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RESEARCH PAPER

The peptide Phα1β, from spider venom, acts as a TRPA1 channel antagonist with antinociceptive effects in mice

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BACKGROUND AND PURPOSE

Peptides from venomous animals have long been important for understanding pain mechanisms and for the discovery of pain treatments. Here, we hypothesized that Phα1β, a peptide from the venom of the armed spider *Phoneutria nigriventer*, produces analgesia by blocking the TRPA1 channel.

EXPERIMENTAL APPROACH

Cultured rat dorsal root ganglion (DRG) neurons, human fetal lung fibroblasts (IMR90) or HEK293 cells expressing the human TRPA1 (hTRPA1-HEK293), human TRPV1 (hTRPV1-HEK293) or human TRPV4 channels (hTRPV4-HEK293), were used for calcium imaging and electrophysiology. Nociceptive responses induced by TRPA1, TRPV1 or TRPV4 agonists or by bortezomib were investigated in mice.

KEY RESULTS

Phα1β selectively inhibited calcium responses and currents evoked by the TRPA1 agonist, allyl isothiocyanate (AITC), on hTRPA1-HEK293, IMR90 fibroblasts and DRG neurons. Phα1β did not affect calcium responses evoked by selective TRPV1 (capsaicin) or TRPV4 (GSK 1016790A) agonists on the various cell types. Intrathecal (i.t.) and intraplantar (i.pl.) administration of low doses of Phα1β (up to 300 pmol per paw) attenuated acute nociception and mechanical and cold hyperalgesia evoked by AITC (i.t. or i.pl.), without affecting responses produced by capsaicin or hypotonic solution. Notably, Phα1β abated the TRPA1-dependent neuropathic pain-like responses induced by bortezomib. *In vitro* and *in vivo* inhibition of TRPA1 by Phα1β was reproduced by a recombinant form of the peptide, CTK 01512-2.

CONCLUSIONS AND IMPLICATIONS

Phα1β and CTK 01512–2 selectively target TRPA1, but not other TRP channels. This specific action underlines the potential of Phα1β and CTK 01512-2 for pain treatment.

Abbreviations

AITC, allyl isothiocyanate; BTZ, bortezomib; CIPN, chemotherapy-induced peripheral neuropathy; DRG, dorsal root ganglia; i.pl., intraplantar; IMR90, human fetal lung fibroblasts; PWT, paw withdrawal threshold; VGCCs, voltage-gated calcium channels

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Tables of Links

TARGETS
GPCRs[a]
PAR2
Voltage-gated ion channels[b]
TRPA1
TRPV1
TRPV4

LIGANDS
AITC, allyl isothiocyanate
BTZ, bortezomib
Capsaicin
Capsazepine
GSK1016790A
HC030031
HC067047

These Tables list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b]Alexander *et al.*, 2015a,b)

Introduction

The poison of venomous animals is a rich source of potent and selective ion channel blockers with potential analgesic effects (Gomez *et al.*, 2002; Estrada *et al.*, 2007). Phα1β is a peptide purified from the venom of the armed spider *Phoneutria nigriventer,* that reversibly inhibits voltage-gated calcium channels (VGCCs), with some selectivity for N-type VGCCs (Vieira *et al.*, 2005). This peptide exerts antinociceptive effects in several preclinical pain models, such as post-surgical, neuropathic and cancer-related pain (Souza *et al.*, 2008; Rigo *et al.*, 2013a,b). Phα1β seems to have a good tolerability (Rigo *et al.*, 2013b), with a higher therapeutic index than the selective N-type VGCC blocker, ω-conotoxin MVIIA, suggesting that additional mechanisms besides VGCC blockade contribute to its antinociceptive effect.

The TRPA1 channel, a non-selective cation channel activated by reactive endogenous and exogenous substances and noxious cold, is now considered to represent a major pain transduction pathway (Andrade *et al.*, 2012, Nassini *et al.*, 2014). TRPA1 channels are co-expressed with other TRP channels, including the capsaicin receptor, (TRPV1 channels), in a specific subset of primary sensory neurons, with cell bodies located in trigeminal, vagal and dorsal root ganglia (DRG) (Story *et al.*, 2003, Andrade *et al.*, 2012). A variety of studies in classical rodent models indicated that TRPA1 channels contributed to signal acute nociception and hyperalgesia in models of inflammatory and neuropathic pain (Baraldi *et al.*, 2010; Andrade *et al.*, 2012; Nassini *et al.*, 2014). Among several examples, in a model of neuropathic pain by chemotherapeutic agents, such as oxaliplatin (Nassini *et al.*, 2011) or bortezomib (BTZ) (Trevisan *et al.*, 2013), mechanical and cold hyperalgesia were found to be totally mediated by TRPA1 channels. Thus, these channels are potential targets for the development of new analgesic drugs.

Some animal venoms contain substances that target TRP channels, including APHC1, which antagonizes TRPV1, and the tarantula venom peptide, ProTx-I, which inhibits TRPA1 channels (Andreev *et al.*, 2008; Gui *et al.*, 2014). Phα1β was found to reduce nociceptive responses evoked by capsaicin administration (Castro-Junior *et al.*, 2013). Notably, the reduction was obtained by using very high local doses of the toxin, while *in vitro* 2 μM Phα1β failed to inhibit TRPV1 channels. The present investigation aimed at identifying additional mechanisms, likely to be produced by small concentrations or doses of Phα1β, which might contribute to the analgesic properties of the toxin. We report here that Phα1β is a potent and selective antagonist of TRPA1 channels and does not target other TRP channels expressed by nociceptors.

Methods

Animals

All animal care and experimental procedures were carried out according to the current European Communities Council-ECC guidelines for the care of laboratory animals, the Italian legislation (DL 116/92) application of the ECC directive (86/609/EEC) and ethical guidelines for investigations of experimental pain in conscious animals. All protocols were conducted under the University of Florence research permit #204/2012-B. In addition, the number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the treatments used. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Male C57BL/6 mice (20–25 g, Harlan Laboratories, Milan, Italy) or Sprague Dawley rats (150–200 g, male, Harlan Laboratories) were used inthese experiments. Animals were housed in a controlled-temperature environment in individually ventilated cages: 10 per cage (mice) or five per cage (rats), with wood shaving bedding and nesting material, maintained at 22 ± 1°C. Animals were housed with a 12 h light/dark cycle (lights on at 0700 h) and fed with rodent chow (Global Diet 2018, Harlan, Lombardy, Italy) and tap water *ad libitum*. Animals were allowed to acclimatize to their housing environment for at least 7 days prior to experimentation and to the experimental room for 1 h before experiments.

General procedures

BTZ-induced neuropathic pain in mice is considered a relevant model of neuropathic pain caused by chemotherapy treatment (Trevisan *et al.,* 2013). In fact, chemotherapy-induced peripheral neuropathy (CIPN) has emerged as a major complication of BTZ therapy in patients, which usually appears in the first courses of therapy with a number of sensory and painful symptoms, including a lower threshold to mechanical and cold stimuli (Cata *et al.,* 2007). No effective treatment exists for BTZ-evoked CIPN.

Allocation concealment was performed using a randomization procedure (http://www.randomizer.org/). Experimenters were blinded to the drug treatment when performing tests. No animals were excluded from the analysis. Each experiment was repeated two to three times (using two or three animals in each repetition) between 0800 and 1700 h.

For the *in vivo* experiments, the primary outcome was acute spontaneous nociception, and secondary outcomes were mechanical and cold hyperalgesia. The group size for each experiment was determined through sample size estimation (Armitage and Berry, 1987) based on the primary outcome. Expected standard deviation values were based on pilot results. The minimum effect size was considered as a prevention of at least 30% of the nociception response, for a significance level of 5%, with a test power of 90% and a two-tailed hypothesis test. Thus, we estimated a group size of six mice for each experimental group.

