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Bactridine 2 effect in DRG neurons. Identification of NHE as a second target

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ABSTRACT

Bactridine 2 (Bact-2) is an antibacterial toxin from *Tityus discrepans* venom which modifies isoforms 1.2, 1.4 and 1.6 voltage-dependent sodium (Na_v) channels. Bactridine-induced Na^+ outflow in *Yersinia enterocolitica* was blocked by amiloride, suggesting that Bact-2 effect was mediated by an amiloride sensitive sodium channel. In this study we show that Bact-2 increases also an outward rectifying current in rat dorsal root ganglia (DRG) sensory neurons; therefore, the nature of the outward rectifying currents was characterized and then the effect of Bact-2 on these currents was studied. These currents are enhanced by amiloride, are decreased by Na^+ when an outward pH gradient is present and its reversal potential coincides with that of a Cl^-/H^+ exchanger, suggesting that rectifying currents are produced by the electrogenic Cl^-/H^+ exchanger modulated by the Na^+/H^+ antiporter. Bact-2 also leads to an increase of the outward currents in a similar way to the produced by the inhibition of the Na^+/H^+ exchanger. Additionally, the subsequent application of Bact-2 after blocking the Na^+/H^+ exchanger does not produce any further effect, suggesting that Bact-2 modifies the outward current by modulating the activity of the Na^+/H^+ exchanger. The effect of Bact-2 on pH_i regulation was determined using the pH indicator BCECF. The results show that the Na^+/H^+ exchanger is blocked by amiloride and Na^+ free solutions and is modulated by Bact-2 in a similar way as cariporide. This study validates that besides Na_v channels, Bact-2 modulates the activity of the Na^+/H^+ exchanger.

1. Introduction

Bactridine 2 (Bact-2) is a toxin that belongs to a group of six antibacterial peptides isolated from *Tityus discrepans* scorpion venom. Structural studies revealed that Bact-2 is a hydrophilic, non-amphipathic and positively charged polypeptide comprising 64 amino acids, with a molecular mass of 7362 and stabilized by four disulfide bridges (Díaz et al., 2009). Bactridines induce leakage of Na^+ ions from the gram negative bacteria strain *Yersinia enterocolitica,* which is prevented by the Na^+ and Ca^{+2} channel blockers amiloride and mibefradil, respectively (Díaz et al., 2009). Additionally, these toxins affect the activation and inactivation kinetics of voltage-dependent Na^+ channels (Na_v) (Peigneur et al., 2012).

Na^+ is the most abundant cation in the extracellular space, which can enter cells through several routes, affecting cell volume, acid-base balance and nerve transmission (reviewed by Yu et al., 2010). The membrane transporter protein SLC9 (Solute Carrier) (Hediger et al., 2013) also known as Na^+/H^+ antiporters (NHE), which can be blocked by amiloride, are ubiquitous membrane proteins that play a major role in Na^+ and pH homeostasis of cells from bacteria to mammalians and higher plants. They are electroneutral in nature, exchanging 1 Na^+ for 1 H^+ down their respective concentration gradients (Brett et al., 2005; Orlowski and Grinstein, 2004). In addition, its activity has been coupled to separated H^+ and Cl^- conductive pathways (Binder et al., 2000; DeCoursey and Cherny, 1994; Demaurex et al., 1995; Rajendran et al., 1999). NHE isoform 1 (NHE-1) is a ubiquitously expressed plasma membrane protein and the most abundant NHE isoform in the central nervous system (Ma and Haddad, 1997). NHE-1 has been identified in dorsal root ganglia (DRG) neurons (Rocha-Gonzalez et al., 2009), where a role in inflammatory pain has been proposed. Besides its well-known participation in intracellular pH and volume regulation, NHE1 is also a key modulator of cellular growth and motility in a variety of non-

Abbreviations: DRG, dorsal root ganglia; TTX, tetrodotoxin; BSA, bovine serum albumin

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neuronal cell types (Cardone et al., 2005; Meima et al., 2007; Simchowitz and Cragoe, 1986; Stock et al., 2008), and in the early formation, extension, and branching of neuronal processes (Sin et al., 2009).

