Pflugers Arch - Eur J Physiol (2012) 463:561–569
DOI 10.1007/s00424-011-1071-x

ION CHANNELS, RECEPTORS AND TRANSPORTERS

TRPA1 and TRPV4 mediate paclitaxel-induced peripheral neuropathy in mice via a glutathione-sensitive mechanism

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Received: 20 December 2011 / Accepted: 28 December 2011 / Published online: 19 January 2012

Abstract Paclitaxel produces a sensory neuropathy, characterized by mechanical and cold hypersensitivity, which are abated by antioxidants. The transient receptor potential vanilloid 4 (TRPV4) channel has been reported to contribute to paclitaxel-evoked allodynia in rodents. We recently showed that TRP ankyrin 1 (TRPA1) channel mediates oxaliplatin-evoked cold and mechanical allodynia, and the drug targets TRPA1 via generation of oxidative stress. Here, we have explored whether TRPA1 activation contributes to paclitaxel-induced mechanical and cold hypersensitivity and whether this activation is mediated by oxidative stress generation. Paclitaxel-evoked mechanical allodynia was reduced partially by the TRPA1 antagonist, HC-030031, and the TRPV4 antagonist, HC-067047, and was completely abated by the combination of the two antagonists. The reduced paclitaxel-evoked mechanical allodynia, observed in TRPA1-deficient mice, was completely abolished when mice were treated with HC-067047. Cold allodynia was abated completely by HC-030031 and in TRPA1-deficient mice. Exposure to paclitaxel of slices of mouse esophagus released the sensory neuropeptide, calcitonin gene-related peptide (CGRP). This effect was abolished by capsaicin desensitization and in calcium-free medium (indicating neurosecretion from sensory nerve terminals), partially reduced by either HC-030031 or HC-067047, and completely abated in the presence of glutathione (GSH). Finally, the reduced CGRP release, observed in esophageal slices of TRPA1-deficient mice, was further inhibited by GSH. Paclitaxel via oxygen radical formation targets TRPA1 and TRPV4, and both channels are key for the delayed development of mechanical allodynia. Cold allodynia is, however, entirely dependent on TRPA1.

Keywords Paclitaxel · TRPA1 · Cold and mechanical hyperalgesia · Primary sensory neurons · Oxidative stress

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Introduction

Paclitaxel (Taxol) is a microtubule-targeting agent labeled for the treatment of a wide variety of solid neoplasms, including ovarian, breast and prostate cancer, currently under investigation to assess its efficacy to treat additional malignant tumors. Peripheral neuropathy (PN) represents a dose-limiting adverse reaction, which negatively affects the quality of life of a relevant portion of patients and, importantly, results in therapy interruption or discontinuation [16]. As described by treated patients, PN by paclitaxel is characterized by various sensory symptoms including mechanical allodynia, spontaneous pain, cold allodynia, ongoing burning pain, tingling, and numbness in a "stocking and glove" distribution [16]. Not infrequently, these symptoms do not resolve with the cessation of paclitaxel therapy and become chronic

for months or years [12, 43]. The mechanism underlying paclitaxel-evoked PN is poorly understood, although a series of studies have focused on a subpopulation of peptidergic primary sensory neurons expressing specific ion channels as the primary target [1, 2, 33, 40].

A subset of primary sensory neurons expresses several members of the transient receptor potential (TRP) family of ion channels, which convey different sensory modalities, including thermo-, mechano-/osmo-, and chemical sensations [8]. These include the vanilloid 1 (TRPV1, the so called "capsaicin receptor"), 2 (TRPV2), 3 (TRPV3), and 4 (TRPV4) channels, the TRPM8 (the "menthol receptor"), and TRPA1 (the ankyrin 1) channels. There is evidence that TRPV4, which has been implicated in the process of osmo-mechanical transduction, mediates part of the mechanical hyperalgesia produced by treatment of rats or mice with paclitaxel [1, 2]. We have recently reported that, in mice and rats, platinum-derived drugs produce a long-lasting mechanical and cold hypersensitivity, by a mechanism that is entirely mediated by TRPA1 [34]. We also showed that both cisplatin and oxaliplatin cause acute TRPA1 activation, an effect that is not mediated by direct channel targeting, but rather is due to the generation of reactive oxygen species (ROS) that eventually gate TRPA1 [34]. Indeed, TRPA1 is activated not only by exogenous irritants, such as allyl isothiocyanate (mustard oil) or cinnamaldehyde (cinnamon) [28, 36], but also by a structurally diverse series of oxidative stress byproducts, including hydrogen peroxide, nitrooleic acid [5, 11], and the unsaturated aldehydes, 4-hydroxy-trans-2-nonenal (4-HNE) [41], 4-oxononenal [5], and acrolein [9].