Drug treatments. Phα1β (10–300 pmol per site or pmol per paw), CTK 01512–2 (100 pmol per site or 300 pmol per paw), HC-030031 (HC03, 30 nmol per site or 300 nmol per paw), ω-conotoxin MVIIA (100 pmol per site or 300 pmol per paw) and allyl isothiocyanate (AITC, 0.01–3 nmol per site or 10 nmol per paw) were injected intrathecally (i.t.) in a volume of 5 μL per site (Hylden and Wilcox, 1980) or intraplantarly (i.pl.) in a volume of 10 μL per paw (Andrade *et al.,* 2012). Hypotonic solution (0.27% NaCl), capsaicin (0.1 nmol per paw), capsazepine (1 nmol per paw) and HC-067047 (300 nmol per paw) were injected i.pl. in a volume of 10 μL per paw. BTZ (1 $mg \cdot kg^{-1}$) was injected i.p. Phα1β and CTK 01512–2 were dissolved in PBS; HC-030031, HC-067047 and capsazepine were dissolved in DMSO 5%; BTZ and AITC were dissolved in DMSO 1%; capsaicin was dissolved in 0.005% ethanol; and NaCl 0.27% was dissolved in Milli-Q water. The drug doses were based on pilot experiments and on previous studies (Andrade *et al.,* 2012; Castro-Junior *et al.*, 2013; Trevisan *et al.,* 2013; Rigo *et al.,* 2013b; Nassini *et al.,* 2015).

Cell culture and isolation of primary sensory neurons. Human fetal lung fibroblasts (IMR90; ATCC, Manassas, VA), used for the study of cells constitutively expressing the human TRPA1 channel, were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin. Untransfected HEK293 cells (ATCC, Manassas, VA) and HEK293 cells stably transfected with the cDNA of human TRPA1 (hTRPA1-HEK293), or with the cDNA of human TRPV1 (hTRPV1-HEK293), or with the cDNA of human TRPV4 channels (hTRPV4-HEK293), were cultured as previously described (Nassini *et al.,* 2012). All cells were cultured in an atmosphere of 95% air and 5% CO_2 at 37°C.

Primary sensory neurons were isolated from Sprague–Dawley rats and cultured as previously described (Materazzi *et al.,* 2012). Briefly, ganglia were bilaterally excised under a dissection microscope and transferred to HBSS containing 2 $mg \cdot mL^{-1}$ of collagenase type 1A and 1 $mg \cdot mL^{-1}$ of trypsin for enzymic digestion (30 min, 37°C). Ganglia were then transferred to warmed DMEM containing 10% FBS, 10% horse serum, 2 mM·L-glutamine, 100 $U \cdot mL^{-1}$ penicillin and 100 $mg \cdot mL^{-1}$ streptomycin and dissociated into single cells by several passages through a series of syringe needles (23–25 G). Medium and ganglia cells were filtered to remove debris and centrifuged. The pellet was suspended in DMEM with added 100 $ng \cdot mL^{-1}$ mouse-NGF and 2.5 mM cytosine-b-D-arabinofuranoside free base. Neurons were then plated on 25 mm-diameter glass coverslips coated with poly-L-lysine (8.3 μM) and laminin (5 μM).

Calcium imaging assay. Intracellular calcium was measured in transfected and untransfected HEK293 and IMR90 cells, or in rat DRG neurons, as previously reported (Materazzi *et al.,* 2013). Plated cells were loaded with 5 μM Fura-2 AM-ester (Alexis Biochemicals; Lausen, Switzerland) added to the buffer solution (37°C) containing the following (in mM): 2 $CaCl_2$; 5.4 KCl; 0.4 $MgSO_4$; 135 NaCl; 10 D-glucose; 10 HEPES and 0.1% bovine serum albumin at pH 7.4. After loading (40 min), cells were washed and transferred to a chamber on the stage of a Nikon Eclipse TE-2000 U microscope for recording. Cells were excited alternately at 340 and 380 nm to indicate relative intracellular calcium changes by the $Ratio_{340/380}$ ($R_{340/380}$) recorded with a dynamic image analysis system (Laboratory Automation 2.0; RCSoftware, Florence, Italy). To evoke a TRPA1 channel-dependent calcium response, cells and neurons were challenged with the selective agonist, AITC (1–10 μM). Buffer solution containing DMSO 0.3% was used as vehicle. Capsaicin (0.1 μM) was used to identify TRPV1-expressing neurons. Cells or neurons were pre-exposed to Phα1β, CTK 01512–2, HC-030031 or vehicle (DMSO 0.3%) before (10 min) the acute addition of the TRPA1 channel agonist. Results are expressed as the percentage of the increase in $R_{340/380}$ over baseline, normalized to the maximum effect induced by ionomycin (5 μM) added at the end of each experiment (% Change in $R_{340/380}$), or as the percentage of the inhibitory effect on the calcium response evoked by AITC (% AITC response) for constructing the concentration–response curves in the presence of PDs.

Electrophysiology. Whole-cell patch-clamp recordings were performed in IMR90 cells on a poly-Llysine-coated 13 mm-diameter glass coverslips (Nassini *et al.,* 2015). Each coverslip was transferred to a recording chamber (1 mL volume) mounted on the platform of an inverted microscope (Olympus CKX41, Milan, Italy) and superfused at a flow rate of 2 $mL \cdot min^{-1}$ with a standard extracellular solution containing (in mM): HEPES 10, D-glucose 10, NaCl 147, KCl 4, $MgCl_2$ 1 and $CaCl_2$ 2 (pH adjusted to 7.4 with

NaOH). Borosilicate glass electrodes (Harvard Apparatus, Holliston, MA, USA) were pulled with a Sutter Instruments puller (model P-87) to a final tip resistance of 4–7 MΩ. Pipette solution used contained the following (in mM): K-gluconate 134, KCl 10, EGTA 11 and HEPES 10 (pH adjusted to 7.4 with KOH). Data were acquired with an Axopatch 200B amplifier (Axon Instruments, CA, USA), stored and analysed with a pClamp 9.2 software (Axon Instruments, CA, USA). Cells were voltage-clamped at a holding potential of −60 mV, and currents were detected as inward currents activated on cell superfusion with AITC (30 and 100 μM) or KCl (90 mM) in the absence or presence of CTK 01512–2 (3 μM) or HC-030031 (50 μM). Cell membrane capacitance was calculated in each cell throughout the experiment by integrating the capacitive currents elicited by a ±10mV voltage pulse. Peak currents activated by each compound were normalized to cell membrane capacitance and expressed as mean of the current density (pA/pF) in averaged results.

Behavioural studies. For behavioural experiments, after habituation, C57BL/6 mice were randomized into treatment groups, and an investigator blinded to treatments recorded the responses. In a first series of experiments, C57BL/6 mice were previously (10 min) treated with Phα1β (30–300 pmol per paw), CTK 01512–2 (300 pmol per paw) or their vehicle (PBS), *via* i.pl. injection (10 μL). Nociceptive response, as well as mechanical and cold hyperalgesia induced by i.pl. injection of the TRPA1 agonist, AITC (10 nmol per paw), were recorded. Mechanical and cold hyperalgesia were assessed from 0.25 to 2 h after i.pl. AITC treatment. The nociceptive response (total time spent in lifting/licking) or the mechanical and cold hyperalgesia produced by AITC in naïve mice was not affected by the vehicle (PBS). Some C57BL/6 mice were pretreated with HC-030031 (TRPA1 antagonist, 300 nmol per paw, 10 min before) as a control. In addition, to test the ability of the peptide to selectively inhibit TRPA1 channels, the effect of pretreatment with Phα1β (30–300 pmol per paw, 10 min before) was evaluated on the nociceptive response and mechanical hyperalgesia induced by i.pl. (10 μL) capsaicin (TRPV1 channel agonist, 0.1 nmol per paw), hypotonic solution (TRPV4 channel stimulator, 0.27% NaCl) or their vehicles (0.005% ethanol and 0.9% NaCl, respectively) 0.25 h after capsaicin or 0.27% NaCl injection. Some mice were pretreated with capsazepine (TRPV1 channel antagonist, 1 nmol per paw, 10 min before capsaicin) and HC-067047 (TRPV4 channel antagonist, 300 nmol per paw, 10 min before 0.27% NaCl) or their vehicle (DMSO 5%).