The proteins that transport Cl^- comprise: (i) chloride channels (ClCs) which represent a group of proteins that include ions channels that mediate voltage-dependent transport of Cl^- ions across cell membranes and Cl/H exchangers, (ii) the cystic fibrosis transmembrane conductance regulator (CFTR) with its related channels and (iii) the ligand-gated Cl^- channels opened by GABA and glycine. (iv) Also, the protein family TMEM16A/ANO1, conducts chloride currents and are activated by calcium (Cho et al., 2012; Pedemonte and Galietta, 2014; Picollo et al., 2015). The CICs are expressed in both plasma and intracellular membranes in all phyla, with nine isoform reported in mammals (ClC-1 to ClC-7, ClC-Ka, and ClC-Kb) (reviewed by (Poroca et al., 2017). Despite the fact that all mammals CLC proteins share the same basic architecture, this channel family can be divided into two main groups regarding their ion transport properties. The first group (ClC-1, ClC-2, ClC-Ka, and ClC-Kb), is strictly expressed in the plasma membrane and work as voltage-gated Cl^- channels, being the permeant ion Cl^- itself the responsible for the voltage-dependent gating (Adachi et al., 1994; Fahlke et al., 1996; Niemeyer et al., 2004; Pusch et al., 1995; Richard and Miller, 1990). The second group (ClC-3 to ClC-7), is expressed in both the plasma and intracellular membranes and operates as Cl^-/H^+ exchanger. These antiporters have a conserved $2Cl^+/1H^+$ stoichiometry and share the hallmark of displaying strong outwardly rectifying currents that are modulated by Cl^- and H^+ ions in both sides of the membrane (Accardi and Miller, 2004; Friedrich et al., 1999; Guzman et al., 2013; Leisle et al., 2011; Matsuda et al., 2008; Neagoe et al., 2010). CLC-3 has been suggested to be also a plasma membrane antiporter (Huang et al., 2001; Pang et al., 2016; Weylandt et al., 2001), and in DRG neurons has been shown to play a role controlling pain sensibility, (Pang et al., 2016). In contrast, ClC-6 and ClC-7 isoforms have been shown to have a different subcellular distribution, although ClC-6 mRNA has been detected in many tissues (Brandt and Jentsch, 1995), they are predominantly expressed in lysosomes and endosomes of developmental and adult sensory neurons (Kornak et al., 2001; Poët et al., 2006) and CLC-7 in osteoclasts (Kornak et al., 2001).

In this work we describe that in DRG neurons, Bact-2 increases an outward rectifying current that depends on Na^+ and H^+ concentration. Our results suggest that this current is produced by the activity of Cl^-/H^+ exchangers and modulated by the activity of the Na^+/H^+ antiporter. Bact-2 would be modifying the Na^+/H^+ exchanger activity but its effect would be reflected in an increase of electrogenic Cl^- currents. If Bact-2 modifies the Na^+/H^+ antiporter, we should detect an effect on the regulation of intracellular pH (pH_i). The investigation of this hypothesis constitutes the main purpose of the present study.

2. Materials and methods

2.1. Venom source

Tityus discrepans scorpions were collected in the metropolitan area surrounding Caracas, Venezuela. Scorpions kept alive in the laboratory were anesthetized once a month with CO_2 and milked for venom by means of electrical stimulation. Venom was dissolved in double distilled water and centrifuged at 15,000 g for 15 min. The supernatant was freeze-dried and stored at −80 °C until used.

2.2. Bactridine 2 purification procedures

Soluble venom (100 mg), was loaded on a Sephadex G-50 column (200 × 1 cm) as described previously (D'Suze et al., 1995). Fraction II was collected, freeze dried and subjected to an analytical C18 reverse phase column (250 × 10 mm) (Vydac, Hesperia, CA, USA). Bactridin-1 to −6 were isolated and purified as described previously by Díaz et al. (2009). Briefly, components were separated using a linear gradient from solution A [0.12% trifluoroacetic acid (TFA) in water] to 45% solution B (0.10% TFA in acetonitrile), in 45 min, at a flow rate of 1 mL/min, monitored by absorbance at 230 nm. Bactridine 2 was purified through the same column, using a linear gradient from 25 to 35% solution B in 60 min at a flow rate of 1 mL/min. The peak was collected manually, aliquots were prepared accordingly and dried using a Savant Speed-Vac dryer. For imaging experiments the toxin was resuspended in working solution with 0.1% BSA.