In vitro studies have shown that paclitaxel-evoked oxidative stress, and the resultant production of hydrogen peroxide and formation of DNA oxidative adducts [38], are associated with the drug cytotoxicity in breast cancer cells [4, 25]. In agreement with this observation, susceptibility to paclitaxel by breast cancer cells was found to be reduced by antioxidant treatments [21], and resistance to paclitaxel has been associated with the total antioxidant cell capacity in a large series of different cancer cell lines [38], suggesting that oxidative stress contributes to the antineoplastic mechanism of action of this drug. Patients subjected to chemotherapy with paclitaxel present immediate systemic oxidative stress and red blood cell oxidative injury associated with the development of anemia [37]. Thus, we have hypothesized that, in addition to TRPV4, TRPA1 also contributes to paclitaxel-induced mechanical and thermal (cold) hypersensitivity and targets these TRP channels via generation of oxidative stress byproducts. Data show that in mice both TRPV4 and TRPA1 contribute to the delayed mechanical allodynia, whereas only TRPA1 mediates the delayed cold hypersensitivity evoked by paclitaxel. In addition, paclitaxel acutely induces neuropeptide release from sensory nerve terminals by activation of TRPA1 and TRPV4, apparently via ROS generation.

Materials and methods

Animals

All animal experiments were carried out in accordance with the European Union Community Council guidelines and approved by the local ethics committee. C57BL/6 mice (male, 25 g) (Harlan Laboratories, Milan, Italy) wild-type (*Trpa1*$^{+/+}$), or TRPA1-deficient mice (*Trpa1*$^{-/-}$), generated by heterozygous mice on a C57BL/6 background [9] were used. Animals were housed in a temperature- and humidity-controlled vivarium (12-h dark/light cycle, free access to food and water). Behavioral experiments were done in a quiet, temperature-controlled room (20°C to 22°C) between 10 a.m. and 4 p.m. and were performed by an operator blinded to the genotype and the status of drug treatment. Animals were sacrificed with a high dose of intraperitoneal (i.p.) sodium pentobarbital (200 mg/kg).

Paclitaxel-induced painful neuropathy models and drugs administration

After habituation and baseline measurements of pain sensitivity, animals were randomized into treatment groups. C57BL/6, *Trpa1*$^{+/+}$, or *Trpa1*$^{-/-}$ mice were treated with a single i.p. administration of paclitaxel (6 mg/kg) or its vehicle (ethanol and Cremophore EL, 50:50, *v*/*v*) [1]. No weight loss was observed in mice throughout the duration of the experiments after paclitaxel treatment. Paclitaxel was formulated at a concentration of 1 mg/ml and was first dissolved in a vehicle containing absolute ethanol and Cremophore EL (50:50, *v*/*v*) because of its poor aqueous solubility. Final solution (10% of this stock solution) was made in sterile saline (NaCl 0.9%) at the time of injection, and the volume was adjusted to 10 ml/kg for the i.p. administration [2]. Intragastric (i.g.) HC-030031 (300 mg/kg) or its vehicle (0.5% carboxymethyl cellulose, CMC), and HC-067047 (10 mg/kg, i.p.) or its vehicle (2.5% DMSO), were administered at day 8 after the administration of paclitaxel or its vehicle. In another experimental setting, HC-030031 (300 mg/kg, i.g.) or its vehicle (0.5% CMC), and HC-067047 (10 mg/kg, i.p.) or its vehicle (2.5% DMSO), were coadministered at day 8 after the administration of paclitaxel or its vehicle.

Tactile allodynia (Von Frey hair test)

Paclitaxel-induced mechanical allodynia was measured in C57BL/6, *Trpa1*$^{+/+}$, or *Trpa1*$^{-/-}$ mice by using the up-and-

down paradigm [13]. Mechanical nociceptive threshold was determined before (basal level threshold) and after drug administration. The effect of paclitaxel was tested for 20 days after treatment. Data are expressed as the mean threshold values (in grams).

