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RESEARCH PAPER

Spinal blockage of P/Q- or N-type voltage-gated calcium channels modulates functional and symptomatic changes related to haemorrhagic cystitis in mice

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BACKGROUND AND PURPOSE

Spinal voltage-gated calcium channels (VGCCs) are pivotal regulators of painful and inflammatory alterations, representing attractive therapeutic targets. We examined the effects of epidural administration of the P/Q- and N-type VGCC blockers Tx3-3 and Phα1β, respectively, isolated from the spider *Phoneutria nigriventer*, on symptomatic, inflammatory and functional changes allied to mouse cyclophosphamide (CPA)-induced haemorrhagic cystitis (HC). The effects of *P. nigriventer*-derived toxins were compared with those displayed by MVIIC and MVIIA, extracted from the cone snail *Conus magus*.

EXPERIMENTAL APPROACH

HC was induced by a single i.p. injection of CPA (300 mg·kg^{-1}). Dose- and time-related effects of spinally administered P/Q and N-type VGCC blockers were assessed on nociceptive behaviour and macroscopic inflammation elicited by CPA. The effects of toxins were also evaluated on cell migration, cytokine production, oxidative stress, functional cystometry alterations and TRPV1, TRPA1 and NK_1 receptor mRNA expression.

KEY RESULTS

The spinal blockage of P/Q-type VGCC by Tx3-3 and MVIIC or N-type VGCC by Phα1β attenuated nociceptive and inflammatory events associated with HC, including bladder oxidative stress and cytokine production. CPA produced a slight increase in bladder TRPV1 and TRPA1 mRNA expression, which was reversed by all the toxins tested. Noteworthy, Phα1β strongly prevented bladder neutrophil migration, besides HC-related functional alterations, and its effects were potentiated by co-injecting the selective NK_1 receptor antagonist CP-96345.

CONCLUSIONS AND IMPLICATIONS

Our results shed new light on the role of spinal P/Q and N-type VGCC in bladder dysfunctions, pointing out Phα1β as a promising alternative for treating complications associated with CPA-induced HC.

Abbreviations

CPA, cyclophosphamide; HC, haemorrhagic cystitis; MDA, malondialdehyde; MPO, myeloperoxidase; VGCCs, voltage-gated calcium channels

Tables of Links

TARGETS	
GPCRs[a]	**Ion channels**[c]
CGRP receptor	TRPA1
Muscarinic ACh receptor	TRPV1
NK_1 receptor	Voltage-gated Ca2+ channels
Ligand-gated ion channels[b]	
P2X receptors	

LIGANDS	
ω-conotoxin MVIIC	IL-1β
ACh	IL-4
Acrolein	IL-10
ATP	Substance P
CGRP	TNF
Cyclophosphamide	Phα1β

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

Voltage-gated calcium channels (VGCCs) are a family of integral membrane calcium-selective proteins that are classified as low voltage- (T type) and high voltage-activated (L, N, P/Q and R types) channels, according to their electrophysiological and pharmacological properties (Stock *et al.*, 2013). VGCCs have been recognized as potential targets to control inflammatory pain, mainly by modulating the calcium influx, and the consequent release of neurotransmitters, such as glutamate and substance P (SP) from primary afferent neurons (Vink and Alewood, 2012). The selective N-type VGCC blocker MVIIA peptide (named Prialt®, Jazz Pharmaceuticals, Palo Alto, CA, USA), isolated from the cone snail *Conus magus*, was approved by the Food and Drug Administration (FDA) as an intrathecal (i.t.) analgesic for managing chronic intractable pain (Adams *et al.*, 2012). Nevertheless, MVIIA has been associated with non-specific side effects, including memory impairment, dizziness or speech disorders. In addition to MVIIA, the ω-conotoxin MVIIC has been demonstrated to display marked spinal antinociceptive effects in diverse preclinical models, an effect mostly related to the inhibition of P/Q-type calcium currents (Nimmrich and Gross, 2012).

A series of peptides derived from the Brazilian armed spider *Phoneutria nigriventer* has been investigated, particularly concerning their ability to modulate VGCCs in nociceptive and inflammatory processing (Gomez *et al.*, 2002). One example is the purified fraction Tx3-3 that attenuated nociceptive effects associated with neuropathic pain in rodents, through P/Q-type VGCC blockage in the spinal cord (Dalmolin *et al.*, 2011). Of high interest, the patented toxin Tx3-6 (named Phα1β), a selective N-type VGCC blocker, was characterized as displaying long-lasting analgesic effects in several animal models of nociception when compared with MVIIA (de Souza *et al.*, 2011).

Haemorrhagic cystitis (HC) is the main adverse effect associated with cyclophosphamide (CPA) chemotherapy, and is due to the formation of the urinary metabolite acrolein, which causes bladder fibrosis, oedema, severe haemorrhage, as well as pain. Currently, Mesna (Eurofarma, São Paulo, Brazil) is the only reference drug used to prevent acute HC by limiting uroepithelial exposure to acrolein (Emadi *et al.*, 2009).

It is well established that the CNS has an important integrative role with the urinary bladder, by mediating distinct functions, such as mechanosensation, bladder filling, micturition reflex and pain (Birder and Andersson, 2013). Furthermore, several receptors and ion channels are known to be expressed throughout bladder tissues (urothelium, nerve endings, detrusor muscle and lamina propria), including VGCCs, transient receptor potential (TRP) channels and neurokinin (NK) receptors (Moran *et al.*, 2011; Pailleux *et al.*, 2012; Jiang *et al.*, 2014). Noteworthy, Su *et al.* (2008) previously showed that spinal blockage of P/Q-type VGCC by ω-conotoxin MVIIC reduced acute bladder nociception induced by mechanical distention in rats.

In the present study we evaluated the effects of spinal administration of the P/Q- and N-type VGCC blockers Tx3-3 and Phα1β, respectively, isolated from the spider *P. nigriventer*, on painful, inflammatory and functional alterations associated with HC induced by CPA. Additionally, the effects of *P. nigriventer*-derived toxins were compared with those induced by MVIIC and MVIIA extracted from the marine cone snail *C. magus*.

Methods

Experimental animals

Male Swiss mice (25–30 g; total number: 282 mice) were used throughout this study. Animals were obtained from Federal University of Pelotas (UFPEL; Pelotas, Brazil). They were housed in groups of five per cage and maintained in controlled temperature (22 ± 1°C) and humidity (60–70%), under

a 12 h light–dark cycle (lights on 0800 h), with food and water available *ad libitum*. The experimental procedures reported in this manuscript followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications no. 85-23, revised 1996), the ARRIVE Guidelines Checklist and Ethical Guidelines for the Investigation of Experimental Pain in Conscious Animals (Zimmermann, 1983; Kilkenny *et al*., 2012). The protocols were approved by the Local Animal Ethics Committee (CEUA-PUCRS, protocol number: 12/00292). Killing was carried out under deep anaesthesia by isoflurane inhalation.

Pharmacological treatments

HC was induced by a single i.p. administration of CPA (300 mg·kg^{-1}) as described by Martins *et al*. (2012). The animals were treated i.t. according to the technique described by Quintao *et al*. (2008) with some modifications. The needle (26 G1/2) was connected to a 10 µL microsyringe and introduced through the skin, a volume of 5 µL of PBS, toxins obtained from the spider *P. nigriventer* Phα1β (50, 100 or 200 pmol per site), Tx3-3 (10, 30 or 50 pmol per site) or toxins from the marine cone snail *C. magus* MVIIA (10 pmol per site) or MVIIC (10, 30 or 50 pmol per site) were injected between the L5 and L6 vertebral spaces. In some cases, the animals received the selective NK_1 receptor antagonist CP-96345 (50 µg per site). The reference drug Mesna (60 mg·kg^{-1}, i.p.) was given in two doses, 30 min before and 4 h after injection of CPA. The toxins Phα1β, Tx3-3, MVIIA and MVIIC were administered as a single i.t. dose, at 1, 2 or 3 h after CPA administration. CP-96345 was co-administrated with Phα1β via a single i.t. injection, 2 h after CPA-evoked HC. The control groups received the respective vehicle used to dissolve the drugs. The schemes of administration were selected on the basis of literature data (Chien *et al*., 2003; Dalmolin *et al*., 2011; de Souza *et al*., 2011; Martins *et al*., 2012). The experimenters were unaware of the different pharmacological treatments.

Behavioural assessment of nociception

These experiments were performed between 0830 and 1230 h to minimize the potential circadian variations in the behavioural responses. The nociceptive spontaneous behaviour was measured for 2 min, every 30 min, over a total period of 4 h. During the 2 min period, each animal was evaluated for (i) activity (walking, rearing, climbing and grooming); (ii) immobility; and (iii) behaviours indicative of visceral nociception ('crises'). The nociception behaviour was scored according to Olivar and Laird (1999). For nociception assessment, six to eight Swiss mice per group were used. The same animals were employed for evaluating macroscopic inflammation and myeloperoxidase (MPO) activity.

Evaluation of bladder macroscopic inflammation

For this analysis, the animals were killed 6 h after CPA administration. The haemorrhage and oedema examination was based on criteria previously established by Gray *et al*. (1986). All bladders were dissected free from connecting tissues, and transected at the bladder neck. Each bladder was macroscopically assessed, by two examiners with experience in this technique blinded to the experimental groups. As an additional index of oedema, the wet weight of each bladder was registered and expressed as mg 100 g^{-1} of animal body weight (Gray *et al*., 1986).

MPO activity

Neutrophil recruitment to the mouse bladder was quantified indirectly by tissue MPO activity, according to the method described by Martins *et al*. (2012), with minor modifications. Bladders were removed at 6 h after CPA injection. The absorbance was measured at 595 nm, and the results are expressed as optical density mg^{-1} tissue.

Cytokine production in bladder tissues

The procedure used was similar to the method described by Fernandes *et al*. (2005). The animals were treated with CPA (300 mg·kg^{-1}, i.p.), and the bladders were collected at 6 h. TNF, IL-1β, IL-4 and IL-10 levels were analysed by sandwich ELISA using DuoSet® ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The results are expressed in pg mg^{-1} tissue. For these experiments, five to six animals per group were used.