In another set of experiments, we assessed the hyperalgesia evoked by i.t. injection of AITC. Mechanical and cold hyperalgesia induced by AITC (0.01–3 nmol per site) were measured at 0.5 to 6 h after treatment. Next, the effects of both Phα1β and CTK 01512–2 were evaluated on mechanical and cold hyperalgesia induced by i.t. injection (5 μL per site) of the TRPA1 channel agonist, AITC (1 nmol per site). Mice were treated 10 min before AITC treatment with Phα1β (10–100 pmol per site, i.t.), CTK 01512–2 (100 pmol per site, i.t.), HC-030031 (30 nmol per site, i.t.) or their vehicle, and 0.5 to 2 h after treatment, the mechanical and cold hyperalgesia were measured.

A single administration of BTZ (1 mg·kg^{-1}, i.p.) has previously been shown to produce persistent (15–20 days) mechanical and cold hyperalgesia in mice (Trevisan *et al.*, 2013). At day 7 after BTZ injection (1 mg·kg^{-1}, i.p.), mechanical and cold hyperalgesia were measured from 0.5 to 4 h after treatment with Phα1β (10–100 pmol per site, i.t.), CTK 01512–2 (100 pmol per site, i.t. or 300 pmol per paw), HC-030031 (30 nmol per site, i.t. or 300 nmol per paw), ω-conotoxin MVIIA (100 pmol per site, i.t. 300 pmol per paw) or their vehicle.

Behavioural tests

Acute spontaneous nociception test. Immediately after i.pl. injection with AITC, capsaicin and 0.27% NaCl, mice were placed inside a plexiglass chamber, and the total time spent in lifting/licking of the injected hind paw was recorded for 10 min, as an indicative parameter of nociception (Trevisani *et al.*, 2007). The i.pl. injection with vehicles of AITC, capsaicin and 0.27% NaCl produced nociceptive behaviour for a maximum of 2 s.

Von Frey test. Mechanical nociceptive pain was assessed through the measurement of paw withdrawal threshold (PWT) using the 'Up-and-Down' paradigm, as previously described (Tonello *et al.*, 2014). Mechanical nociceptive threshold was determined before (basal level threshold) and after different treatments. The 50% mechanical PWT (in g) response was then calculated from resulting scores as previously described by Dixon (1980).

Cold stimulation. Cold hyperalgesia was assessed in mice by measuring the acute nocifensive response to the acetone-evoked evaporative cooling as previously reported (Trevisan *et al.*, 2013). Briefly, a droplet (50 μL) of acetone, formed on the flat-tip needle of a syringe, was gently touched to the plantar surface of the mouse hind paw, and the time spent in elevation and licking of the plantar region over a 60 s period was measured.

Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). The results are expressed as the means ± SEM, with the exception of the ED_{50} or IC_{50} values, which were reported as the geometric mean accompanied by the corresponding 95% CIs. The values of IC_{50} and ED_{50} for concentration and dose–response curves were calculated with non-linear regression using a sigmoidal dose–response curve. The percentages of maximum effect (E_{max}) and maximum inhibition (I_{max}) were reported as the means ± SEM for each individual experiment in relation to the control values. Differences among three or more groups at one time point were analysed by one-way ANOVA followed by Newman–Keuls' *post hoc* test. Differences among three or more groups at time course curves were analysed by two-way ANOVA (treatment and time as factors, time as repeated measure) followed by Bonferroni's *post hoc* test. *P* values less than 0.05 were considered significant. To meet the ANOVA assumptions, the mechanical hyperalgesia data

were log transformed prior to statistical analysis. Statistical analysis was performed using GraphPad Software 5.0 (GraphPadSoftware, San Diego, CA, USA).

Materials

Native Phα1β was purified as previously described and had the following amino acid sequence: ACIPRGEICT DDCECCGCDN QCYCPPGSSL GIFKCSCAHA NKYFCNRKKE KCKKA (Cordeiro *et al.*, 1993). The recombinant forms of Phα1β (CTK 01512-2) and ω-conotoxin MVIIA were purchased from Giotto Biotech S.r.l. (Florence, Italy) and Latoxan (Portes lès Valence, France). The stock solutions of drugs were prepared with PBS (pH 7.4) in siliconized plastic tubes, maintained at −20°C and diluted to the desired concentration just before use. The TRPA1 channel-selective antagonist HC-030031 was synthesized as previously described (Andre *et al.*, 2008), and BTZ was purchased from LC Laboratories. Unless otherwise indicated, all reagents were from Sigma (St Louis, MO, USA) and were dissolved in appropriate vehicle solutions.

Results

Phα1β is a selective antagonist of TRPA1 channels

The ability of Phα1β or CTK 01512–2 to affect calcium responses evoked by stimulation of TRPA1 channels was investigated in hTRPA1-HEK293 cells. Similar to the TRPA1 channel antagonist HC-030031, Phα1β and CTK 01512–2 inhibited responses evoked by AITC (Figure 1A and Table 1).

Table 1

The concentration inhibiting 50% of the maximum response (with corresponding CIs) of Phα1β and CTK 01512–2 on calcium responses evoked by AITC

Cell type	Phα1β (nM)	CTK 01512–2 (nM)
TRPA1-HEK293	681 (472–983)	506 (414–619)
IMR90	40 (22–74)	28 (15–51)
DRG neurons	32 (21–47)	34 (11–101)