Cold stimulation

Cold allodynia was assessed in C57BL/6, *Trpa1*$^{+/+}$, or *Trpa1*$^{-/-}$ by measuring the acute nocifensive responses to the acetone-evoked evaporative cooling as previously described [22]. Briefly, the animal was held in the hand and a droplet (50 μl) of acetone, formed on the flat-tip needle of a syringe, was gently touched to the plantar surface of the hind paw. The mouse was immediately put in a cage with a transparent floor, and the time spent in elevation and licking of the plantar region over a 60-s period was measured. Acetone was applied three times at a 10–15-min interval, and the average of elevation/licking time was calculated. Cold allodynia was measured in mice before (baseline) and for 20 days after drug treatment.

Isolation of primary sensory neurons

Primary dorsal root ganglia (DRG) from *Trpa1*$^{+/+}$ or *Trpa1*$^{-/-}$ adult mice were cultured as previously described [31]. Briefly, lumbosacral (L5–S2) ganglia were bilaterally excised under a dissection microscope. Ganglia were digested using 2 mg/ml of collagenase type 1A and 1 mg/ml of papain in HBSS (25 min, 37°C). Neurons were pelleted and resuspended in Ham's-F12 containing 10% FBS, 100 U/ml of penicillin, 0.1 mg/ml of streptomycin, and 2 mM glutamine, dissociated by gentle trituration, and plated on glass coverslips coated with poly-L-lysine (8.3 μM) and laminin (5 μM). Neurons were cultured for 3–4 days.

Calcium imaging experiments

Cells were incubated with 5 μM Fura-2 AM ester for 30 min at 37°C. Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured on Nikon Eclipse TE2000U microscope. Fluorescence was measured during excitation at 340 and 380 nm for 5 min before and 10 min after stimulus administration, and after correction for the individual background fluorescence signals, the ratio of the fluorescence at both excitation wavelengths (F_{340}/F_{380}) was monitored. Experiments were performed using a buffer solution containing (in millimolars): 150 NaCl, 6 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, and 10 HEPES and titrated to pH 7.4 with 1 N NaOH. Cells were exposed to paclitaxel (10 and 50 μM), allyl isothiocyanate (AITC, 30 μM), or their respective vehicles (0.1%, 0.5%, and 0.03% DMSO). DRGs were challenged with capsaicin (0.1 μM) and by KCl (50 mM) to identify nociceptive neurons and at the end of each experiment with ionomycin (5 μM).

Calcitonin gene-related peptide release

Slices (0.4 mm) of esophagus taken from C57/BL6, *Trpa1*$^{+/+}$, or *Trpa1*$^{-/-}$ were superfused with paclitaxel (10-30-50 μM), or the vehicle (2.5% DMSO), dissolved in a modified Krebs solution at 37°C, and oxygenated with 95% O_2 and 5% CO_2, containing (in millimolars): 119 NaCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.5 $MgSO_4$, 2.5 $CaCl_2$, 4.7 KCl, 11 D-glucose, 0.1% BSA, phosphoramidon (1 μM), and captopril (1 μM). Some tissues were preexposed to capsaicin (10 μM) for 20 min to desensitize TRPV1-expressing sensory nerve terminals. Some experiments were performed in a calcium-free medium, containing EDTA (1 mM). Other experiments were performed in the presence of HC-030031 (30 μM) and HC-067047 (3 μM) or in the presence of the unsaturated aldehyde and ROS scavenger, glutathione monoethylester (GSH, 1 mM). Calcitonin gene-related peptide (CGRP) immunoreactivity (CGRP-IR) was assayed in 10-min fractions (two before, one during, and one after exposure to the stimulus) according to the methods previously reported [41]. The detection limit was 5 pg/ml. CGRP-IR release was calculated by subtracting the mean pre-stimulus value from those obtained during or after stimulation. Stimuli did not cross react with CGRP antiserum.

Reagents

If not otherwise indicated, all reagents were from Sigma-Aldrich (Milan, Italy). HC-030031 was synthesized as previously described [6]. HC-067047 was from Tocris Bioscience (Bristol, United Kingdom), and paclitaxel was from Ascent Scientific Ltd (Bristol, UK).