Malondialdehyde (MDA) assay

The levels of MDA were measured as indicative of oxidative stress, according to the method described by Boeira *et al*. (2011). The bladders of five mice per group were collected 6 h after CPA injection. The protein content in the supernatant was determined with a commercial kit (Labtest, Lagoa Santa, Brazil). MDA levels were calculated from the standard curve using the 1,1,3,3-tetraethoxy propane (97%) and expressed in nmol mg^{-1} protein.

Expression of TRPV1, TRPA1 and NK_1 receptors by RT-PCR analysis

Bladders and spinal cords were dissected 6 h after CPA-induced HC, and stored in 300 µL of TRIzol Reagent® (Sigma, St. Louis, MO, USA). Total RNA was quantified by spectrophotometry, and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA), in accordance with manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen, Carlsbad, CA, USA). Reactions were performed in a volume of 25 µL using 12.5 µL of diluted cDNA (1:50) and 200 nM of each reverse and forward primers (Table 1). Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems, Carlsbad, CA, USA), and the $2^{-\Delta\Delta ct}$ method was used for data analysis. The efficiency per sample was calculated using LinRegPCR 11.0 software (http://LinRegPCR.nl) and the stability of the reference genes Tbp and Hprt (M-value) (Pernot *et al*., 2010) and the optimal number of reference genes according to the pair-wise variation (*V*) were analysed by GeNorm 3.5 software (http://medgen.ugent.be/genorm/). For the expression assays, the experimental *n* was eight per group.

In vivo cystometric parameters

The urodynamic functional analysis was performed 6 h after CPA-evoked HC, following the method described in rats by Andrade *et al*. (2011), and adapted for mice (*n* = 4–6 per

Table 1
Quantitative PCR primers

Primers	Sequences (5′–3′)		PCR product (bp)	GenBank accession number
Tbp[a]	F	CCGTGAATCTTGGCTGTAAACTTG	118	NM_013684
	R	GTTGTCCGTGGCTCTCTTATTCTC		
Hprt[a]	F	CTCATGGACTGATTATGGACAGGAC	123	NM_013556
	R	GCAGGTCAGCAAAGAACTTATAGCC		
TRPV1[b]	F	GGCAAGGATGACTTCCGGTGGTG	125	NM_001001445
	R	AAGCTCAGGGTGCGCTTGACG		
TRPA1[b]	F	GTATCATCTTCGTGTTGCCCTTGTTC	196	NM_177781
	R	AGGAAGATAAACACTCCGGTCGATC		
NK_1[b]	F	AATGACAGGTTCCGTCTGGGCTTC	133	NM_009313
	R	GGCTGACCTTGTACACGCTGCTCTG		

[a]According to Pernot *et al.* (2010).
[b]Designed by authors, using Oligos.

group). The animals were anaesthetized with urethane (1.2 g·kg^{-1}, i.p.). After 30 min, a polyethylene catheter-10 (Clay Adams, Parsippany, NJ, USA) was inserted via a midline abdominal incision through the bladder dome. The intravesical catheter was connected via a three-way stopcock to a pressure transducer (ADInstruments, Castle Hill, New South Wales, Australia) and to a micro-infusion pump (Insight Scientific Devices, São Paulo, Brazil) to record intravesical pressure and to infuse saline into the bladder respectively. Intravesical pressure was recorded continuously using data acquisition software (PowerLab 8/30, ADInstruments). After catheter implantation, mice were left untouched for 30 min for bladder stabilization. After this period, the animals received a continuous infusion of 0.9% NaCl at a rate of 20 µL·min^{-1}, for 30 min.

We assessed the basal pressure (BP; the lowest bladder pressure between micturitions), micturition pressure (maximum bladder pressure during micturition) and the intercontraction interval (ICI). The mean amplitude (maximum bladder pressure, less, threshold pressure) and the number of non-voiding contractions (NVCs) were also measured. NVCs were defined as rhythmic intravesical pressure increases greater than 5 mmHg from baseline pressure without release of fluid from the urethra. Saline voided from urethral meatus was collected, and the voided volume (VV) was measured. To determine the residual volume (RV), saline infusion was stopped at the beginning of the voiding contraction, and the RV was measured by withdrawing saline through the intravesical catheter and then manually expressing the remaining intravesical contents by exerting pressure on the bladder abdominal wall. The bladder capacity (BC) was calculated as mean VV plus RV. The voiding efficiency (VE) was estimated as a percentage using the following equation: $VE = [(VV/BC) \times 100]$.

Statistical analysis

The results are presented as the mean ± SEM of four to eight animals per group, depending on the experimental protocol. The percentages of inhibition were calculated as the mean of inhibitions obtained for each individual experiment. Statistical comparison of the data was performed by one-way ANOVA followed by Newman–Keuls *post hoc* test. *P*-values less than 0.05 ($P < 0.05$) were considered significant. All tests and the production of graphs were performed using the GraphPad 5 software (San Diego, CA, USA).

Drugs and reagents

The following drugs were used: CPA (Genuxal, Baxter Oncology GmbH, Halle/Westfalen, Germany) and 2-mercaptoethanol sodium sulfonate (Mesna) were purchased from Medilar (Porto Alegre, Brazil); ω-conotoxins MVIIA and MVIIC were purchased from Latoxan (Valence, France); Phα1β and Tx3-3 were purified as previously described by Cordeiro Mdo *et al.* (1993). All of the samples used in this study presented a purity superior to 95%. Phα1β and Tx3-3 have molecular weights of 6044.39 and 6300.00 Da, respectively, and their amino acid sequences are ACIP RGEICTDDCECCGCDNQCYCPPGSSLGIFKCSCAHANKYFCN RKKEKCKKA and GCANAYKSCNGPHTCCWGYNGYKKACI CSGXNWK, respectively (Gomez *et al.*, 2002); (2S,3S)-*N*-(2-methoxyphenyl)methyl-2-diphenylmethyl-1-azabicyclo[2.2.] octan-3-amine (CP-96345) was purchased from Tocris Bioscience (Bristol, UK). CPA and Mesna were diluted in distilled water. All toxins (MVIIA, MVIIC, Phα1β and Tx3-3) were prepared in PBS in siliconized plastic tubes and maintained at –18°C. CP-96345 was solubilized in 1% dimethyl sulfoxide (Sigma, St Louis, MO, USA) with gentle warming.

Results

Antinociceptive effects of P/Q and N-type VGCC inhibitors in HC

A single i.p. administration of CPA (300 mg·kg^{-1}) led to marked nociceptive behaviour in mice (as evaluated through

