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The spider toxin Phα1β recombinant possesses strong analgesic activity

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ABSTRACT

The native Phα1β – a Voltage-Gated Calcium Channel (VGCC) blocker – and its Recombinant Version – were both tested in rodent pain models with an intraplantar injections of capsaicin or formalin, a chronic constriction injury, and melanoma cancer related pain. The formalin nociceptive behaviour in the neurogenic phase was not affected by the toxin pre-treatments, while in the inflammatory phase, Phα1β and the Recombinant form caused a significant reduction. The nociception that was triggered by capsaicin, an agonist of the TRPV1 vanilloid receptor, was totally blocked by 100 pmol/site, i.t. of Phα1β or the recombinant version. For the neuropathic pain that was induced by a chronic constriction injury of the sciatic nerve, Phα1β and its Recombinant reduced the allodynia that was induced by the CCI procedure in the rats and the hypersensitivity lasted for 4 h. Fourteen days after the inoculation of the B16-F10 melanoma cells in the mice, a marked hyperalgesia was induced in the melanoma cancer pain model. Phα1β and the Recombinant form reduced the hyperalgesia with a full reversion at 100 pmol/site i.t. The inhibitory effects of the nociception that was induced by native Phα1β and the Recombinant in the studied pain models were not statistically different and they developed with no side effects.

1. Introduction

The spider neurotoxin PhTx3-6, a peptide having 55 amino acids and 6 S-S bridges, was patented as an antinociceptive agent (US 8,383,162 B 2 and PI 0605484-6, Brazil) with the name of Phα1β. By using electrophysiological methods, the peptide reversibly inhibits the high-voltage-activated Ca2+ channels, namely L-(Cav1.2), N-(Cav2.2), P/Q-(Cav2.1), and R-(Cav2.3), with varying potencies (N > R > P/Q > L) expressed in heterologous and native systems (Vieira et al., 2005). An intrathecal injection (i.t) of Phα1β has had an antinociceptive action in several models of rodents' pain (Souza et al., 2008; de Souza et al., 2013; Rigo et al., 2013b; Diniz et al., 2014; Tonello et al., 2014), with a therapeutical window (DT50/DI50) of 16, four times greater than ω-conotoxin MVIIA (ziconotide). The better analgesic profile of Phα1β, when compared with ω-conotoxin can be explained by several factors, including binding in other types of VSCCs and cation channels, such as TRP receptors. In fact, differently from ω-conotoxin MVIIA, Phα1β, can also act on other types of VSCCs that are involved in pain transmission to the spinal cord, such as P/Q and L, that could improve its analgesic actions (Vieira et al., 2005).Moreover, we have recently demonstrated that Phα1β is a selective TRPA1 receptor antagonist, but not a TRPV1 or a TRPV4 receptor antagonist (Tonello et al., 2017). TRPA1 receptor blockers are well known for producing effective antinociception with reduced side effects. It is noteworthy that Phα1β is

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able to produce maximal analgesia with doses that do not induce potential side effects.

In contrast, the maximal analgesia that is induced by ω-conotoxin MVIIA (ziconotide) could only be observed in doses close to DT50, causing severe side effects (Penn and Paice, 2000; Staats et al., 2004; Souza et al., 2008; Schmidtko et al., 2010). Thus, Phα1β has a higher therapeutical index and has a superior efficacy for the relief of pain, suggesting that this toxin has the potential to be used as a drug in the control of persistent pathological pain.

Given the high cost and the complexity of the synthetic processes of the disulfide-rich peptides, a genetic recombinant approach may simplify the development for this potent therapeutical agent. Thus, we have generated a Recombinant form of Phα1β expressed in *E. coli* that shares the analgesic properties of the native toxin. As a result, the aim of this manuscript was to compare, in different models of rodent's pain, the analgesic and side effects of native Phα1β and its Recombinant version.

2. Materials and methods

2.1. Native and the recombinant form of Phα1β

Native Phα1β was purified from the venom of the *P. nigriventer* spider by a combination of gel filtration, reverse phase FPLC/FPLC and ion exchange HPLC, as previously has been described (Cordeiro Mdo et al., 1993). The Recombinant version of Phα1β was synthesised by Giotto Biotech (www.giottobiotech.com) and was expressed in *E. coli*. It was purified through a proprietary production process, with a combination of ion exchange and size exclusion chromatography. The yield of the process was 0.5 mg/ml. The peptide MW is 6045 kDa. Both the native and the Phα1β Recombinant have the same 55 amino acids and the sequence is ACIPRGEICTDD-CECCGCDNQCYCPPGSSLGIFKCSCAHANKYFCNRKKE KCKKA. The sequence of the Recombinant and the natural Phα1β peptides are identicals, with the exception of a methionine that is added at the N-terminal portion of the Recombinant peptide (the addition of the starting methionine is a common practice in the heterologous protein expression). The stock solutions of the drugs were prepared in phosphate-buffered saline (composed of 137 mmol/L NaCl, 2.7 mmol/L KCl, and 10 mmol/L phosphate buffer) and in siliconised plastic tubes that were maintained at −20 °C. They were then diluted to the desired concentration just before use. All of the used drugs were of an analytical grade.