Statistical analysis

Data are presented as mean±SEM. Statistical analyses were performed by the unpaired two-tailed Student's *t* test for comparisons between two groups, the one-way analysis of variance, followed by the post-hoc Bonferroni's test for comparisons of multiple groups. $p<0.05$ was considered statistically significant.

Results

TRPA1 and TRPV4 receptors activation contributes to the mechanical allodynia evoked by paclitaxel in mice

We first investigated the involvement of TRPA1 in the mechanical allodynia induced by paclitaxel in mice. As

previously reported [1], administration of a single dose of paclitaxel (6 mg/kg, i.p.) produced a delayed reduction in mechanical nociceptive threshold as assayed by the Von Frey hair test in C57BL/6 mice. Reduction from baseline value was significant at day 2, peaked at day 8, and returned to baseline about 20 days after paclitaxel administration (Fig. 1a). A role for the TRPV4 channel in paclitaxel-induced sensory hypersensitivity has been previously reported by using TRPV4 knockout mice and antisense-mediated TRPV4 knockdown [1, 14]. Here we confirm that administration of the selective TRPV4 antagonist, HC-067047 (10 mg/kg, i.p.) [18], 8 days after paclitaxel injection partially reverted paclitaxel-evoked mechanical allodynia. In agreement with previous reports in a different pain model [18], maximum inhibition by HC-067047 was evident 30 min post dosing. HC-067047 did not affect the baseline threshold for mechanical stimulation in naïve animals (Fig. 1c). In the present study, we also investigated TRPA1 contribution to mechanical allodynia induced by paclitaxel. Eight days after paclitaxel administration, systemic administration of the TRPA1 selective antagonist, HC-030031 (300 mg/kg, i.g.) [32], reverted partially mechanical allodynia. In keeping with previous data obtained in different models of hyperalgesia [17], the effect of HC-030031 was evident 60 min post dosing. HC-030031 did not affect the threshold in mechanical allodynia in naïve animals (Fig. 1b). Finally, we found that treatment with a combination of the TRPA1 antagonist, HC-030031 (300 mg/kg, i.g.) and the TRPV4 antagonist, HC-067047 (10 mg/kg, i.p.), 8 days after paclitaxel injection completely reverted paclitaxel-evoked mechanical allodynia (Fig. 1d).

In another series of experiments, we treated *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice following the same protocol used in C57BL/6 mice (one single dose of paclitaxel, 6 mg/kg, i.p.). In *Trpa1*$^{+/+}$ mice, the reduction in mechanical nociceptive threshold from baseline value was already significant at day 2, peaked at day 8, and returned to baseline about 20 days after paclitaxel administration. *Trpa1*$^{-/-}$ mice treated with paclitaxel developed a similar, although less pronounced, mechanical allodynia than that observed in *Trpa1*$^{+/+}$ mice. In particular, at days 7, 8, and 9 after paclitaxel administration, the threshold in the mechanical nociceptive response was significantly reduced in *Trpa1*$^{+/+}$ compared to *Trpa1*$^{-/-}$ mice (Fig. 1e). To further investigate the relative contribution of TRPV4 and TRPA1 in mechanical allodynia induced by paclitaxel, the effect of HC-067047 was studied in *Trpa1*$^{-/-}$ mice at day 8 after drug injection. Thirty minutes after treatment with HC-067047 (10 mg/kg, i.p.), mechanical allodynia induced by paclitaxel was completely reverted (Fig. 1f). Thus, present pharmacological and genetics data indicate that, in addition to TRPV4 [1], TRPA1 contributes to paclitaxel-evoked mechanical allodynia.