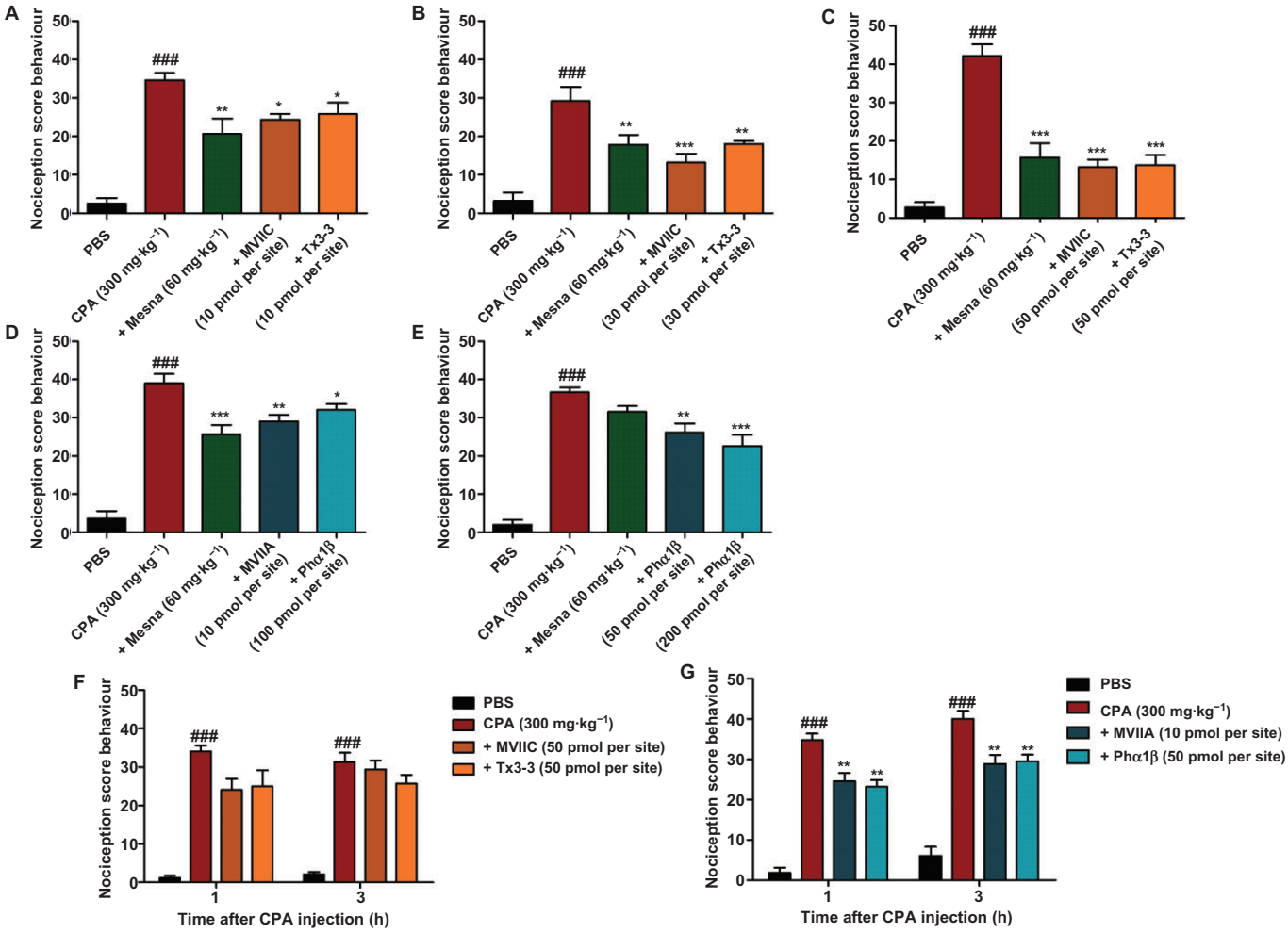

Figure 1

Nociception behaviour was measured for 2 min, at 30 min intervals, over a total period of 4 h after i.p. injection of CPA. (A–C) Effects of treatment with the reference drug Mesna (60 mg·kg^{-1}, i.p., given 30 min before CPA), P/Q-type VGCC blockers MVIIC from *Conus magus*, or Tx3-3 from *Phoneutria nigriventer* (10, 30 or 50 pmol per site, respectively, 2 h post-CPA), given by i.t. route, on the nociceptive responses in the model of CPA-evoked HC in Swiss mice. (D, E) Effects of treatment with the reference compound Mesna (60 mg·kg^{-1}, i.p., given 30 min before CPA), (D) N-type VGCC blockers MVIIA from *Conus magus* (10 pmol per site, i.t., 2 h post-CPA), or (D, E) Phα1β from *P. nigriventer* (50, 100 or 200 pmol per site, i.t., 2 h post-CPA) on the nociceptive responses in CPA-induced HC. (F) Time-related effects of MVIIC or Tx3-3 (50 pmol per site, i.t.), given 1 or 3 h after CPA injection, on the nociceptive responses in CPA-induced HC in Swiss mice. (G) Time-related effects of MVIIA (10 pmol per site, i.t.) or Phα1β (50 pmol per site, i.t.), given 1 or 3 h after CPA injection, on the nociceptive responses in CPA-induced HC in Swiss mice. Differences in the nociceptive scores of behaviour were determined by ANOVA, followed by Newman–Keuls *post hoc* test. Each column represents the mean of six to eight animals, and the vertical lines show the SEM. ###$P < 0.001$ significantly different from PBS values. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ significantly different from CPA alone values.

4 h), which was inhibited by treating animals with the reference drug Mesna (30 min before CPA) (Figure 1). Of note, the i.t. administration of P/Q-type VGCC inhibitors MVIIC from *C. magus*, or Tx3-3 from *P. nigriventer* (given 2 h post-CPA, at 10, 30 and 50 pmol per site) produced a significant and dose-related inhibition of CPA-elicited visceral nociception in mice. The observed percentages of inhibition were 30 ± 4, 55 ± 8 and 69 ± 5% for MVIIC, and 25 ± 9, 38 ± 3 and 68 ± 6% for Tx3-3 toxin respectively (Figure 1A–C).

Next, the i.t. administration of MVIIA (10 pmol per site, 2 h post-CPA) promoted a significant decrease of CPA-elicited nociception, with 26 ± 5% of inhibition (Figure 1D). When the animals received Phα1β by i.t. route (50, 100 or 200 pmol per site, 2 h post-CPA), the nociceptive behaviour responses were reduced by 29 ± 6, 18 ± 4 and 39 ± 8%, respectively, with no clear dose-dependent effects (Figure 1D and E).

Additional assessment of time-related effects of P/Q- and N-type VGCC inhibitors revealed that both MVIIC and Tx3-3 (50 pmol per site) lacked significant effects when administered at 1 or 3 h post-CPA (Figure 1F). Conversely, the N-type blockers MVIIA (10 pmol per site) and Phαβ (50 pmol per site) also displayed significant inhibitory effects on HC-related nociception, when given 1 or 3 h after CPA (Figure 1G).

Modulation of macroscopic inflammation

As can be observed from Figure 2, CPA-induced HC was associated with high scores of macroscopic oedema and haemorrhage, as well as increase in bladder wet weight, in comparison with PBS control groups, according to the evaluations at 6 h after HC induction. These inflammatory alterations were sensitive to the administration of Mesna (60 mg·kg^{-1}, i.p.), given 30 min before and 4 h after CPA. The P/Q-type inhibitors MVIIC or Tx3-3 failed to significantly alter any of the indicators of macroscopic inflammation evaluated, when administered i.t., at 10 pmol per site, 2 h after CPA (Figure 2A, D and G). A lack of effect was also observed for MVIIC, given at 30 pmol per site, although the same dose of Tx3-3 significantly inhibited both macroscopic oedema (60 ± 10%) and bladder wet weight increase (38 ± 5%) (Figure 2B, E and H). Both MVIIC and Tx3-3 (50 pmol per site) produced significant reductions in oedema scores (by 49 ± 9%, for both toxins) (Figure 2F), whereas only Tx3-3 was able to significantly decrease the haemorrhage index at this dose (63 ± 12%; Figure 2C). Nonetheless, 50 pmol per site of both P/Q-type VGCC inhibitors visibly brought the bladder wet weight near to PBS control values, although this effect was not statistically significant (Figure 2I).

We also tested the potential anti-inflammatory effects of three doses of the *P. nigriventer*-derived toxin Phα1β (50, 100 or 200 pmol per site, 2 h post-CPA), using MVIIA (10 pmol

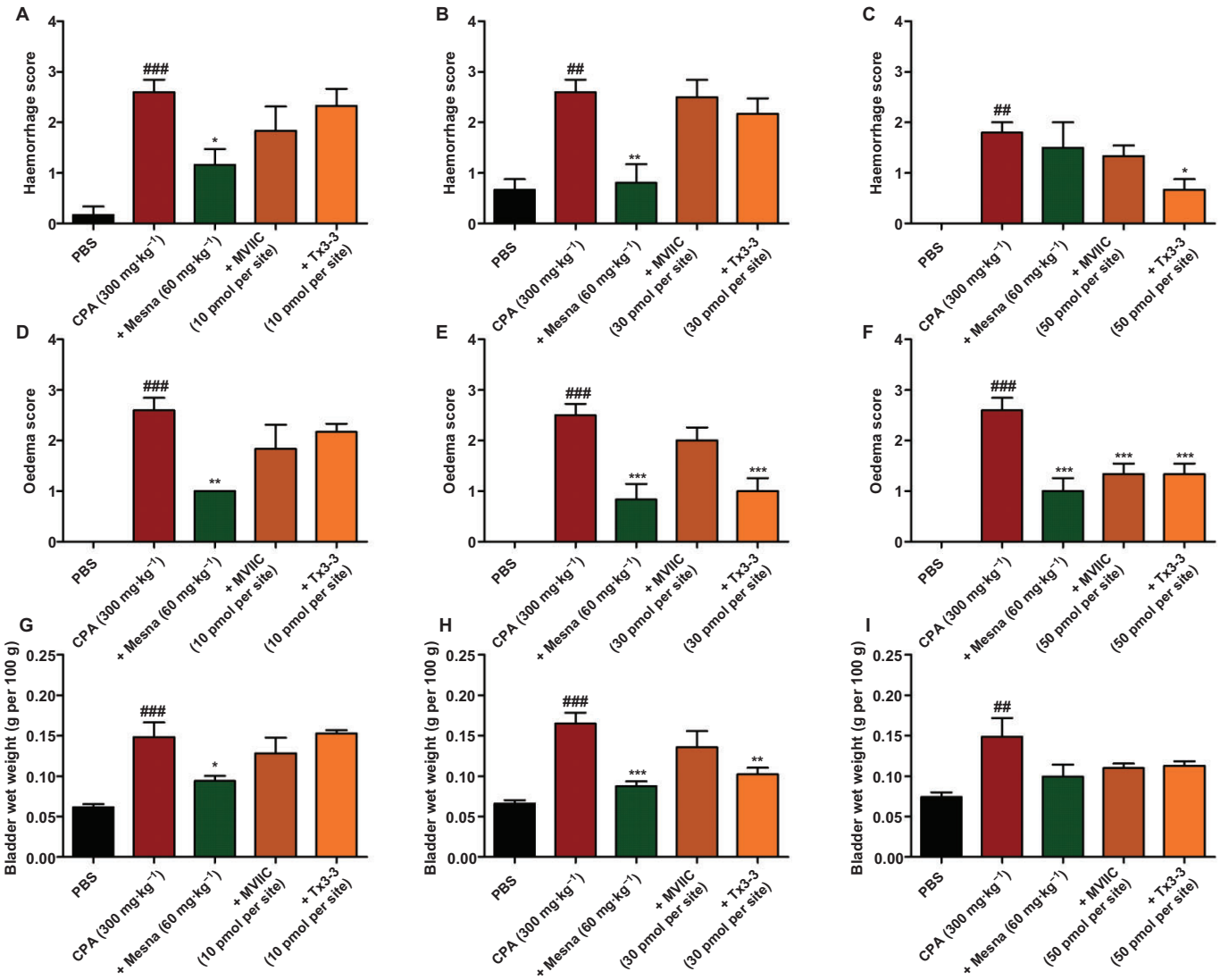

Figure 2

Macroscopic inflammation was assessed 6 h after i.p. administration of CPA. Effects of treatment with the reference drug Mesna (60 mg·kg^{-1}, i.p., given 30 min before and 4 h post-CPA), P/Q-type VGCC blockers MVIIC from *Conus magus* or Tx3-3 from *Phoneutria nigriventer* (10, 30 or 50 pmol per site, 2 h post-CPA), by i.t. route, on macroscopic haemorrhage (A–C), oedema (D–F) and on bladder wet weight (G–I), respectively, in the model of CPA-evoked HC in Swiss mice. Differences in the macroscopic inflammation scores were determined by one-way ANOVA followed by Newman–Keuls *post hoc* test. Each column represents the mean of five to six animals, and the vertical lines show the SEM value. ##P < 0.01 and ###P < 0.001 significantly different from PBS values. *P < 0.05, **P < 0.01 and ***P < 0.001 significantly different from CPA values.