2.2. Animals

Male adult Swiss and C57Bl/6 mice (30–40 g) or Wistar rats (180–250 g) were acclimatised in the laboratory for at least 2 h before testing and they were used only once throughout the experiments. While in the home cage environment, the animals were allowed free access to water and food. The room temperature was maintained at 22 ± 1 °C and the room illumination was on a 12/12 light/dark cycle. The experiments were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for the investigations of experiments in conscious animals (Zimmermann, 1983). The experiments were authorized by the Ethics Committee of the Federal University of Minas Gerais, Protocol 347/2012. In addition, the number of animals and the intensity of the noxious stimuli used were the minimum levels necessary in order to demonstrate the consistent effects of the drug treatments.

2.3. Administration of the native and the recombinant version of Phα1β

In order to investigate the spinal effects of the Phα1β or Recombinant version peptides (0.1–100 pmol/site), they were administered by an intrathecal (i.t) route in accordance with the method as previously described by Hylden and Wilcox (1980) and Mestre et al., 1994.

The injections were made with a 28-gauge needle that was connected to a Hamilton micro syringe with volumes of 2.5 μl/site (for the capsaicin test) or 5.0 μl/site (for the other pain models). Before injecting the intrathecal toxins and in order to validate the method, our experimenters had to perform a previous training with the i.t. administration of an anaesthetic (lidocaine 1%) following by an observation for the development of a spinal blockade that was indicated by paralysis of both hind limbs. We only accepted those experimenters who were able to perform an i.t. injection of toxins and those that could achieve more than 90% accuracy in all of their injections. The correct puncture of the dura was indicated by a slight flick of the tail.

2.4. Mechanical hyperalgesia - Von Frey test

The mechanical nociceptive pain was assessed by a measurement of the paw withdrawal threshold (PWT) by using the up-down paradigm as described previously by Chaplan et al. (1994) with minor modifications. Briefly, the mice were first acclimatised (1–2 h) in individual clear Plexiglass boxes (9 × 7 × 11 cm) on an elevated wire mesh platform, in order to allow for an access to the plantar surfaces of the hind paws. Von Frey filaments of increasing stiffness (0.02–10 g) were applied to the hind paw plantar surfaces of the mice with enough pressure to bend the filament. The absence of a paw being lifted after 5 s led to the use of the next filament with an increased weight, whereas with the paw being lifted, this indicated a positive response and led to the use of next weaker filament. The 50% mechanical paw withdraw threshold (PWT) response was then calculated from the resulting scores, as previously described by Dixon (1980). The PWT responses were expressed in grams (g), and they were evaluated several times after the intrathecal administrations of native Phα1β, Phα1β Recombinant or PBS.

2.5. Chronic constriction of a sciatic nerve injury (CCI)

Adult male Wistar rats (180–250 g) that were bred in-house were used. A Chronic Constriction Injury (CCI) of the sciatic nerve was used as the neuropathic pain model. Nociception was assessed by detecting the mechanical hyperalgesia, when considering a significant reduction in the 50% paw withdrawal threshold values after the CCI, when compared with the baseline values. First of all, we assessed the antinociceptive effects of a single i.t. injection of Phα1β (10, 30, or 100 pmol/site) in a model of neuropathic pain, 8 days after the nerve injury. In order to induce the neuropathic pain, the animals were anaesthetised with a mixture of ketamine (90 mg/kg) and xylazine (30 mg/kg) by an intraperitoneal (i.p.) injection. A chronic constriction injury (CCI) of the sciatic nerve was performed according to a previously described method, with minor modifications, as related by Bennett and Xie (1988). Briefly, the sciatic nerve was exposed in the midline of the thigh and close to its trifurcation, where it was held with 3 loose ligatures, 1 mm distant from each other, using nylon thread (4.0). The incision was closed by using a nylon suture (6.0). As a control sham-operated animals were used, in which the sciatic nerve was exposed, but no ligation or injury of the nerve was performed.

The 50% mechanical paw withdraw threshold (PWT) responses were then calculated from the resulting scores, as described previously by Dixon (1980). The 50% PWT responses were expressed in grams (g). These were evaluated several times after the intrathecal injection of Phα1β (100pmole/site), the Phα1β Recombinant