TRPA1 activation mediates the paclitaxel-induced cold hypersensitivity in mice

Next, by using the same treatment protocol, we addressed whether paclitaxel produced cold hypersensitivity by assaying the time spent licking the hind paw following acetone application for cooling stimulation, and the relative contribution of TRPA1 and TRPV4 activation in this response. A single dose of paclitaxel (6 mg/kg, i.p.) significantly increased the behavioral responses evoked following acetone application for cooling stimulation in C57BL/6 mice from day 4 to day 12 after paclitaxel administration (Fig. 2a). Peak increase was seen at day 8 (Fig. 2a). This effect of paclitaxel was completely reverted by treatment with HC-030031 (300 mg/kg, i.g.), 60 min post dosing. It should be underlined that time course of inhibition by HC-030031 of either mechanical or cold hypersensitivity was similar. HC-030031 did not affect cold sensitivity in naïve animals (Fig. 2b). Treatment with HC-067047 (10 mg/kg, i.p.) 8 days after paclitaxel injection did not affect the cold allodynia induced by the drug (Fig. 2c). Like C57BL/6 mice, *Trpa1*$^{+/+}$ mice treated with paclitaxel developed a cold hypersensitivity that started at day 2, peaked at day 8, and returned to baseline 18 days after paclitaxel administration (Fig. 2d). The increased response to the cold stimulus observed in *Trpa1*$^{+/+}$ mice was completely absent in *Trpa1*$^{-/-}$ mice, which responded to the stimulus in a manner superimposable to vehicle-treated animals. Pharmacological and genetic findings indicate that TRPA1, but not TRPV4, contributes to paclitaxel-evoked cold allodynia.

Paclitaxel does not directly activate TRPA1 or TRPV4 in dorsal root ganglion neurons but releases CGRP from peripheral nerve endings via glutathione-sensitive mechanism

Exposure to AITC (30 μM) of mouse DRG evoked a calcium response in neurons obtained from *Trpa1*$^{+/+}$ mice (24 cells of the 48 capsaicin (0.1 μM) sensitive neurons responded to AITC), an effect that was completely absent in DRG neurons taken from *Trpa1*$^{-/-}$ mice (0 cells of the 52 responding to capsaicin). Exposure to paclitaxel (50 μM) failed to evoke any significant calcium response in the 68 neurons tested, taken from *Trpa1*$^{+/+}$.

TRPA1 activation of peripheral terminals of capsaicin-sensitive primary sensory neurons is associated with the release of sensory neuropeptides, including CGRP [23]. Several peripheral tissues, including the esophagus [42], have been previously used to study the release of sensory neuropeptides. Paclitaxel increased the basal outflow of CGRP from slices of C57BL/6 mouse esophagus in a concentration-dependent manner (Fig. 3a), a response that was markedly reduced (>80% inhibition) by preexposure of