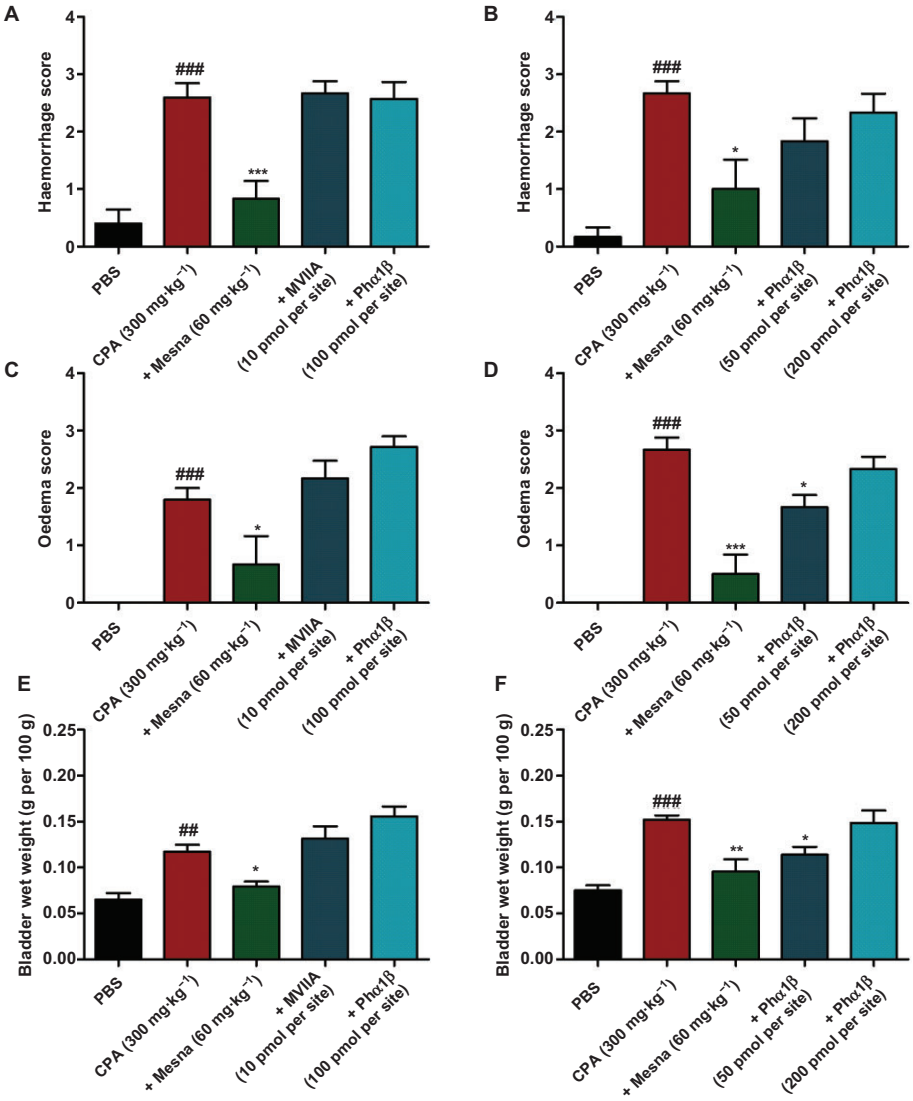

Figure 3

Macroscopic inflammation was evaluated 6 h after i.p. administration of CPA. Effects of treatment with the reference drug Mesna (60 mg·kg^{-1}, i.p., given 30 min before and 4 h post-CPA), N-type VGCC blockers MVIIA from *Conus magus* (10 pmol per site, 2 h post-CPA) or *Phoneutria nigriventer*-derived Phα1β toxin (50, 100 or 200 pmol per site, 2 h post-CPA), by i.t. route, on macroscopic haemorrhage (A, B), oedema (C, D) and on bladder wet weight (E, F), respectively, in the model of CPA-evoked HC in Swiss mice. Differences in the macroscopic inflammation scores were determined by one-way ANOVA followed by Newman–Keuls *post hoc* test. Each column represents the mean of five to seven animals, and the vertical lines show the SEM value. ##$P < 0.01$ and ###$P < 0.001$ significantly different from PBS values. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ significantly different from CPA values.

per site, 2 h post-CPA) as a positive control for N-type VGCC inhibition. Interestingly, mice treated i.t. with Phα1β, at 50 pmol per site, displayed a significant reduction in macroscopic oedema and bladder wet weight (37 ± 8 and 25 ± 6%, Figure 3D and F, respectively), although haemorrhage remained unaffected (Figure 3B). However, this toxin was not effective in reducing any of the macroscopic inflammatory markers evaluated, when administered i.t., at doses of 100 and 200 pmol per site. Likewise, a lack of effect was observed for *C. magus* toxin MVIIA (10 pmol per site) against CPA-induced macroscopic inflammation (Figure 3A, C and E). Additional time course evaluation showed that neither toxin displayed significant effects on CPA-elicited macroscopic inflammation, when administered 1 and 3 h after CPA (Figure 4A–F).

Assessment of neutrophil migration

CPA administration was associated with a striking increase in MPO activity, compared with the PBS control group. The pretreatment with Mesna (60 mg·kg^{-1}, i.p., 30 min before and 4 h after CPA) produced a significant reduction in MPO activity. Of high interest, the treatment with N-type VGCC blocker Phα1β (50 pmol per site, 2 h post-CPA) caused a marked and significant inhibition of MPO activity (60 ± 9%).

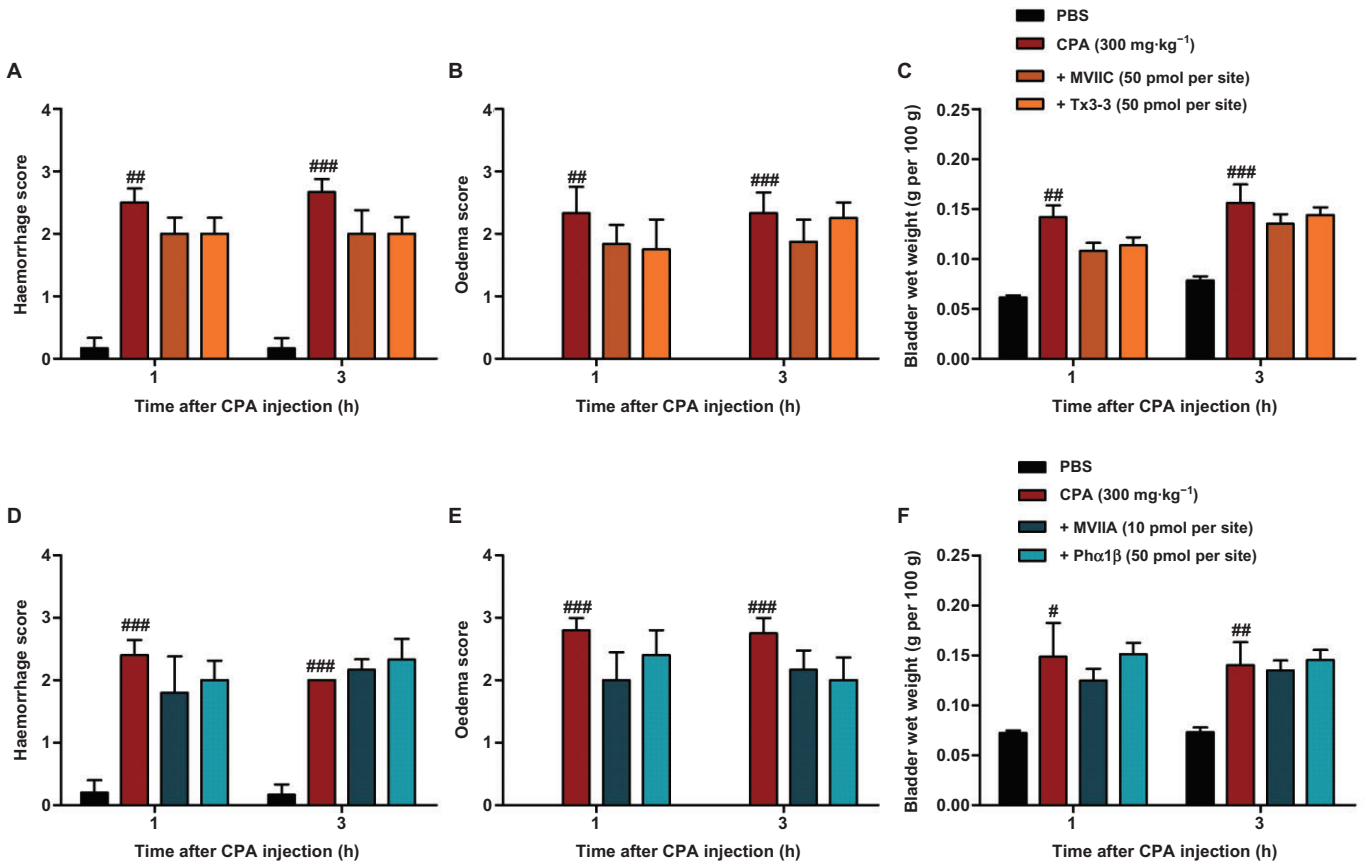

Figure 4

Time course profile of VGCC blockers on macroscopic inflammation induced by i.p. injection of CPA. Effects of treatment with P/Q-type VGCC blockers MVIIC from *Conus magus*, or *Phoneutria nigriventer*-derived Tx3-3 toxin (50 pmol per site), given by i.t. route 1 or 3 h after CPA, on macroscopic haemorrhage (A), oedema (B) and on bladder wet weight (C), in the model of CPA-evoked HC in Swiss mice. Effects of treatment with N-type VGCC blockers MVIIA from *C. magus* (10 pmol per site, i.t.), or *P. nigriventer*-derived Phα1β toxin (50 pmol per site, i.t.), given 1 or 3 h after CPA, on macroscopic haemorrhage (D), oedema (E) and on bladder wet weight (F), in the model of CPA-evoked HC in Swiss mice. Differences in the macroscopic inflammation scores were determined by one-way ANOVA followed by Newman–Keuls *post hoc* test. Each column represents the mean of six to eight animals, and the vertical lines show the SEM value. #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ significantly different from PBS values.

However, the other tested toxins, namely MVIIC and Tx3-3 (50 pmol per site) and MVIIA (10 pmol per site), did not alter CPA-related MPO activity in a significant manner (Figure 5A).

