Toxicon 57 (2011) 217–224

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Antiarrhythmogenic effects of a neurotoxin from the spider *Phoneutria nigriventer*

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ARTICLE INFO

Article history:
Received 4 August 2010
Received in revised form 17 November 2010
Accepted 22 November 2010
Available online 27 November 2010

Keywords:
Cardiac arrhythmias
Isolated heart
Atropine
Acetylcholine

ABSTRACT

In this study, we evaluated the effects of PhKv, a 4584 Da peptide isolated from the spider *Phoneutria nigriventer* venom, in the isolated rat heart and in isolated ventricular myocytes. Ventricular arrhythmias were induced by occlusion of the left anterior descending coronary artery for 15 min followed by 30 min of reperfusion. Administration of native PhKv (240 nM) 1 min before or after reperfusion markedly reduced the duration of arrhythmias. This effect was blocked by atropine, thereby indicating the participation of muscarinic receptors in the antiarrhythmogenic effect of PhKv. Notably, recombinant PhKv (240 nM) was also efficient to attenuate the arrhythmias (3.8 ± 0.9 vs. 8.0 ± 1.2 arbitrary units in control group). Furthermore, PhKv induced a significant reduction in heart rate. This bradycardia was partially blunted by atropine and potentiated by pyridostigmine. To further evaluate the participation of acetylcholine on the PhKv effects, we examined the release of this neurotransmitter from neuromuscular junctions. It was found that Phkv (200 nM) significantly increased the release of acetylcholine in this preparation. Moreover, PhKv (250 nM) did not cause any significant change in action potential or Ca^{2+} transient parameters in isolated cardiomyocytes. Altogether, these findings show an important acetylcholine-mediated antiarrhythmogenic effect of the spider PhKv toxin in isolated hearts.

1. Introduction

Spider venoms contain polypeptide toxins capable of producing a variety of cardiovascular responses, including increased vascular permeability (Antunes et al., 1992), enhanced contraction of vascular smooth muscle (Antunes and Málaque, 2003), effects on blood pressure (Costa et al., 1996), activation of the tissue kallikrein-kinin system (Marangoni et al., 1993) and increased nitric oxide (NO) release in cavernosum tissue (Nunes et al., 2008). In severe spider envenomations, cardiovascular alterations such as hypertension, tachycardia and arrhythmias have been described (Antunes and Málaque, 2003).

Phoneutria nigriventer (Ctenidae, Labidognatha), popularly known as the "armed" spider, is an aggressive venomous spider found in South America (Lucas, 1988), responsible for

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doi:10.1016/j.toxicon.2010.11.013

approximately 40% of the spider bites in humans in Brazil (Bucaretchi et al., 2000). Its venom contains a cocktail of toxins that affect ionic channels (see review Gomez et al., 2002) including voltage gated sodium (Na^+), calcium (Ca^{2+}) and potassium (K^+) channels.

We have previously shown that one component of the venom, a neurotoxic peptide originally named Tx3-1 (Cordeiro et al., 1993) blocks voltage activated A-type potassium currents in the GH3 neuroendocrinal cell line. In the interest of large scale testing of this peptide, we subsequently produced recombinant Tx3-1 which maintained its channel blocking activity (Carneiro et al., 2003). In light of its potassium channel blocking activity, this toxin was recently renamed PhKv. In the present study, we describe large scale production of recombinant PhKv and investigated the effects of native and recombinant PhKv on cardiac function using an isolated heart preparation and isolated ventricular cardiac myocytes.

2. Materials and methods

2.1. Drugs and toxins

PhKv toxin was purified from the PhTx3 fraction of the *P. nigriventer* venom, according to Cordeiro et al. (1993). PhKv, previously named Tx3-1, contains 40 amino acids and a molecular weight of 4584 Da. All other chemical reagents were of analytical grade. The toxin was dissolved in deionized water and work solutions were prepared by dilution of frozen 1 mM stock solutions immediately before use.