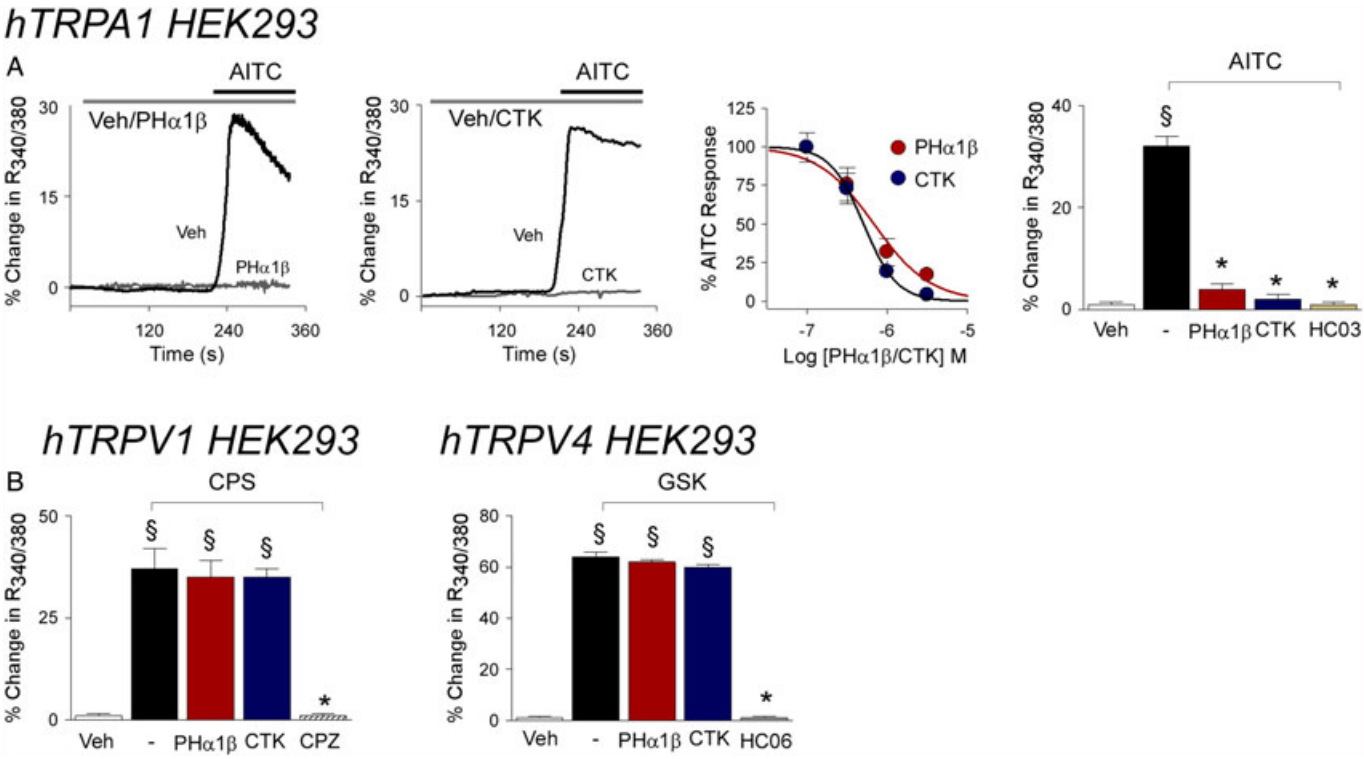

Figure 1

Phα1β and its recombinant form (CTK 01512–2) selectively inhibit the calcium response evoked by stimulation of human TRPA1 channels. (A) Typical traces of the inhibitory effect of pre-exposure (10 min) to Phα1β (3 μM) and CTK 01512–2 (3 μM) on the calcium response evoked by the TRPA1 channel agonist, AITC (30 μM), in HEK293 cells transfected with the cDNA for human TRPA1 channels (hTRPA1-HEK293). Concentration–-response curve of the inhibitory effect of Phα1β and CTK 01512–2 on the calcium response evoked by AITC (AITC concentrations are 30 μM). Phα1β (3 μM) and CTK 01512–2 (3 μM) and the selective TRPA1 channel antagonist HC-030031 (HC03, 30 μM) inhibit calcium response evoked in hTRPA1-HEK293 cells by AITC. (B) Phα1β (3 μM) and CTK 01512–2 (3 μM) do not affect responses evoked by capsaicin (CPS, 0.1 μM) in HEK293 cells transfected with the cDNA for human TRPV1 channels (hTRPV1-HEK293) and by GSK1016790A (GSK, 50 nM) in HEK293 cells transfected with the cDNA for human TRPV4 channels (hTRPV4-HEK293). Values are mean ± SEM of $n > 25$ cells from at least three different experiments for each condition. $^{§}P < 0.05$, significantly different from vehicle (Veh); $^{*}P < 0.05$, significantly different from AITC.

In hTRPV1-HEK293 cells, calcium responses evoked by the selective TRPV1 agonist, capsaicin, were abated by the selective channel antagonist, capsazepine, but were unaffected by Phα1β or CTK 01512–2 (Figure 1B). Likewise, calcium responses evoked by the selective TRPV4 channel agonist, GSK1016790A, were abated by the corresponding channel antagonist, HC-067047, in hTRPV4-HEK293 cells, but were unaffected by Phα1β or CTK 01512–2 (Figure 1B).

The ability of Phα1β or CTK 01512–2 to antagonize TRPA1 channels was also studied in IMR90 fibroblasts, a cell line from which TRPA1 channels were originally cloned (Jaquemar *et al.*, 1999), and in rat DRG neurons that constitutively express TRPV1, TRPV4 and TRPA1 channels. Pre-exposure to Phα1β, CTK 01512–2 or HC-030031 inhibited responses evoked by AITC in IMR90 cells and in rat DRG neurons (Figure 2 and Table 1).