2.3. DRG neurons culture

Adult male Sprague-Dawley rats (~250 gm) were used and were kept and sacrificed following the regulations of the Instituto de Estudios Avanzados, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23), revised in 1996. Care was taken to use the minimal amount of animals and all precautions were taken to minimize animal suffering. DRG cells were obtained as previous reported by Castillo et al. (2011). Cells were seeded on Thermanox coverslips (Nunc Inc., Naperville, IL) covered with poli-D-lysine (37–50 μg/ml) (Sigma-Aldrich) and allowed to attach for 20 min in an incubator (humidified atmosphere, 5% CO2, 37 °C). After cell attachment, 2 ml of Neurobasal A medium (Gibco-Invitrogen, Waltham, MA), supplemented with 2% B27 (Gibco-Invitrogen), 0.5 mM Glutamax (Gibco-Invitrogen), penicillin (100 U/ml), streptomycin (100 μg/ml) were added and maintained in an incubator (5% CO2 at 37 °C). Electrophysiological experiments were performed after at least 16 h to 3 days of neuron plating. For imaging experiments cells were seeded on 15 mm glass coverslips (Warner Instruments).

2.4. Electrophysiological assays

Whole-cell recordings were performed according to standard techniques with a commercially available patch-clamp amplifier (Axopatch 200B, Molecular Devices, Sunnyvale, CA, USA) in an Axiovert 10 inverted microscope [Carl Zeiss AG (Oberkochen), Germany]. Data were acquired with pClamp software 10.0 (Molecular Devices) with a Digidata 1322 interface (Molecular Devices). Patch pipettes were pulled from borosilicate glass (Sutter Instruments, Novato, CA) with a Narishige puller and were fired-polished to a tip diameter yielding a resistance between 1 and 2 MΩ. The pipettes were filled with (in mM): NaCl 20, CsF 100, EGTA 10, TEA-Cl 20, ATP 2, HEPES-CsOH 10, pH 7.2, 310 mOsm adjusted with mannitol. The external solution had the following composition (in mM): NaCl 60, KCl 5, $CaCl_2$ 2, TEA-Cl 20, 4-Aminopyridine 5, glucose 5, $CdCl_2$ 0.2, $NiCl_2$ 0.2, $MgCl_2$ 1 and 0.1% BSA, HEPES-NaOH 10, pH adjusted to 7.2, 320 mOsm adjusted with mannitol. For 0 mM Na^+, external Na^+ was replaced with $NMDG^+$ and KCl was ommited. Outward rectifying currents were measured in the presence of 300 nM-1 μM TTX and calcium and potassium channel blockers.

Cells were voltage-clamped using the tight-seal whole-cell patch-clamp method. (Hamill et al., 1981). Most of the capacitive transients were canceled with the electronic circuitry provided with the amplifier, some records were corrected using a P/6 protocol but for most of them leak subtraction was performed offline using the Clampfit program of the pClamp 10.6 software. Recordings were always started 5 min after establishing the whole-cell configuration Series resistance was usually compensated between 70 and 80% in order to minimize voltage errors. All measurements were done at room temperature ~22 °C. Toxins were resuspended with 4 μl of double distilled water and were added directly with a pipette into the chamber with a final volume of 60 μl. Toxins were prepared and aliquoted in individual plastic tubes containing the mass required and lyophilized with a Savant Speed-Vac dryer so when diluted and added to the chamber yielded the concentration to be studied (from 0.1 mM to 22 μM). Chambers were thoroughly cleaned after each experiment. Membrane currents were filtered at 5 kHz and