2.2. Recombinant PhKv

2.2.1. Plasmid construction

The coding region for the toxin was produced by PCR using the Tx3-1-ISEF clone (Carneiro et al., 2003) as template. Serial PCR reactions were used in order to change some of the spider cDNA codons to *Escherichia coli* preferential codons. The oligonucleotides Tx31F (5′GCA GAA TGC GCA GCT GTT TAT GAA CGT TGC GGT AAA G 3′) and Tx31R (5′TTT GCA CGG ACG TTC TTC ACA G 3′) were used to amplify a fragment that codes for the 5′ region of the spider cDNA and the oligonucleotides Tx31F2 (5′TGA AGA ACG TCC GTG CAA ATG C 3′) and Tx31R2 (5′ AAT TCT GCA GTC ATT CGC TGA TAA ATT TTT TGC 3′) were used to amplify a fragment that codes for the 3′ region of the spider Pskov cDNA. Both fragments were used as template in a third PCR reaction that used as primers Tx31FECO (5′ AAT TGA ATT CAT CGA GGG AAG GGC AGA ATG CGC AGC TG 3′) and Tx31R2. This third PCR reaction produced a 156 bp fragments that codes for the entire mature peptide. The primer Tx31FECO had a sequence that codes for Factor Xa cleavage site immediately after the EcoRI restriction site that allowed separation of the recombinant mature Tx3-1 peptide from the maltose binding protein with no extra amino acids attached. Primers were synthesized by IDT-Integrated DNA Technologies. Amplification reaction contained primers in a 1 μM concentration, 250 μM of each deoxynucleotide triphosphate and 2 units of the thermostable recombinant Taq polymerase. The reactions were run in a programmable heat block manufactured by BioRad (USA). Each cycle consisted of denaturing the DNA at 94 °C for 1 min, annealing the primers for 1 min at 55 °C, and then extending the primers at 72 °C for 1 min. This cycle was repeated 40 times. After the final cycle samples were chilled at 4 °C.

The 156 bp PCR band was purified using the QIAquick TM Gel Extraction kit (Qiagen, USA), digested with EcoRI and PstI and cloned into pMAL (New England Biolabs, USA). This plasmid (pMAL-PhKv) encodes a 48 KDa recombinant PhKv protein which is tagged at the N-terminus with the maltose binding protein (MBP). The plasmid was purified using the Qiagen Plasmid Maxi Kit (Qiagen, USA). To ensure that no mutation had been introduced by the polymerase, clones had their sequence determined by automatic sequencing using the dideoxynucleotide chain-termination reaction (Sanger et al., 1977).

2.2.2. Expression and purification of the fusion protein

Expression of the fusion protein was induced by 0.6 mM IPTG at 37 °C. After 3 h of growth in the presence of IPTG, cells were harvested by centrifugation at 4000 × *g* for 20 min, suspended in 10 ml of column buffer (20 mM Tris/HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA), lysed by sonication and cell debris were removed by centrifugation. The soluble fusion protein was affinity-purified from the bacterial lysates using amylose resin and eluted with 50 mM maltose in column buffer.

2.2.3. Cleavage of MBP-PhKv fusion protein and purification of the recombinant PhKv protein

Fractions containing MBP-PhKv were combined and treated with Factor Xa protease which recognizes a specific amino acid sequence between MBP and PhKv. Fusion protein solution at a concentration of 1 mg/ml was incubated with 20 mM Tris–HCl pH 8 and Factor Xa (1% mass/mass; New England Biolabs) for 36 h at room temperature. The recombinant toxin was then concentrated using Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-30 membrane and purified by FPLC Sephadex 75 chromatography using column buffer. Fractions were analyzed by SDS/PAGE and pooled.

2.3. Isolated perfused heart

All the animal experiments were carried out in accordance with current guidelines for the care of laboratory animals and were authorized by the Ethics Committee of Federal University of Minas Gerais.

Male Wistar rats (230–260 g body weight) were decapitated 10–15 min after intraperitoneal injection of 400 IU heparin. The thorax was opened and the heart was carefully dissected and perfused through a 1.0 ± 0.3 cm aortic stump with Krebs–Ringer solution (KRS) containing (in mmol/l) 118.4 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4 \cdot 7H_2O$, 2.5 $CaCl_2 \cdot 2H_2O$, 11.7 glucose and 26.5 $NaHCO_3$ (pH 7.4). The perfusion fluid was maintained at 37 ± 1 °C with a pressure of 65 mmHg and constant oxygenation (5% CO_2/95% O_2). A force transducer (model FT3 – Grass) was attached through a heart clip to the apex of the ventricles to record the contractile force (tension, *g*) on a computer using a data acquisition system (Biopac System, CA, USA). A diastolic tension of 1.0 g was applied to the hearts. Electrical activity