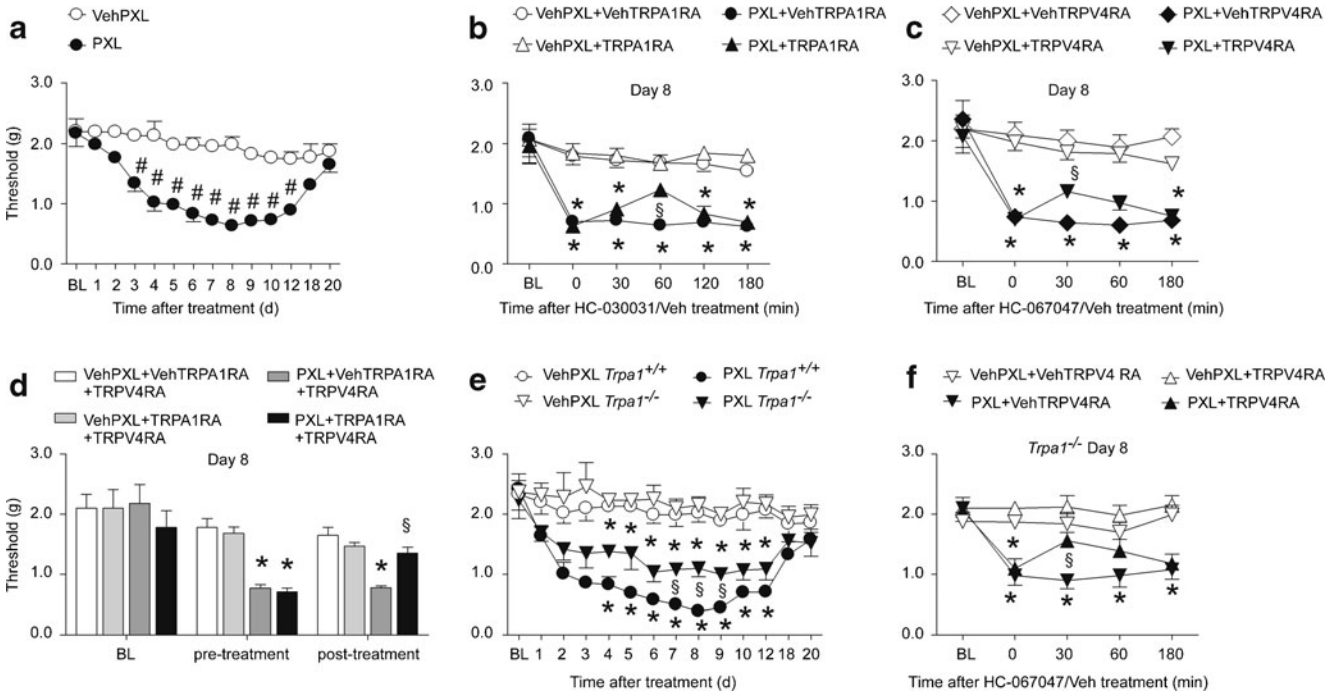

Fig. 1 Paclitaxel induces mechanical allodynia via TRPA1 and TRPV4 activation in mice. **a** The administration of a single dose of paclitaxel (*PXL*; 6 mg/kg, i.p.) in C57BL/6 mice induces a time-dependent reduction in mechanical nociceptive threshold (Von Frey test), with a maximum effect at day (*d*) 8 after PXL administration. At day 8 after PXL administration, treatment with TRPA1 receptor antagonist, HC-030031 (TRPA1RA; 300 mg/kg i.g), significantly reduces mechanical allodynia 60 min post dosing (**b**). A similar significant reduction in mechanical allodynia is visible after treatment with the TRPV4 receptor antagonist, HC-067047 (TRPV4RA; 10 mg/kg, i.p.), 30 min post dosing (**c**). At day 8 after PXL, treatment with a combination of TRPA1 and TRPV4 receptor antagonists HC-030031 and HC-067047 (TRPA1RA+ TRPV4RA) completely reverses the mechanical allodynia at the time of the maximum effect of inhibition for each antagonist (post-treatment; 60 and 30 min post HC-030031 and HC-067047 administration, respectively) (**d**). The administration of the same dose of PXL (6 mg/kg, i.p.) induces a time-dependent reduction in mechanical nociceptive threshold *Trpa1*$^{+/+}$ mice (**e**). The development of mechanical allodynia observed in *Trpa1*$^{+/+}$ mice after PXL treatment is not completely absent in *Trpa1*$^{-/-}$ mice. A significant difference in the reduction of mechanical nociceptive threshold between *Trpa1*$^{+/+}$ and T*rpa1*$^{-/-}$ mice is visible at days 7, 8, and 9 after PXL treatment. At day 8 after PXL administration, treatment with the TRPV4 antagonist HC-067047 (TRPV4RA; 10 mg/kg, i.p.) significantly reduces mechanical allodynia developed by *Trpa1*$^{-/-}$ mice after PXL treatment (**f**). Values are mean ± SEM of $n=8$–10 mice. #$p<0.05$ vs. VehPXL in **a**; Student's *t* test; *$p<0.05$ vs. VehPXL-VehTRPA1RA and VehPXL-TRPA1RA in **b**, or VehPXL-VehTRPV4RA and VehPXL-TRPV4RA in **c** or VehPXL-VehTRPA1 + TRPV4RA and VehPXL-TRPA1RA + TRPV4RA in **d** or VehPXL-*Trpa1*$^{+/+}$ and Veh PXL-*Trpa1*$^{-/-}$ in **e** or VehPXL-Veh TRPV4RA and VehPXL-TRPV4RA in **f**; $^{§}p<0.05$ vs. PXL-Veh TRPA1RA in **b**, or PXL-VehTRPV4RA in **c** and **f**, or PXL-Veh TRPA1RA + TRPV4RA in **d** or PXL *Trpa1*$^{-/-}$ in **e**; one-way ANOVA and Bonferroni's test. *BL* baseline withdrawal threshold

the tissue to a high capsaicin concentration (a procedure known to cause desensitization of sensory nerve terminals) or by removal of extracellular calcium ions from the bath solution (Fig. 3a). Thus, paclitaxel evokes a calcium-dependent neurosecretory process of CGRP from capsaicin-sensitive sensory neurons. Paclitaxel-evoked CGRP-IR release was reduced, but not abolished, in the presence of each individual antagonist of TRPA1 (HC-030031) or TRPV4 (HC-067047) channel (Fig. 3b). However, pretreatment of the tissue with GSH (1 mM) abated completely the paclitaxel-evoked increase in CGRP-IR outflow (Fig. 3b).