Effects of P/Q and N-type blockers on bladder cytokines

It is possible to observe that i.p administration of CPA (300 mg·kg^{-1}) resulted in a prominent increase in the levels of both pro-inflammatory cytokines TNF (Figure 5B) and IL-1β (Figure 5C), whereas the levels of anti-inflammatory cytokines IL-4 (Figure 5D) and IL-10 (Figure 5E) remained unchanged. Interestingly, the treatment with Mesna (60 mg·kg^{-1}), (MVIIC 50 pmol per site), Tx3-3 (50 pmol per site) or Phα1β (50 pmol per site) reversed TNF to the basal levels (Figure 5B). Furthermore, CPA-elicited IL-1β production was significantly diminished by all the inhibitors tested, except MVIIA. The percentages of inhibition were 38 ± 10, 36 ± 12 and 58 ± 6%, for MVIIC, Tx3-3 and Phα1β respectively (Figure 5C). Neither VGCC inhibitor tested was able to modify IL-4 levels (Figure 4C), although IL-10 production in bladder tissues was markedly increased in the Phα1β-treated group (50 pmol per site, i.t.). This effect occurred in a significant manner, with a raise percentage of 62 ± 4% (Figure 5E).

Analysis of spinal and peripheral oxidative stress

HC induced by CPA was accompanied by a moderate but significant elevation of MDA production in bladder tissues, according to evaluations obtained 6 h post-CPA administration. Of interest, the treatment with Phα1β (50 pmol per site, 2 h post-CPA), by i.t. route, was able to reduce the MDA levels in bladder tissues by 30 ± 6%, similarly to the reference compound Mesna (29 ± 4%) (Figure 5F). In contrast, no significant changes in MDA levels were detected in spinal cord tissues, either at 2.5 or 6 h following CPA administration (results not shown).

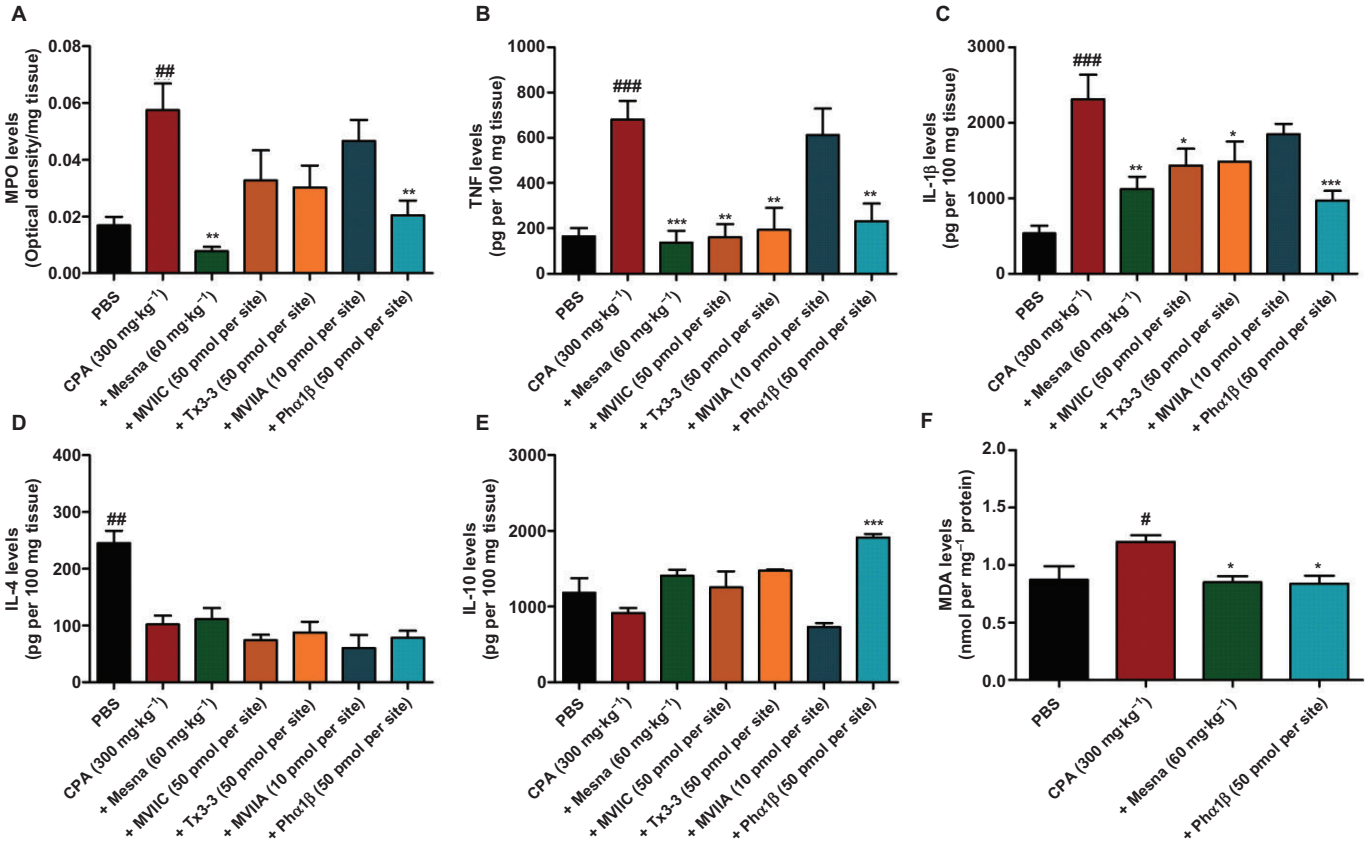

Figure 5

Neutrophil migration, cytokine formation and oxidative stress were measured in bladder tissues at 6 h after i.p. administration of CPA. Effects of treatment with the reference drug Mesna (60 mg·kg^{-1}, i.p., given 30 min before and 4 h post-CPA), P/Q-type VGCC blockers MVIIC from *Conus magus*, given by i.t. route, *Phoneutria nigriventer*-derived Tx3-3 toxin (50 pmol per site, i.t.), N-type VGCC blockers MVIIA from *C. magus* (10 pmol per site, i.t.) or *P. nigriventer*-derived Phα1β toxin (50 pmol per site, i.t.), on MPO activity (A), generation of TNF (B), IL-1β (C), IL-4 (D) and IL-10 (E), or production of MDA levels (F), in the model of CPA-caused HC in Swiss mice. Differences in the MPO levels, cytokine production and MDA formation were determined by one-way ANOVA followed by Newman–Keuls *post hoc* test. Each column represents the mean of five to six animals, and the vertical lines show the SEM value. #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ significantly different from PBS values. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ significantly different from CPA values.

Expression of genes related to neurogenic inflammation

Data depicted in Figure 6A and B demonstrate that HC caused by CPA was associated with a slight increase in TRPV1 and TRPA1 mRNA receptor expression, in the urinary bladder, according to the evaluations at 6 h. The increased expression of TRPV1 mRNA in bladders was reversed by pre-treating animals with Tx3-3 (50 pmol per site), MVIIC (50 pmol per site), Phα1β (50 pmol per site) or MVIIA (10 pmol per site), 2 h after HC induction (Figure 6A). The toxins tested, except MVIIC, also produced a visible reduction in TRPA1 mRNA expression in the bladder tissues (Figure 6B) in CPA-treated mice, although significant differences were not observed. Nevertheless, bladder NK_1 receptor expression was not modified by CPA administration or by any of the compounds tested (Figure 6C). Furthermore, no marked changes in TRPV1, TRPA1 and NK_1 receptor expression were observed in the spinal cords in either experimental condition tested, in relation to the basal expression levels (Figure 6D–F).

Assessment of bladder functional parameters

The i.p. administration of CPA resulted in a marked and significant decrease in the following urodynamic parameters: mean amplitude (Figure 7B), intercontraction interval (ICI; Figure 7C), voided volume (VV; Figure 7D), bladder capacity (BC; Figure 7F) and voiding efficiency (VE: Figure 7G). In addition, CPA caused a significant increase in basal pressure (Figure 7A) and number of NVCs (Figure 7E). Notably, the i.p. treatment with the reference drug Mesna (60 mg·kg^{-1}, 30 min before and 4 h post-CPA), or the i.t. administration of the toxin Phα1β (50 pmol per site, 2 h post-CPA) was able to strikingly reverse all the urodynamic parameters evaluated (Figure 7A–G), except the ICI in Phα1β-treated animals (Figure 7C). Representative traces for this set of experiments are shown in Figure 7H–K.

Interplay between N-type VGCC inhibition and NK_1 receptor antagonism

In an attempt to examine the effects of Phα1β on NK1 receptor activation, the selective antagonist of this receptor,