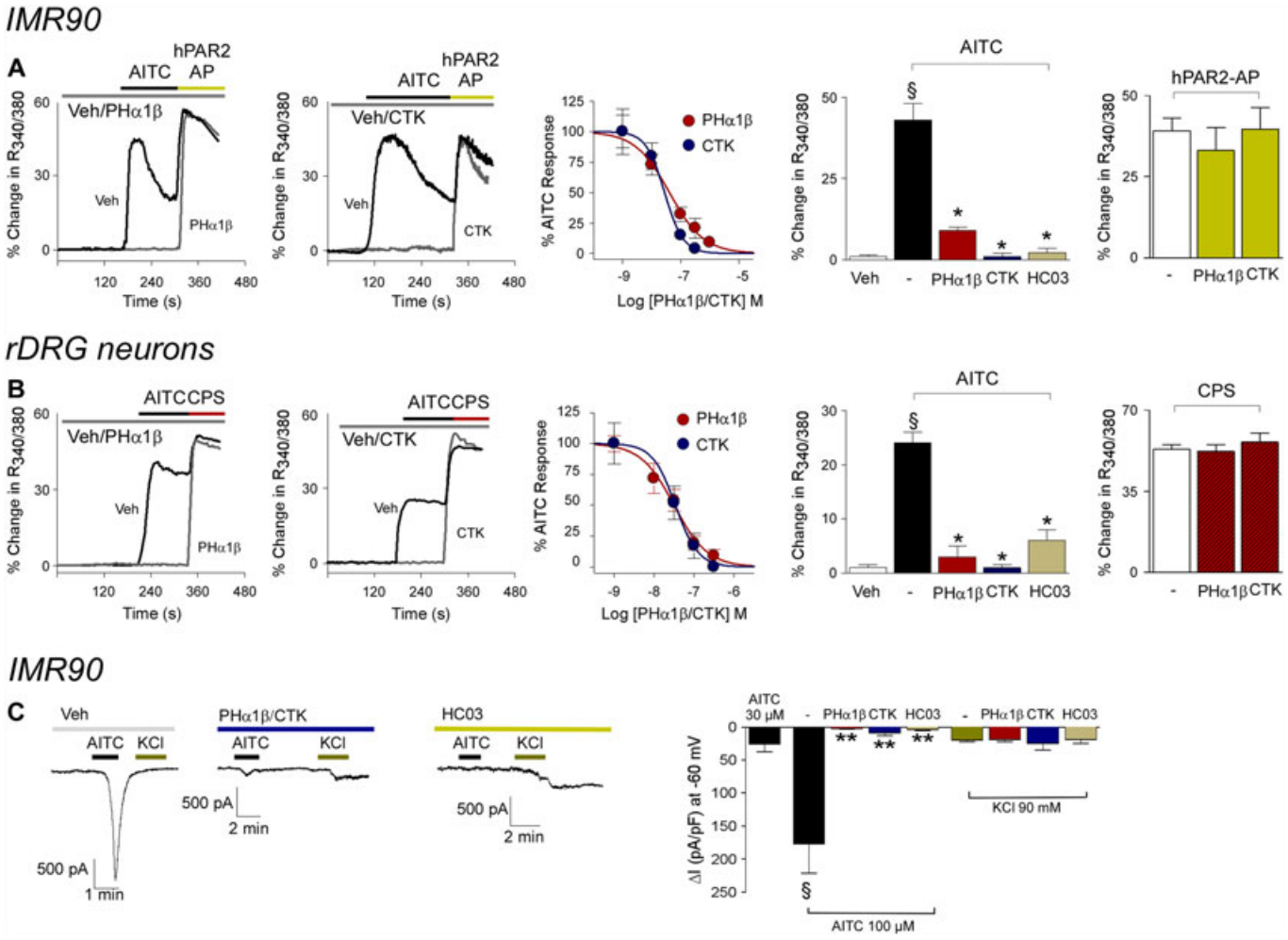

Figure 2

Phα1β and its recombinant form (CTK 01512–2) selectively inhibit calcium response or currents evoked by stimulation of TRPA1 channels. (A) Typical traces of the inhibitory effect of pre-exposure (10 min) to the Phα1β (300 nM) and CTK 01512–2 (300 nM) on the calcium response evoked by the TRPA1 channel agonist, AITC (10 μM), in cultured IMR90 cells. Concentration–response curves of the inhibitory effect of Phα1β and CTK 01512–2 on the calcium response evoked by AITC in IMR90 cells (AITC concentrations are 1 μM). Phα1β (300 nM) and CTK 01512–2 (300 nM) and the selective TRPA1 channel antagonist HC-030031 (HC03, 30 μM) inhibit calcium responses evoked in IMR90 cells by AITC. Phα1β (300 nM) and CTK 01512–2 (300 nM) do not affect responses evoked by the activating peptide of the human PAR2 receptor (hPAR2-AP, 100 μM) in IMR90 cells. TRP, trypsin. (B) Typical traces of the inhibitory effect of pre-exposure (10 min) to Phα1β (300 nM) and CTK 01512–2 (300 nM) on the calcium response evoked by AITC (10 μM), in cultured rat DRG (rDRG) neurons. Concentration–response curves of the inhibitory effect of Phα1β and CTK 01512–2 on the calcium response evoked by AITC in rDRG neurons (AITC concentrations are 10 μM). Phα1β (300 nM) and CTK 01512–2 (300 nM) and HC03 (30 μM) inhibit calcium response evoked in rDRG neurons by AITC. Phα1β (300 nM) and CTK 01512–2 (300 nM) do not affect responses evoked by capsaicin (CPS, 0.1 μM) in rDRG neurons. Values are mean ± SEM of $n > 25$ cells from at least three different experiments for each condition. $^{\S}P < 0.05$, significantly different from vehicle (Veh); $^{*}P < 0.05$, significantly different from AITC. (C) Typical traces and pooled data obtained by whole-cell patch-clamp recordings in cultured IMR90 exposed to the selective TRPA1 channel agonist AITC. The inward currents evoked at −60 mV by AITC (100 μM), but not those evoked by KCl (90 mM), are attenuated by the selective TRPA1 channel antagonist, HC-030031 (HC03; 50 μM), Phα1β or CTK 01512–2 (3 μM). Values are the mean ± SEM of at least five independent experiments. §$P < 0.05$, significantly different from AITC 30 μM; **$P < 0.05$, significantly different from AITC 100 μM alone.

Neither Phα1β nor CTK 01512–2 produced any stimulatory effect nor affected any responses to other excitatory stimuli, such as the activating peptide of the human proteinase activated receptor-2 (hPAR2-AP) in IMR90 cells, or the selective TRPV1 channel agonist, capsaicin, in DRG neurons, thus indicating selectivity (Figure 2).

The ability of Phα1β or CTK 01512–2 to affect inward currents evoked by TRPA1 channel stimulation was investigated in IMR90 cells. In such cells, inward currents evoked by AITC were reduced by HC-030031, Phα1β or CTK 01512–2 (Figure 2C). HC-030031, Phα1β or CTK 01512–2 did not affect the inward currents evoked by high KCl, indicating selectivity.

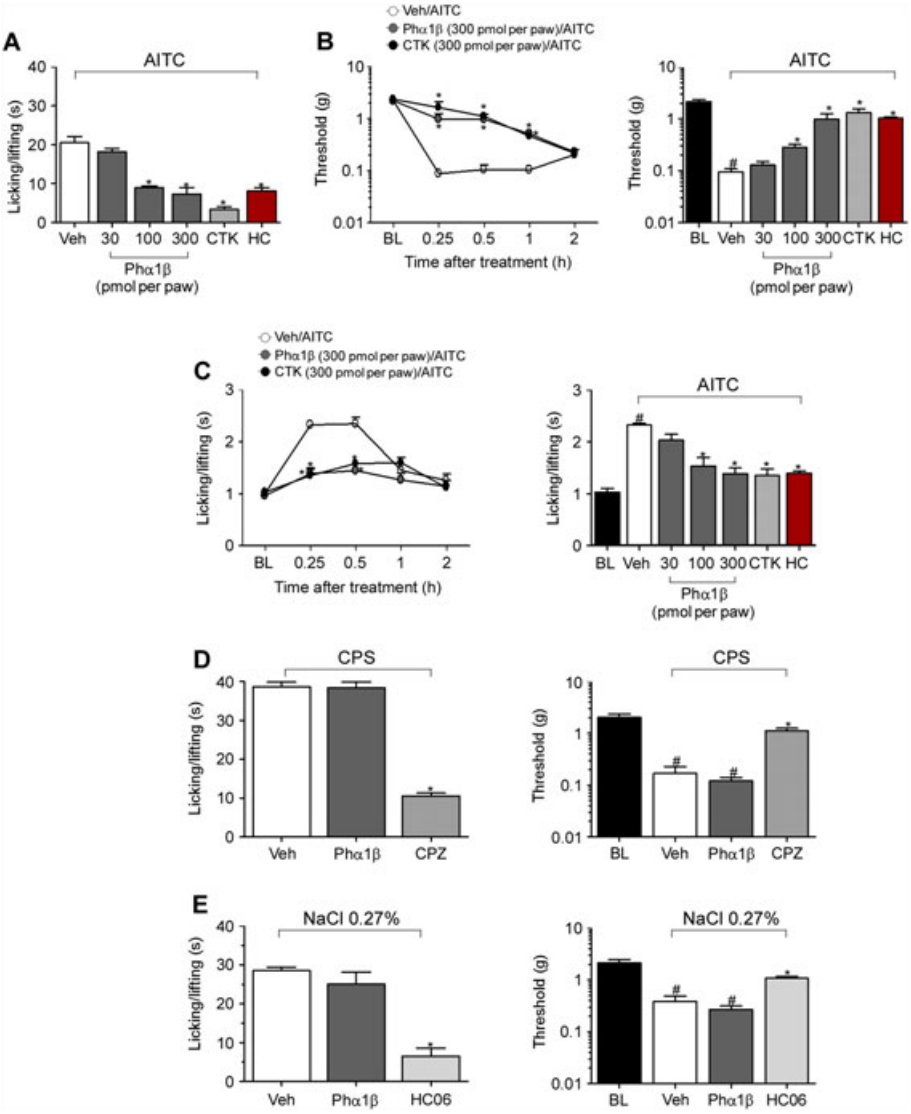

Figure 3

Phα1β and its recombinant form (CTK 01512–2) selectively block nocifensor responses evoked by reactive TRPA1 channel agonist. (A–C) Effect of increasing doses of i.pl. administration of Phα1β (30–300 pmol per paw) or CTK 01512–2 (300 pmol per paw) and the selective TRPA1 channel antagonist, HC-030031 (HC, 300 nmol per paw) on the nociceptive response evoked by the i.pl. injection (10 μL, i.pl.) of AITC (10 nmol per paw) in C57BL/6 mice. (B–C) Time course and dose–response curve (0.25 h after AITC treatment) of Phα1β (30–300 pmol per paw) or CTK 01512–2 (300 pmol per paw) and HC-030031 (HC, 300 nmol per paw) on mechanical (B) and cold (C) hyperalgesia evoked by the i.pl. injection of AITC (10 nmol per paw) in C57BL/6 mice. (D) Effect of i.pl. administration of Phα1β or CTK 01512–2 and the selective TRPV1 channel antagonist, capsazepine (CPZ, 1 nmol per paw) on the nociceptive response and mechanical hyperalgesia evoked by the injection (10 μL, i.pl.) of capsaicin (CPS, 0.1 nmol per paw) in C57BL/6 mice. (E) Effect of i.pl. administration of Phα1β or CTK 01512–2 and the selective TRPV4 channel antagonist, HC-067047 (HC06, 300 nmol per paw) on the nociceptive response and mechanical hyperalgesia evoked by the injection (10 μL, i.pl.) of hypotonic solution (0.27% NaCl per paw) in C57BL/6 mice. Each column represents the mean of six mice, and vertical lines show the SEM. Statistical analysis was performed using one-way or two-way ANOVA followed by Student–Newman–Keuls or by Bonferroni's *post hoc* test respectively. $^{*}P < 0.05$, significantly different from Veh; $^{\#}P < 0.05$, significantly different from BL values; $^{\S}P < 0.05$, significantly different from Phα1β (300 pmol per paw).

