of tissue homogenates (1 mg/ml) were used as source of AChE activity. The AChE assay was monitored at 37 °C for 10 min in a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices) at 405 nm.

Results: PhKv (30 and 100 pmol/site, i.t.) had no effect on the thermal or mechanical sensitivity thresholds. However, in a chronic constriction injury model of pain, PhKv (10 pmol/site, i.t.) caused a robust reduction in mechanical withdrawal with an antinociceptive effect that lasted 4 h. A pretreatment in mice with PhKv (30 pmol/site, i.t.) or neostigmine (100 pmol/site, i.t.) 15 min before an intraplantar injection

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* Corresponding author at: Instituto de Ensino e Pesquisa Santa Casa Belo Horizonte, Rua Domingos Vieira, 590, 30150-240 Belo Horizonte, MG, Brazil.
E-mail address: marcusvgomez@gmail.com (M.V. Gomez).
[1] These authors contributed equally to the development of this project.

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of capsaicin (1 nmol/paw) caused a maximal antinociceptive effect of 69.5 ± 4.9% and 85 ± 2.5%, respectively. A pretreatment in mice with atropine; 5 mg/kg, i.p. or mecamylamine 0.001 mg/kg, i.p. inhibited a neostigimine and PhKv-induced antinociception, suggesting a cholinergic mechanism. Spinal acetylcholinesterase was inhibited by PhKv with ED_{50} of 7.6 (4.6–12.6 pmol/site, i.t.). PhKv also inhibited the *in vitro* AChE activity of spinal cord homogenates with an EC_{50} of 20.8 (11.6–37.3 nM), shifting the Km value from 0.06 mM to 18.5 mM, characterizing a competitive inhibition of AChE activity by PhKv.

Conclusions: Our findings provide, to our knowledge, the first evidence that PhKv caused inhibition of AChE, it increased the ACh content at the neuronal synapses, leading to an activation of the cholinergic system and an antinociceptive response.

Implications: Studies regarding the nociceptive mechanisms and the identification of potential targets for the treatment of pain have become top priorities. PhKv, by its action of stimulating the cholinergic receptors muscarinic and nicotinic system, reduces pain it may be an alternative for controlling the pain processes.

1. Introduction

The agonists of nicotinic [1] and muscarinic [2] acetylcholine receptors (AChRs) are being evaluated as candidate analgesics for the treatment of pain. The antinoceptive effects of (−)-nicotine hydrogen tartrate (nicotine), a relatively non-selective nAChR agonist, have been demonstrated in preclinical and clinical studies [3,4]. In wild-type mice, a systemic intrathecal or an intracerebroventricular administration of centrally active muscarinic agonists resulted in robust analgesic effects, indicating that muscarinic analgesia mediates both the spinal and the supraspinal mechanisms. Centrally active muscarinic agonists display pronounced analgesic effects that are not observed in M2/M4 double-KO mice, indicating that both M2 and M4 mAChRs are involved in mediating a muscarinic analgesia [2].

The hydrolysis of ACh by acetylcholinesterase (AChE) has a key role in limiting the activation of both nicotinic and muscarinic receptors and cholinesterase inhibitors have shown an activity in clinical trials for pain [5]. A neostigmine treatment reversed a non-noxious stimulus of neuropathic pain in the streptozotocin neuropathic pain of rats, suggesting a potential therapeutic role for diabetic neuropathy [6]. A major site of action for cholinomimetics in analgesia is the spinal cord. Intrathecal cholinergic agents cause antinociception by mimicking the release of ACh from the spinal cholinergic nerves. Drugs that modulate the cholinergic system (muscarinic/nicotinic agonists, acetylcholinesterase inhibitors) induce antinociception. Furthermore, the release of ACh acts as a modulator of the nociceptive signals in the spinal cord [7].

Various studies showing that spider venoms and some of its toxins are both nociceptive and antinociceptive [8]. Toxins isolated from spider venoms either inhibit or activate a vast number of targets, such as ion channels, with a high selectivity and an affinity [9]. For this selectivity, these animals have received the help of several million years. Thus, nature has evolved venoms into a huge pharmacological library of active pharmaceuticals with high selectivities and affinities, which can be explored as being therapeutics or to serve as templates for drug design. The spider toxins Phα1β and PhTx3-3 are calcium channel blockers that are effective in different rodent models of pain and in the control of chronic pathological pain, especially neuropathic [10,11] and cancer-related pain [12]. One component of *Phoneutria nigriventer* venom originally named PnTx3-1 [13] blocks voltage activated A-type potassium currents in the GH3 neuroendocrinal cell line [14]. In light of its potassium channel blocking activity, this toxin was renamed PhKv. This toxin reduced the ventricular arrhythmias that were induced by an occlusion of the left anterior descending coronary artery following a reperfusion [15]. This inhibitory effect of PhKv on cardiac arrhythmias was caused by a toxin-induced increase in the ACh release and it was blocked by atropine, a muscarinic receptor antagonist. Since the agonists of muscarinic and nicotinic ACh receptors display pronounced analgesic effects, it was decided to investigate the possible antinociceptive effects of PhKv in the cholinergic system.

2. Material and methods

2.1. Animals

Three-month-old male albino Swiss mice (25–35 g) that were bred in our animal house were used in this study. The animals were housed in a controlled temperature (22 ± 2 °C) with a 12 h light/dark circle, with the lights on at 6 a.m.

They were provided with standard rodent chow and tap water *ad libitum*. The animals were habituated in the experimental room for at least 1 h before the experiments. Each animal was used for only one experiment. The Ethics Committee of the Federal University of Minas Gerais authorised the studies, Protocol 347/2012. The experiments were performed in accordance with the current ethical guidelines for the investigation of experimental pain in conscious animals [16].

2.2. Drugs and chemicals

The PhKv toxin was purified from a PhTx3 fraction of the *Phoneutria nigriventer* spider's venom according to Cordeiro, 1993 [13]. The PhKv toxin, previously named PnTx3-1, contained 40 amino acids (AECAAVYERCGKGYKRCCEERPCKCN IVNDNCTCKKFISE) with a molecular weight of 4575.4 dalton (Da) [17]. Capsaicin, atropine, mecamylamine, neostigmine, acetylthiocholine iodide, and 5,5′-dithiobis-2-nitrobenzoicacid (DTNB), were all purchased from Sigma (St Louis®, MO, USA). The other reagents were of an analytical grade.

2.3. Intrathecal administration

Because of its peptide nature, PhKv is not orally available and it was delivered directly into the central nervous system (CNS) by an intrathecal (i.t.) route as described elsewhere [18]. The injections were made with a 28-gauge needle that was connected to a Hamilton micro syringe with a volume of 2.5 μl/site. Before injecting the toxin and in order to validate the method, the experimenters had to perform a prior training with the i.t. administration of an anaesthetic (lidocaine 1%) followed by an observation for the development of a spinal blockade, indicated by a paralysis of both hind limbs. The experimenters were only accepted if they had previously performed i.t. injections of toxins and had achieved more than 90%