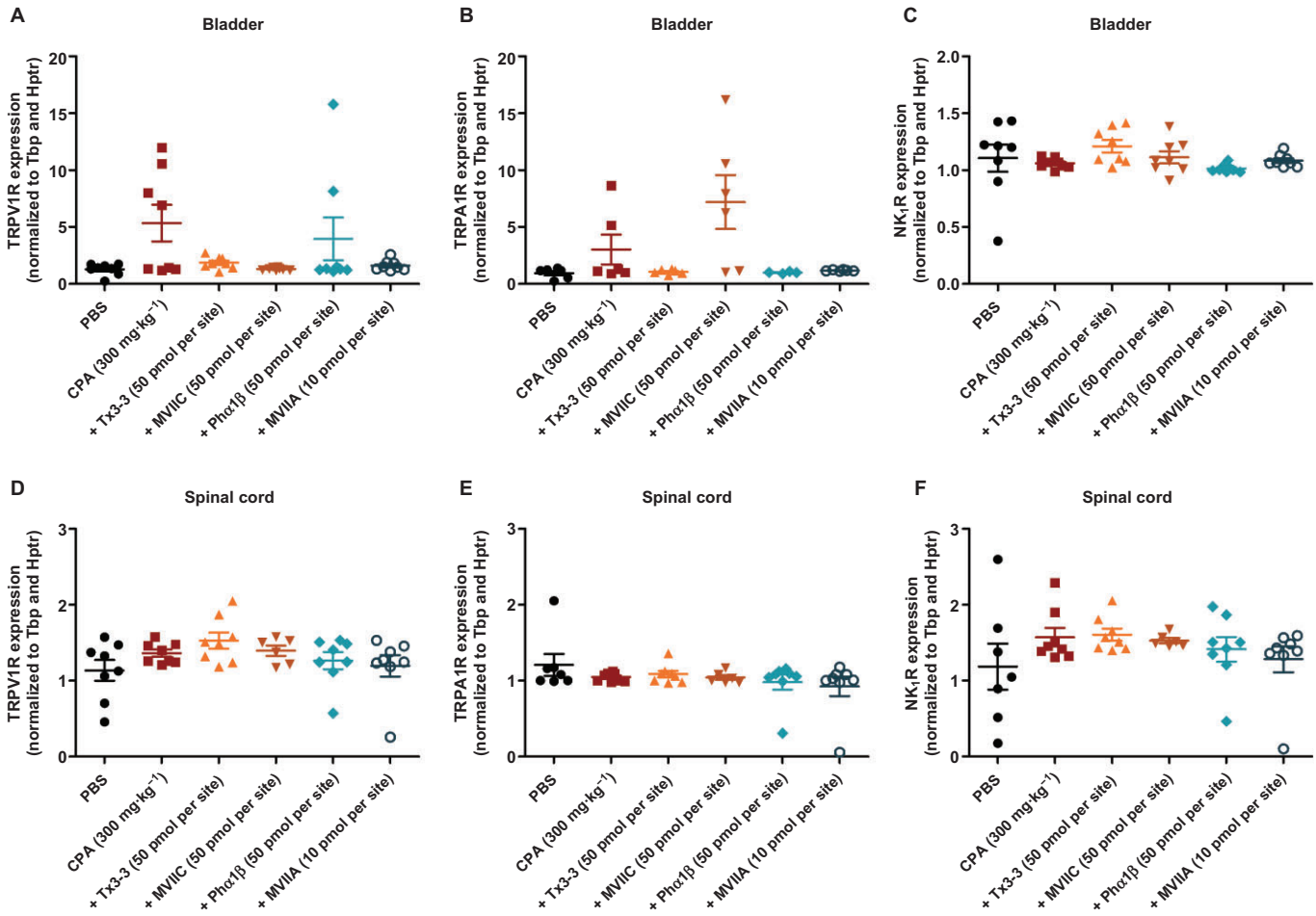

Figure 6

Expression of TRPV1, TRPA1 or NK_1 receptors was measured by quantitative PCR, in bladder and spinal cord tissues, at 6 h after i.p. administration of CPA. Effects of treatment with P/Q-type VGCC blockers MVIIC from *Conus magus*, given by i.t. route, *P. nigriventer*-derived Tx3-3 toxin (50 pmol per site, i.t.), N-type VGCC blockers MVIIA from *C. magus* (10 pmol per site, i.t.) or *P. nigriventer*-derived Phα1β toxin (50 pmol per site, i.t.), on TRPV1 (A, D), TRPA1 (B, E) or NK_1 (C, F) receptor mRNA levels in bladder and spinal cord tissues respectively. Data have been normalized to the levels of Tbp and Hptr expression using the same sample. Each column represents the mean of eight samples, and the vertical lines show the SEM.

namely CP-96345 (50 μg per site), was co-administered with Phα1β toxin (50 pmol per site), via a single i.t. injection, 2 h after CPA-evoked HC. As can be observed from Figure 8, the i.p. administration of CPA (300 mg·kg^{-1}) produced a marked increase in nociception scores, allied to decreases in mouse activity (as evaluated for 4 h) and high scores of macroscopic inflammation (6 h post-induction). The isolated i.t. treatment with either CP-96345 (50 μg per site) or Phα1β toxin (50 pmol per site), given 2 h post-CPA, reversed all the evaluated parameters (Figure 8A–E). Interestingly, the animals that received CP-96345 plus Phα1β displayed a marked reduction in nociception scores, which was significantly different from the inhibition obtained with Phα1β given alone. However, this combined strategy did not produce additive effects on locomotion (measured by activity) (Figure 8B), haemorrhage (Figure 8C), oedema (Figure 8D) or bladder wet weight (Figure 8E), in comparison with the group treated with Phα1β toxin alone.

Discussion and conclusions

HC is the main toxic effect caused by the chemotherapeutic agent CPA, representing a major clinical challenge. Severe pain is one of the most relevant symptoms observed in CPA-elicited HC. It is well known that VGCCs play an important role in peripheral pain transmission to CNS, by facilitating the propagation of action potentials along the primary afferent nerves into sensory neurons in the dorsal root ganglia (Park and Luo, 2010). VGCC-blocking toxins, including Tx3-3, Phα1β, MVIIC and MVIIA, have been widely evaluated as pharmacological targets for development of new analgesic and anti-inflammatory drugs (Gomez *et al.*, 2002; Lewis *et al.*, 2012). However, there is no previous study investigating whether the spinal inhibition of VGCC could interfere with the symptoms related to CPA-induced HC.

Our data revealed that i.t. treatment of mice, 2 h post-CPA injection, with the selective P/Q-type VGCC blockers Tx3-3

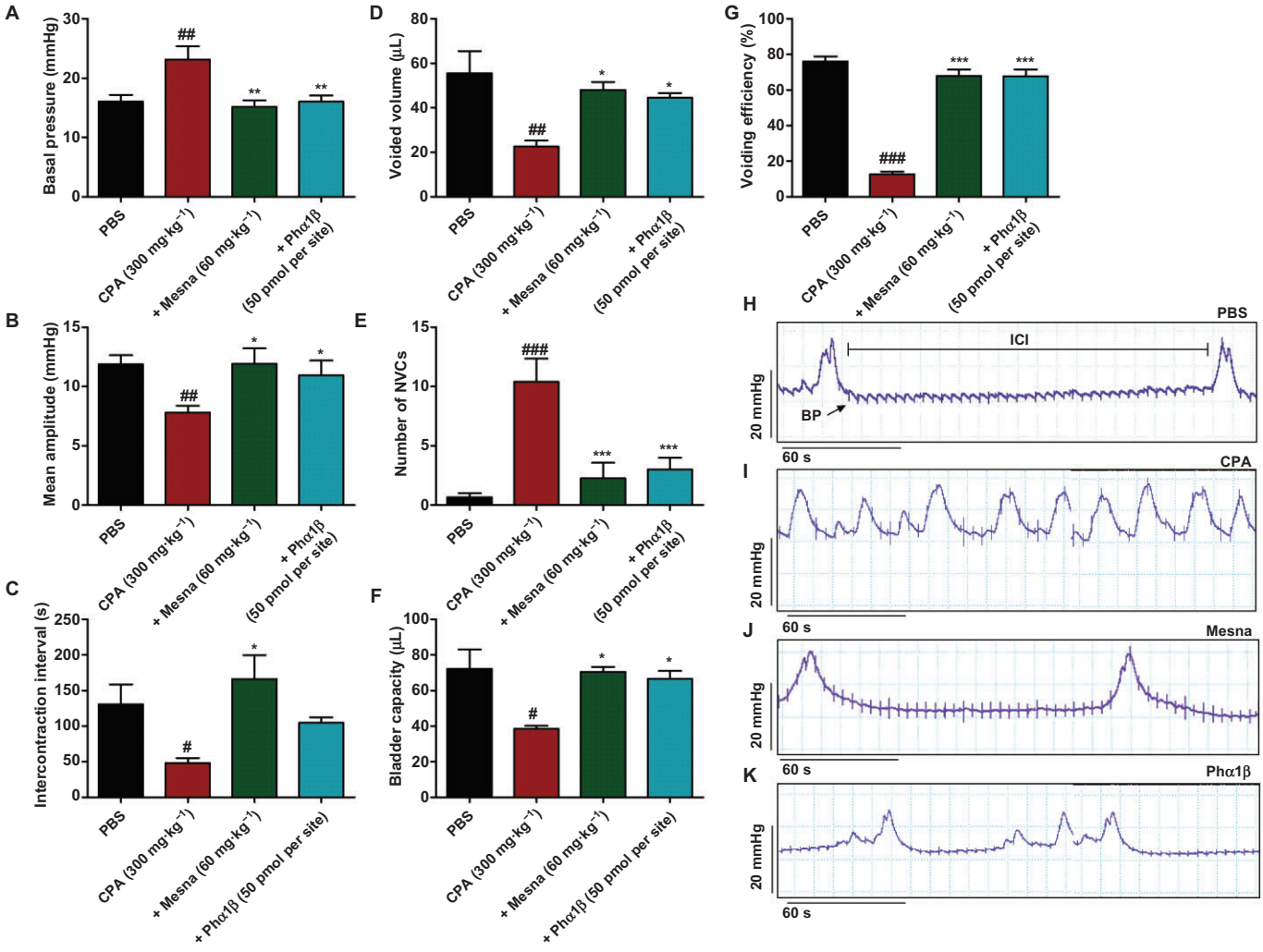

Figure 7

Changes in urodynamic parameters allied to CPA administration in HC model were assessed by functional cystometry assay, for 30 min, 6 h after i.p. administration of CPA. Effects of treatment with the reference drug Mesna (60 mg·kg^{-1}, i.p., given 30 min before and 4 h post-CPA) or N-type VGCC blockers Phα1β from *P. nigriventer* (50 pmol per site), given by i.t. route, on BP (A), mean amplitude (B), ICI (C), VV (D), number of NVCs (E), BC (F) and on VE (G) in the model of CPA-induced HC in Swiss mice. Representative traces of cystometry after administration of PBS (H), CPA (I), Mesna (J) or Phα1β (K) in HC model. Differences in the urodynamic parameters were determined by one-way ANOVA followed by Newman–Keuls *post hoc* test. Each column represents the mean of four to six animals, and the vertical lines show the SEM. #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ significantly different from PBS values. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ significantly different from CPA values.

or MVIIC, clearly prevented the nociceptive behaviour related to CPA-evoked HC, with a dose-related profile of inhibition for Tx3-3 toxin, and an efficacy similar to the reference drug Mesna. A similar dose–response effect was prior demonstrated for both toxins Tx3-3 and MVIIC in mouse models of neuropathic pain (Dalmolin *et al.*, 2011). The visceral pain allied to CPA was also reduced by the spinal administration of the N-type VGCC blockers Phα1β and MVIIA, both given 2 h after HC induction. It has been shown that i.t. administration of Phα1β and MVIIA reduced the acute and chronic pain evoked by paclitaxel in rats, with Phα1β toxin showing less adverse effects, when compared with the FDA-approved drug MVIIA (Rigo *et al.*, 2013). Importantly, we decided to not perform two administrations of toxins, as it was carried out for Mesna, considering the possible tissue damage that might be related to repeated i.t. injections.