Phα1β selectively blocks nocifensive responses evoked by reactive TRPA1 channel agonist

Next, we studied whether Phα1β or CTK 01512–2 produced *in vivo* antinociceptive and antihyperalgesic effects *via* TRPA1channel antagonism. Phα1β administration (i.pl.) dose-dependently reduced spontaneous nociception evoked by i.pl. AITC injection (Figure 3A) with an ED_{50} (CI) of 42 (32–54) pmol per paw. Administration (i.pl.) of CTK 01512–2 also reduced the spontaneous nociception (Figure 3A and Table 2). AITC induced spontaneous nociception, mechanical hyperalgesia and cold-hyperalgesia (Figure 3A–C and Table 2). Moreover, Phα1β and CTK 01512–2 reduced mechanical and cold hypersensitivities evoked by i.pl injection of AITC (Figure 3B–C and Table 2). Phα1β did not affect TRPV1- or TRPV4 channel-mediated spontaneous nociception response and mechanical hyperalgesia evoked by i.pl. capsaicin or a hypotonic stimulus (which is known to activate TRPV4 channels) (Benemei *et al.,* 2015), respectively (Figure 3D–E), thus indicating *in vivo* selectivity.

The i.t. administration of AITC (1 nmol per site) induced mechanical and cold hyperalgesia that lasted 4 h after administration (Figure 4A–B). AITC (0.1–3 nmol per site, i.t.) caused a dose-dependent mechanical and cold hyperalgesia with ED_{50} (CI) of 0.2 (0.1–0.3) and 0.4 (0.2–0.6) nmol per site respectively. Intrathecal administration of Phα1β (10–100- pmol per site, i.t.) reduced in a dose-dependent manner the mechanical and cold hyperalgesia evoked by AITC (1 nmol per site, i.t.), with an ED_{50} (CI) of 28 (20–38) or 19 (15–24) pmol per site respectively (Figure 4C–D and Table 2). Similarly, CTK 01512–2 (100 pmol per site, i.t.) reverted mechanical and cold hyperalgesia induced by AITC (Figure 4C–D and Table 2). As expected, HC-030031 (30 nmol per site) attenuated AITC-induced mechanical and cold hyperalgesia (Figure 4C–D and Table 2).

Phα1β reduced TRPA1 channel-dependent hyperalgesia in a model of neuropathic pain induced by the chemotherapeutic agent BTZ

In mice, mechanical and cold hyperalgesia produced by the administration of BTZ have been reported, and we confirm here (Figure 5) that they are entirely dependent on activation of TRPA1 channels (Trevisan *et al.,* 2013). In our model, Phα1β markedly attenuated mechanical and cold hyperalgesia induced by BTZ (Figure 5A–D). Phα1β (i.t., 10–100 pmol per site) markedly reduced mechanical and cold hyperalgesia (92 and 100% inhibition at a dose of 100 pmol per site respectively) with an ED_{50} (CI) of 19 (13–27) pmol per site and 11 (8–16) pmol per site respectively (Figure 5 A–B). CTK 01512–2 (i.t., 100 pmol per site or i.pl., 300 pmol per paw) also reduced mechanical (94 and 100% inhibition respectively) and cold (97 and 100% inhibition respectively) hyperalgesia induced by i.pl. BTZ (Figure 5A–D). The inhibitor of neuronal VGCC, ω-conotoxin MVIIA, did not affect BTZ-induced hyperalgesia when given by i.pl. (Figure 5C–D) or i.t. (Figure S1) injection.

Discussion

In the present study, we show that Phα1β and CTK 01512–2 behave as TRPA1 channel antagonists. Phα1β showed similar efficacy, but a higher potency than the widely used, low MW antagonist, HC-030031 (McNamara *et al.,* 2007) in inhibiting the AITC-induced calcium response. Of note, the potency of Phα1β in antagonizing TRPA1 channels was higher than that of the tarantula venom peptide ProTx-I (Gui *et al.,* 2014). Phα1β potency was lower in the recombinant human TRPA1 channels than in the native rat (DRG neurons) or human (IMR90 fibroblasts) channels. The reason for the difference cannot be the species as the toxin behaves similarly in the rat and human native channels, but rather the recombinant versus the native nature of the TRPA1 channels. Although the underlying mechanism for the difference remains unknown, similar findings have been reported previously (Shapiro *et al.,* 2013; Nassini *et al.,* 2015). One possible hypothesis is that the constitutive form of TRPA1 channels, but apparently not recombinant TRPA1 channels, is coexpressed with accompanying proteins, such as the Tmem100, which may change the affinity of ligands to the channel (Weng *et al.,* 2015). The failure of Phα1β or CTK 01512–2 to affect TRPV1 or TRPV4 channel-mediated responses, or responses evoked by KCl and hPAR2-AP, indicated *in vitro* selectivity for the TRPA1 channels.

The ability of Phα1β or CTK 01512–2 to selectively target TRPA1 channels *in vitro* was paralleled by the capacity of the two compounds to act as antagonists of these channels *in vivo*. This effect was obtained via both peripheral (i.pl.) and central (i.t.) routes of administration,

Table 2

Inhibitory effect of Phα1β, CTK 01512–2 and HC-030031 on AITC-induced nociception

Intraplantar route	**Phα1β (300 pmol per paw)**	**CTK 01512–2 (300 pmol per paw)**	**HC-030031 (300 nmol per paw)**
AITC-induced spontaneous nociception (%)	64	84	60
AITC-induced mechanical hyperalgesia (%)	71	82	77
AITC-induced cold hyperalgesia (%)	72	75	72
Intrathecal route	**Phα1β (100 pmol per site)**	**CTK 01512–2 (100 pmol per site)**	**HC-030031 (30 nmol per site)**
AITC-induced mechanical hyperalgesia (%)	94	94	94
AITC-induced cold hyperalgesia (%)	91	94	97