We examined the time-related effects of *P. nigriventer*- or *C. magus*-derived toxins in the same experimental parameters of nociception, when these toxins were administered 1 or 3 h after CPA injection. The selective P/Q-type VGCC blockers Tx3-3 and MVIIC did not alter the nociception parameters, when dosed in these time points. Otherwise, the spinal pharmacological inhibition of N-type VGCC by Phα1β or MVIIA greatly decreased CPA-elicited nociception, corroborating previous results that showed antinociceptive effects for Phα1β at different time points in a rat model of inflammatory pain evoked by CFA (de Souza *et al.*, 2013). Thus, the blockage of N-type calcium channels by Phα1β or MVIIA showed a

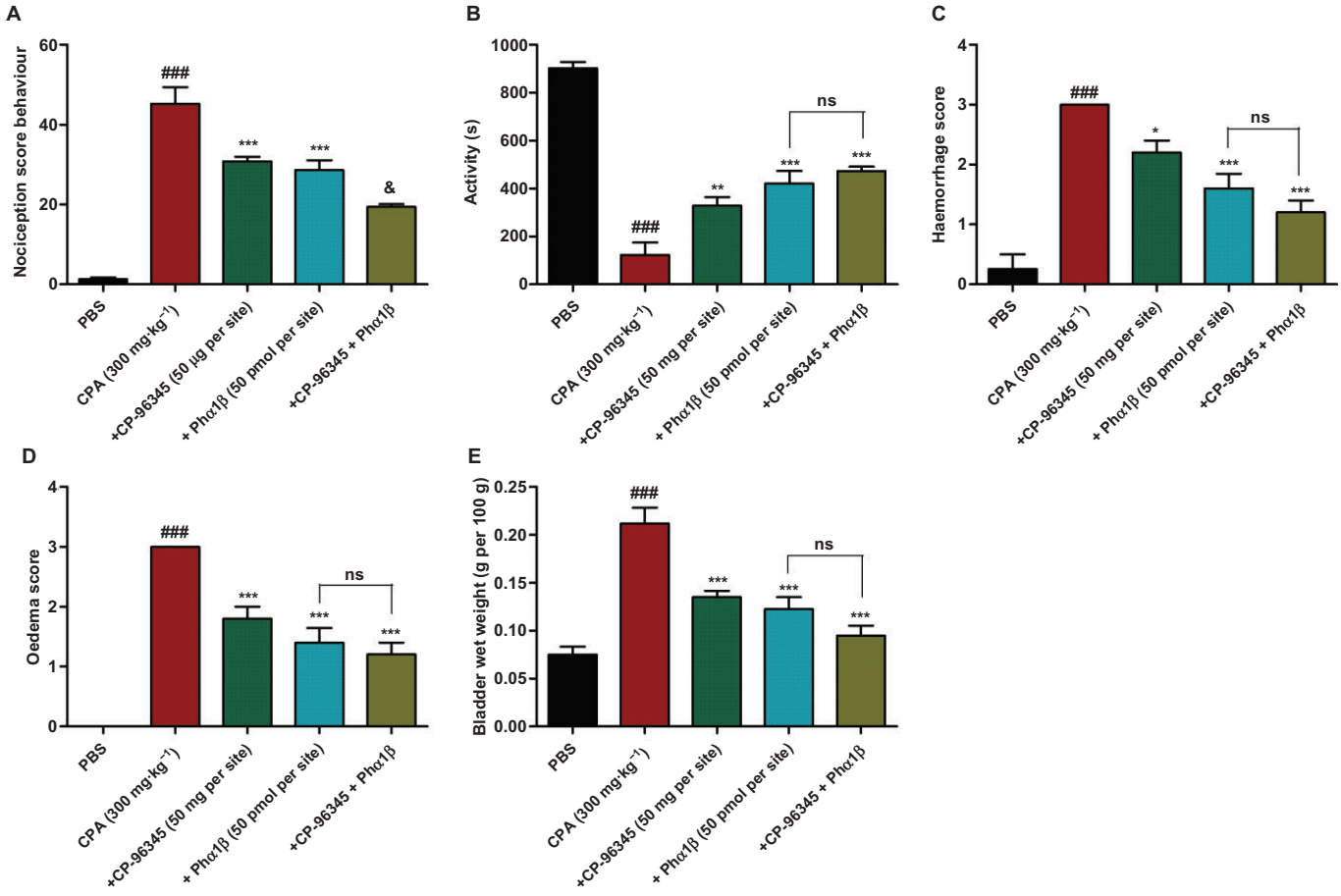

Figure 8

Nociception behaviour and activity were measured for 2 min, at 30 min intervals, over a total period of 4 h after i.p. injection of CPA, whereas macroscopic inflammation was evaluated 6 h post-CPA. Effects of treatment with the selective NK_1 receptor antagonist CP-96345 (50 µg per site, 2 h post-CPA), N-type VGCC blocker Phα1β from *Phoneutria nigriventer* (50 pmol per site) or CP-96345 (50 µg·kg^{-1}) plus Phα1β (50 pmol per site), given by i.t. route, on the nociceptive responses (A), activity (B), haemorrhage (C), oedema (D) and on bladder wet weight (E) in the model of CPA-caused HC. Differences in the nociception score, activity and macroscopic inflammation were determined by one-way ANOVA followed by Newman–Keuls *post hoc* test. Each column represents the mean of four to five animals, and the vertical lines show the SEM. ###$P < 0.001$ significantly different from PBS values. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ significantly different from CPA values. $^{\&}P < 0.05$ significantly different from Phα1β values. ns, not significant.

superior antinociceptive activity, with long-lasting effects. Nonetheless, all the tested toxins were found to be effective at alleviating HC-related bladder nociception, probably by modulating P/Q and N-type calcium currents and peripheral pain transmission to the CNS.

The TRPA1 receptor plays an important role in overactive bladder after spinal cord injury (SCI), and the levels of TRPA1 mRNA are increased in patients with interstitial cystitis (Andrade *et al.*, 2011; Homma *et al.*, 2013). Moreover, Dornelles *et al.* (2013) showed that TRPV1 mRNA is up-regulated in the rat bladder after CPA administration, and the systemic pharmacological inhibition of TRPV1 was capable of diminishing pain behaviour and bladder dysfunction related to CPA. Herein, we demonstrate that CPA-elicited HC was associated to the up-regulation of TRPA1, and mainly TRPV1 mRNA expression in the mouse urinary bladder, although statistically significant differences were not achieved. Relevantly, the increased expression of TRPV1 mRNA in the bladders was virtually prevented by Tx3-3, MVIIC, Phα1β or MVIIA. These toxins, except MVIIC, reduced TRPA1 mRNA expression to control levels. On the other hand, it is relevant to mention that a previous study of our group suggested that Phα1β effects do not rely on TRPV1 receptor activation (Castro-Junior *et al.*, 2013). Despite this, our data indicate that antinociceptive action follow on the spinal inhibition of either P/Q- or N-type VGCC might rely on the peripheral modulation of TRPV1 and TRPA1 receptors. In order to support this hypothesis, further studies are necessary, likely using Western blotting or immunohistochemical studies to determine the regulation of these receptors at post-transcriptional levels. Our data also revealed that NK_1 receptor expression was not modified by CPA administration or by any of the tested compounds, as assessed in bladders or spinal cords. This allows us to suggest that CPA-induced HC and spinal inhibition of VGCC might lead to changes of NK1 receptor function, rather than expression.

It was shown that selective inhibition of N-type VGCC by N-triazole oxindole (TROX-1) prevented arthritis-related hyperalgesia in rats, with an efficacy similar to that seen for a series of non-steroidal anti-inflammatory drugs (Abbadie *et al.*, 2010). The present data revealed, for the first time, the ability of P/Q- and N-type VGCC blockers in preventing bladder inflammation induced by CPA. Accordingly, the haemorrhage or oedema induced by CPA was greatly diminished in mice that had been treated with the selective P/Q-type VGCC blockers Tx3-3 and MVIIC, whereas the N-type VGCC inhibitor Phα1β produced a significant reduction of oedema and bladder wet weight, when these toxins were dosed 2 h after CPA. Of note, the drug clinically used for refractory pain MVIIA failed to alter macroscopic inflammation, what allow us to propose additional beneficial effects for Tx3-3, MVIIC and Phα1β. These latter toxins were effective in preventing both painful and inflammatory alterations associated with CPA-induced HC, supporting their possible therapeutic application for treating cystitis, especially in clinical conditions resistant to Mesna.

Data on macroscopic inflammation prompted us to examine whether the i.t. administration of Tx3-3 and Phα1β from *P. nigriventer*, or MVIIC and MVIIA from *C. magus* might affect neutrophil migration and cytokine production elicited by CPA. The i.t. treatment with the selective N-type VGCC blocker Phα1β caused a marked and significant inhibition of MPO activity, whereas MVIIC, Tx3-3 or MVIIA did not significantly alter this parameter. Indeed, the relevance of N-type VGCC has been extensively reported under inflammatory pain, due to their distribution throughout the central and peripheral terminals, principally in spinally innervated organs, such as the urinary bladder (Waterman, 1996; Pradhan *et al.*, 2013). Our data also revealed that i.t. treatment with MVIIC, Tx3-3 or Phα1β brought TNF to the basal levels. Furthermore, CPA-elicited IL-1β production was significantly diminished by all the tested inhibitors, except by MVIIA, supporting our previous results on oedema and haemorrhage. Previous literature data indicate that IL-4 reduced the severity of HC induced by ifosfamide in mice, probably via inhibition of TNF and IL-β production (Malley and Vizzard, 2002; Macedo *et al.*, 2012). Nevertheless, we failed to demonstrate any significant changes in IL-4 levels in our study, even after treatment with either VGCC-blocking toxin evaluated. Noticeably, IL-10 levels in bladder tissues were found to be markedly increased in the Phα1β-treated group. Previously, it was demonstrated that epidural administration of the neurotoxin HWTX-I, derived from the Chinese bird spider *Ornithoctonus huwena*, produced a marked inhibition of joint inflammatory pain and TNF production, allied to an increase in IL-4 and IL-10 serum levels, in a rat model of rheumatoid arthritis (Wen Tao *et al.*, 2011).