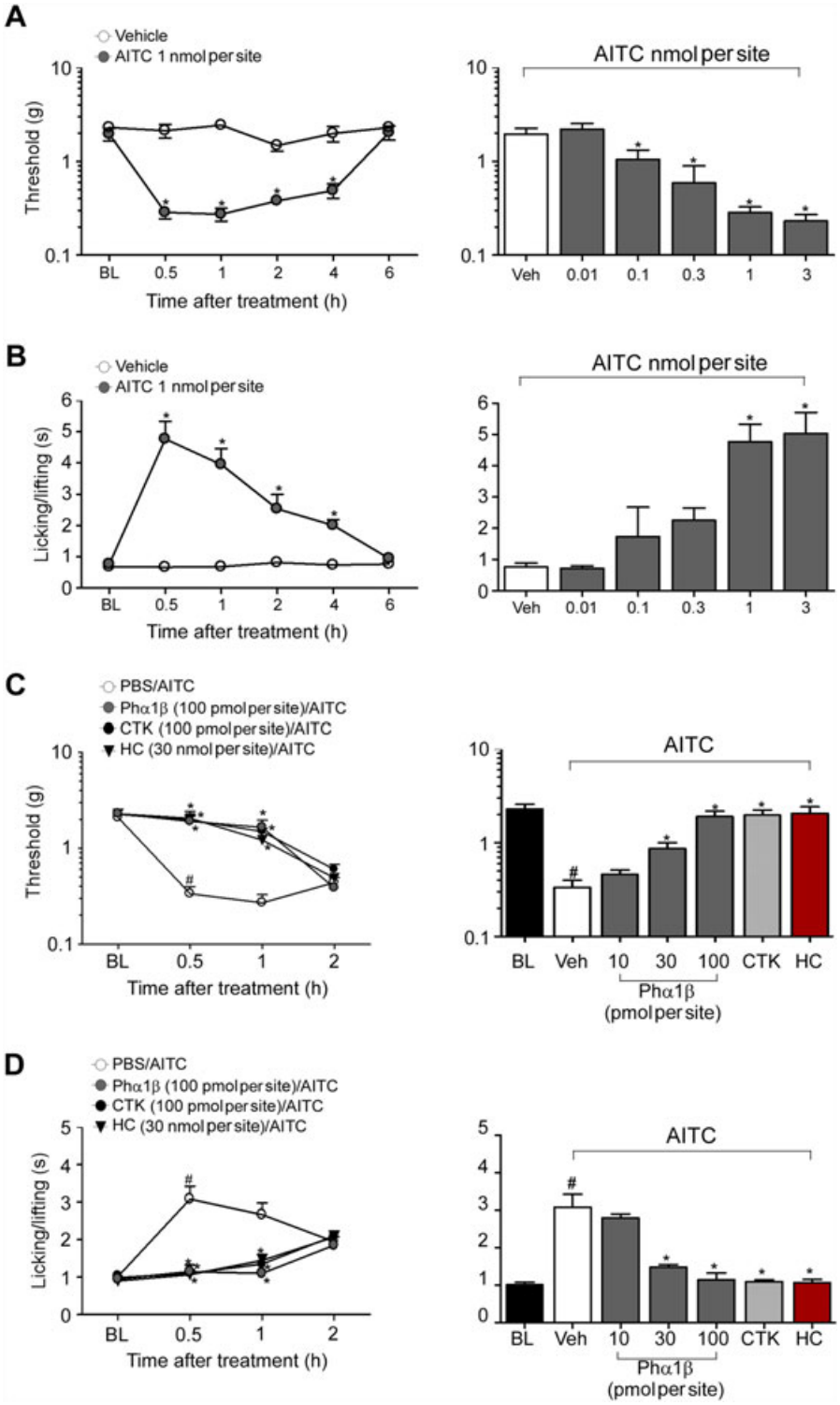

Figure 4

Phα1β and its recombinant form (CTK 01512–2) block the hyperalgesia evoked by i.t. injection of AITC, a TRPA1 channel agonist. (A–B) Time course and dose–response curve of the effect of intrathecal (5 μL, i.t.) administration of AITC (0.01–3 nmol per site) on mechanical (A) and cold (B) nociception. (C–D) Time course and dose–response curve of i.t. administration of Phα1β (10–100 pmol per site), CTK 01512–2 (100 pmol per site) and the selective TRPA1 channel antagonist, HC-030031 (HC, 30 nmol per site) on mechanical (C) and cold (D) hyperalgesia evoked by the injection (5 μL, i.t.) of AITC (1 nmol per site) in C57BL/6 mice. Dose–response curves were performed 0.5 h after AITC treatment. Each point and column represents the mean of six mice, and vertical lines show the SEM. Statistical analysis was performed using one-way or two-way ANOVA followed by Student–Newman–Keuls *post hoc* test or by Bonferroni's *post hoc* test respectively. $^{*}P < 0.05$, significantly different from Veh; $^{\#}P < 0.05$, significantly different from BL values.

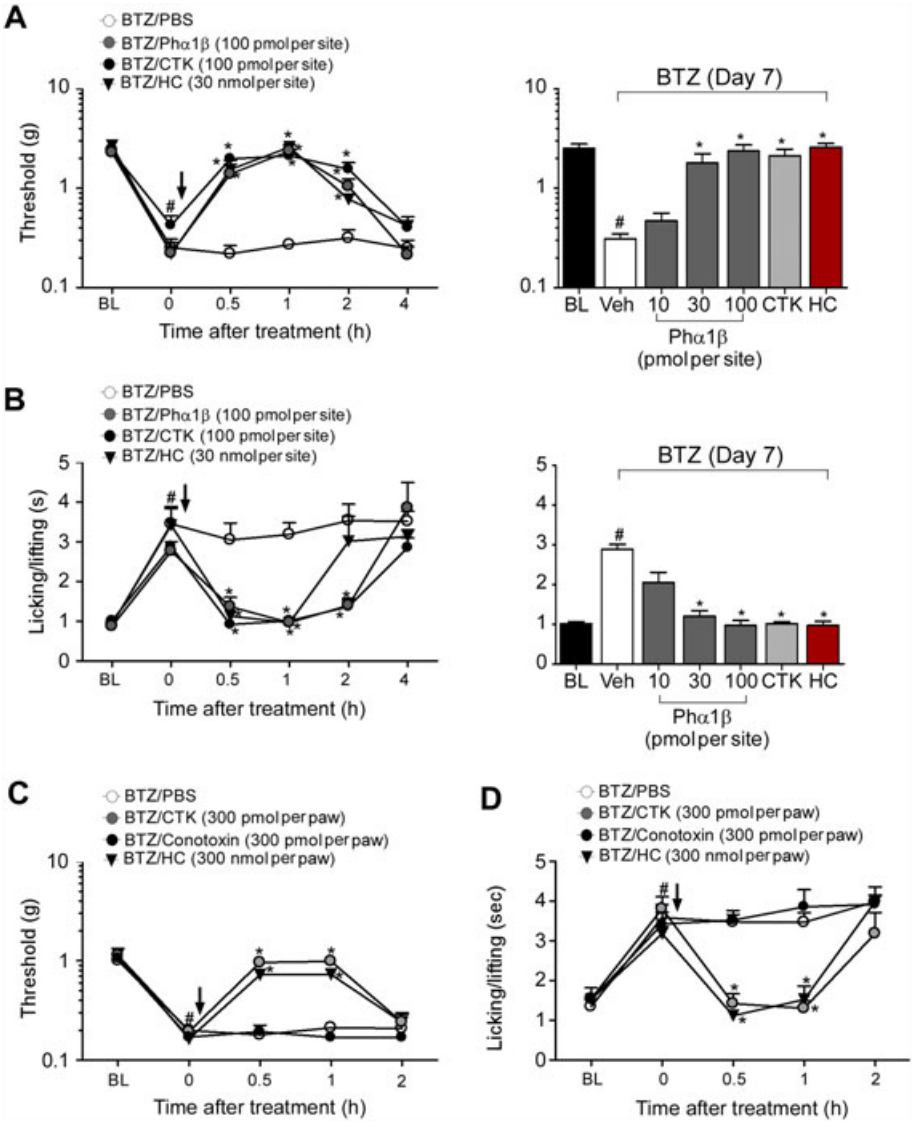

Figure 5

Phα1β and its recombinant form (CTK 01512–2) reduce TRPA1 channel-dependent hyperalgesia in a model of neuropathic pain induced by BTZ. At day 7 after treatment with BTZ (1 mg·kg^{-1}, i.p.), mechanical hyperalgesia and cold hyperalgesia are increased (BL, basal level threshold at day 0 before BTZ). Time course and dose–response curve of i.t. administration of Phα1β (10–100 pmol per site), CTK 01512–2 (100 pmol per site) and the selective TRPA1 channel antagonist, HC-030031 (HC, 30 nmol per site) on mechanical (A) and cold (B) hyperalgesia evoked by BTZ (1 mg·kg^{-1}, i.p.) at day 7 after treatment. Time course of the effect of the i.pl. administration of CTK 01512–2 (300 pmol per paw), ω-conotoxin MVIIA (300 pmol per paw) and HC-030031 (HC, 300 nmol per paw) on mechanical (C) and cold (D) hyperalgesia evoked by BTZ (1 mg·kg^{-1}, i.p.) at day 7 after treatment. Dose–response curves were performed 0.5 h after AITC treatment. Each point and column represents the mean of six mice, and vertical lines show the SEM. Statistical analysis was performed using one-way or two-way ANOVA followed by Student–Newman–Keuls *post hoc* test or by Bonferroni's *post hoc* test respectively. *$P < 0.05$, significantly different from BTZ/PBS; $^{\#}P < 0.05$, significantly different from BL values.

as the drug may target channel-enriched sites, such as the peripheral and central (within the dorsal spinal cord) terminals of nociceptors. The high potency of i.pl. Phα1β to attenuate AITC-mediated acute nociception and mechanical and cold hyperalgesia derives from the observation that maximum inhibition was attained by a dose of Phα1β about 200 times lower than that of HC-030031. Selectivity for TRPA1 channels of Phα1β in *in vivo* experiments is underlined by the fact that the peptide did not affect TRPV1- or TRPV4 channel-mediated acute nociception and mechanical and cold hypersensitivities. In a previous publication (Castro-Junior *et al.*, 2013), we reported that a high dose of Phα1β (3 nmol per paw), but not lower doses (1 and 0.3 nmol per paw), reduced spontaneous nociception and delayed hyperalgesia induced by i.pl. capsaicin (5 nmol per paw) in rats. Present results support these previous findings (Castro-Junior *et al.*, 2013), confirming that low doses of the spider toxin peptide Phα1β (1 or 0.3 nmol per paw) did not affect capsaicin-induced pain-like responses.

Although few studies have investigated the role of the TRPA1 channel in pain transmission in the spinal cord, the ability of the channel to activate nociceptive signals at this central level has received some support (Raisinghani *et al.,* 2011; Koivisto *et al.,* 2014). Accordingly, we evaluated Phα1β in a central paradigm of nociceptive transmission, using the responses induced by i.t. AITC administration. AITC-evoked mechanical and cold hyperalgesia is consistent with recent findings that a TRPA1 channel agonist induces mechanical hyperalgesia when injected i.t. in rodents (Raisinghani *et al.,* 2011; Klafke *et al.,* 2012). Consistent with this finding, HC-030031 and centrally administered Phα1β produced a robust analgesic effect against the i.t. AITC-mediated mechanical and cold hyperalgesia. Notably, the effect of i.t. Phα1β lasted for a longer period of time than that observed after i.pl. administration. A reduced protease activity in the spinal cord as compared with peripheral tissues (King, 2011; Diao and Meibohm, 2013) might be responsible for such a prolonged half-life of the peptide and the consequent antinociceptive and antihyperalgesic action of Phα1β. These findings confirm the antagonistic action of Phα1β towards TRPA1 channel-mediated responses and suggest an additional route of administration for potential clinical applications (Smith *et al.,* 2008; King, 2011) of this venom-derived drug.

The effect of Phα1β was assessed in a mouse model of CIPN, i.e., that evoked by a single injection of BTZ which had been previously identified as entirely dependent on activation of TRPA1 channels (Trevisan *et al.,* 2013). Administration of Phα1β (both i.pl. and i.t.) inhibited mechanical and cold hyperalgesia evoked by BTZ, with a profile in efficacy and duration similar to that produced by HC-030031, thus providing further, although indirect, indication that Phα1β evokes analgesia in the BTZ model of CIPN by targeting TRPA1 channels. However, *in vitro* concentrations of Phα1β required to block TRPA1 channels are similar to those required to block VGCC (Vieira *et al.,* 2005), suggesting that, *in vivo,* the actions of Phα1β could depend on VGCC inhibition. In agreement with a previous report (Kitamura *et al.,* 2014), we failed to observe any protective effect by either i.pl. or i.t. administration of ω-conotoxin MVIIA in BTZ-induced nociceptive hypersensitivities at a dose that was able to produce antinociception (Castro-Junior *et al.,* 2013; Rigo *et al.,* 2013a,b). As both Phα1β and ω-conotoxin MVIIA (both i.t.) have previously been found to reverse mechanical hyperalgesia in a model of post-operative pain (de Souza *et al.,* 2011), whereas a specific TRPA1 channel antagonist (i.t.) was ineffective in such pain models (Wei *et al.,* 2012), the analgesic mechanism of action of Phα1β appears to depend on the pain model under investigation. This analgesic action is likely to derive from inhibition of VGCC in a post-operative pain model and from antagonism of TRPA1 channels in a model of CIPN.

The low yield of Phα1β from spider venom is a limitation to the use of the native peptide as an analgesic agent (Gomez *et al.,* 2002). A possible alternative is the production of a recombinant peptide, such as CTK 01512–2. CTK 01512–2, showing efficacy and potency similar to that of the native peptide, in terms of inhibiting calcium responses evoked by stimulation of TRPA1 channels *in vitro*, and robust antinociceptive/antihyperalgesic effects against AITC-mediated pain-like behaviours, represents a similarly useful and more easily available drug than the native Phα1β. Poisons of venomous animals have long been an important source of new drugs, including analgesic molecules (Gomez *et al.,* 2002; Estrada *et al.,* 2007). Phα1β, the peptide purified from the venom of the armed spider *Phoneutria nigriventer,* which previously has been shown to exhibit antinociceptive effects (Souza *et al.,* 2008; Rigo *et al.,* 2013a,b), and its recombinant form (CTK 01512–2) have now been identified as selective and potent TRPA1 channel antagonists with antihyperalgesic effects in a relevant model of neuropathic pain. These findings, in addition to reinforcing the role of TRPA1 channels in pain transmission, suggest Phα1β and CTK 01512–2 as novel strategies for the treatment of painful conditions where TRPA1 channels might be involved. The dual activity of Phα1β on both TRPA1 channels and VGCC may represent a potential advantage of the two drugs that could result in broader activity in human pain conditions. However, the ability to target TRPA1 channels and VGCC might also increase the chance to cause adverse reactions.

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Author contributions

R.T., R.N., S.M., P.G., J.F., M.V.G., C.C.J. and S.B. designed the experiments and interpreted the results. C.F., S.M., F.D.L., E. C. and R.T. performed the *in vitro* experiments. R.T., R.N., M.C.G., J.F. and I.M.M. performed the *in vivo* experiments.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13652

Figure S1 Effect of ω-conotoxin MVIIA in a model of neuropathic pain induced by bortezomib. Bortezomib (BTZ, 1 mg kg-1, i.p.) at day 7 after treatment increases mechanical hyperalgesia and cold hyperalgesia. Time course of ω-conotoxin MVIIA (100 pmol per site) by intrathecal (i.t.) administration does not affect BTZ-evoked pain-like responses. Each point represents the mean of 6 mice, and vertical lines show the SEM. Statistical analysis was performed using two-way ANOVA, followed by Bonferroni's *post hoc* test. #$P < 0.05$, significantly different from BL values.