Considering the marked anti-inflammatory effects of Phα1β, we further evaluated the effects of this toxin on additional alterations evoked by CPA. HC was accompanied by a moderate but significant elevation of MDA levels in bladder. Interestingly, the treatment with Phα1β, by i.t. route, greatly reduced the MDA levels in bladder tissues, similar to the reference compound Mesna. However, no significant changes of MDA levels were detected in spinal cord tissues, either at 2.5 or 6 h following CPA administration. We surmise that blockage of N-type VGCC by Phα1β reduces neutrophil migration, and consequently attenuates reactive oxygen species (ROS) production. In fact, the ROS generation was found abolished in MPO knockout mice with spinal cord injury (Kubota *et al.*, 2012).

It has been shown that bladder dysfunction can develop in clinics, as a result of several neurological conditions, such as diabetic neuropathy, amyloid neuropathy and SCI (Chen *et al.*, 2011; Burakgazi *et al.*, 2012; Nardulli *et al.*, 2012). Bladder overactivity represents a sensorial and motor syndrome affecting detrusor muscle function, which involves a series of symptoms including urgency, frequency, nocturia and incontinence urinary (Tincello *et al.*, 2014). Herein, we have also assessed the effects of Phα1β on functional cystometry changes caused by CPA in mice. Surprisingly, the epidural administration of Phα1β was able to restore all the evaluated urodynamic parameters altered by CPA administration, indicating a potential participation of spinal N-type VGCC in bladder pathophysiology. Our data emphasize previous evidence on the relevance of N-type VGCC for bladder contraction and reflex responses to noxious distension (Frew and Lundy, 1995; Su *et al.*, 2008). Additionally, the intracellular calcium regulation of bladder smooth muscle is critical for healthy bladder and detrusor function, which plays a central role in filling and VE of the bladder (Landsberg and Yuan, 2004). The present results could open new avenues for drug development, revealing the potential of Phα1β to recover bladder dysfunction, in addition to the anti-inflammatory and analgesic effects shown for this toxin.

Lastly, we demonstrated that spinal administration of CP-96345 plus Phα1β induced a marked reduction in nociception scores, which was significantly higher than the inhibition obtained for Phα1β alone. This strategy also tended to produce additive effects on locomotion and inflammatory parameters, in comparison with the isolated treatment with Phα1β, but this effect was not significant. A recent study conducted by Malykhina *et al.* (2013) showed that SP contributes to nerve damage in bladder outlet obstruction and triggers secondary changes in the contraction/relaxation mechanisms in the lower urinary tract. We could propose that the analgesic effects of Phα1β are mediated, at least partially, by modulation of NK_1 receptor activity.

Altogether, the present results show that spinally administered P/Q-type VGCC (Tx3-3, MVIIC) or N-type VGCC (MVIIA, Phα1β) blockers are effective in controlling nociceptive and inflammatory processing in the mouse model of CPA-induced HC, probably by interfering with TRPV1 and TRPA1 receptor expression, and TNF and IL-1β production. In the case of the N-type inhibitor Phα1β from *P. nigriventer*, the marked analgesic, anti-inflammatory and recovery of functional actions appear to rely on the reduction of neutrophil migration, which in turn might diminish oxidative stress. Moreover, the beneficial effects of Phα1β seem to be dependent on an increase in the anti-inflammatory cytokine IL-10 in the bladder, as well as the modulation of NK_1 receptor activity (Figure 9). Therefore, the epidural administration of P/Q- or N-type VGCC inhibitors would represent a new attractive therapeutic option for treating patients with severe symptoms allied to HC. With particular regard to the N-type VGCC blocker Phα1β, we suggest that it has potential as a treatment

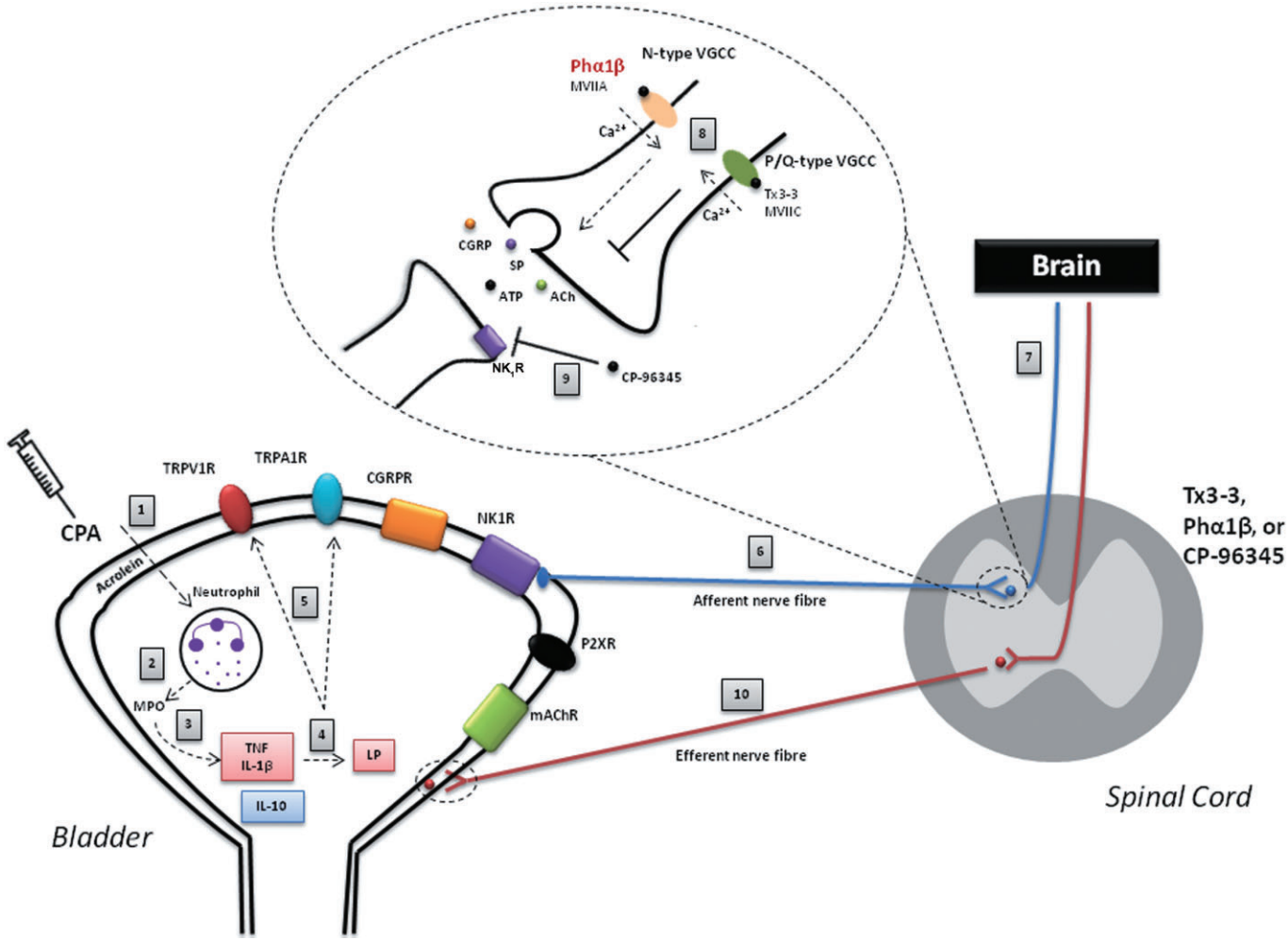

Figure 9

Schematic representation of the inflammatory and nociceptive responses in CPA-evoked HC in mice, and the effects of spinally administered N- and P/Q-type VGCC. (1) An i.p. injection of CPA produces the urinary metabolite acrolein, which recruits inflammatory cells such as (2) neutrophils. (3) The activation of neutrophils on the bladder stimulates the production of pro-inflammatory cytokines, including TNF and IL-1β. (4) Additionally, these inflammatory mediators increase oxidative stress by causing lipid peroxidation (LP). These latter alterations can be inhibited by the production of IL-10, after treatment with the toxins. (5) The inflammatory process might modulate TRPV1 and TRPA1 receptors, leading to afferent sensitization of nerve fibres on bladder. (6) At the spinal cord level, the activation of sensory nerves probably induces calcium-dependent neurotransmitter release, transmitting nociceptive behaviour to the CNS (7). (8) The spinal blockage of P/Q-type VGCC by Tx3-3 and MVIIC or N-type VGCC by Phα1β and MVIIA prevents calcium influx on nerve endings, and consequently attenuates inflammatory visceral nociception. (9) Moreover, the effects of N-type VGCC inhibition can be augmented by i.t. co-administration of the NK_1 receptor antagonist CP-96345, diminishing SP binding to NK_1 receptors on post-synaptic neurons. (10) As the micturition reflex is under control of efferent nerve fibres, the observed effects of the toxins tested on functional bladder activity might be explained by mechanisms similar to that described for inflammatory pain, although further studies are still required to explore this hypothesis. It is tempting to suggest that VGCC blockers might be useful in the cases where efferent pathways are affected (e.g. spinal cord injury and HC). CGRP, calcitonin gene-related peptide; CGRPR, CGRP receptor; mAChR, muscarinic ACh receptor; P2XR, purine P2X receptor; SP, substance P.

for distinct diseases associated with bladder dysfunction, including cystitis.

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Authors contributions

R. B. M. S., N. D. M. S. and M. M. C. designed the experiments, analysed the data and wrote the manuscript. T. C. B. P. and M. R. B. designed the primers and provided qRT-PCR reagents. A. H. S. and M. V. G. provided all toxins. E. L. A. assisted in cystometry experiments. C. E. L. and F. B. M. provided MDA, MPO and cytokine reagents. All authors made a critical review of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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