Exposure to paclitaxel increased the CGRP-IR outflow from slices of esophagus obtained from *Trpa1*$^{+/+}$ mice. This response was significantly, but not completely, reduced in preparations obtained from *Trpa1*$^{-/-}$ mice (Fig. 3c). To further investigate the contribution of the oxidative stress byproducts that eventually target TRPV4 receptor, esophageal slices from *Trpa1*$^{-/-}$ mice were exposed to paclitaxel in the presence of GSH. Under these circumstances, GSH further decreased paclitaxel-evoked CGRP-IR release (Fig. 3c). Thus, paclitaxel evokes a calcium-dependent neurosecretory process from capsaicin-sensitive neurons by a dual TRPA1 and TRPV4 dependent mechanism and in a manner entirely sensitive to GSH.

Discussion

Sensory PN affects a proportion of patients treated with the anticancer drug, paclitaxel, and this adverse reaction is often the cause for drug discontinuation [16]. The experimental counterpart of this clinical condition has been described in a large series of studies in rodents showing that paclitaxel causes mechanical and cold allodynia. Among the various

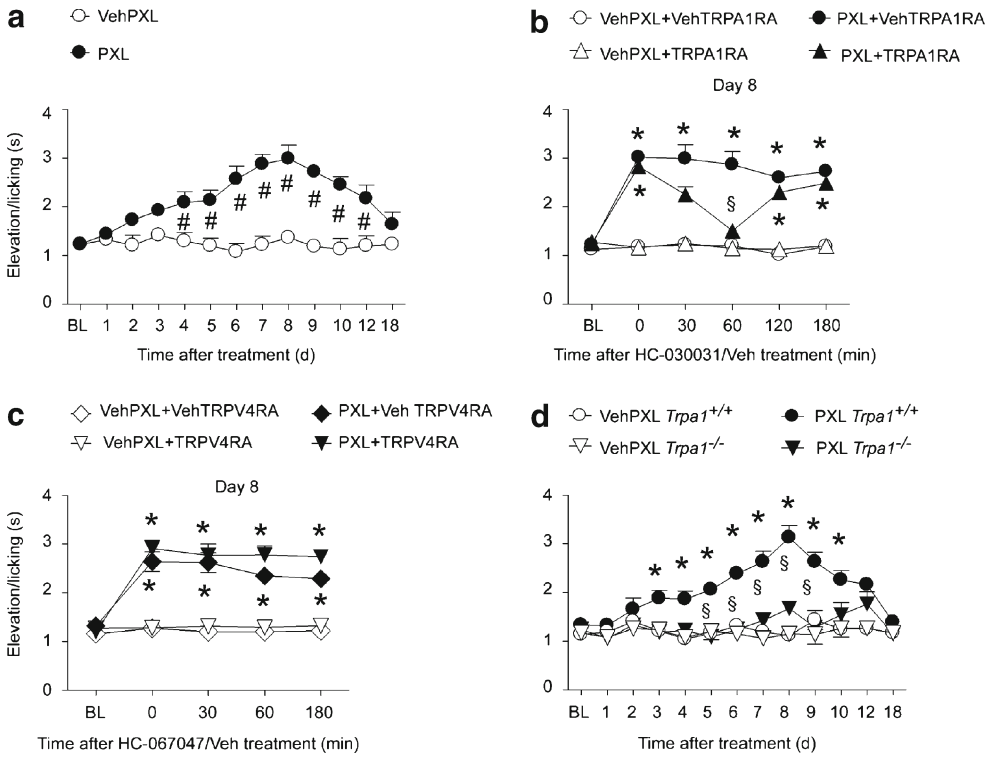

Fig. 2 Paclitaxel-induced cold hypersensitivity is mediated by TRPA1 activation in mice. **a** The administration of paclitaxel (PXL; 6 mg/kg, i.p.) induces in C57BL/6 mice a time-dependent increase in cold hypersensitivity (acetone test) with maximum effect at day (*d*) 8 after PXL administration. At day 8 after PXL treatment, TRPA1 receptor antagonist HC-030031 (TRPA1 RA; 300 mg/kg, i.g.) completely reverses the cold allodynia 60 min post dosing (**b**). Treatment with the TRPV4 receptor antagonist HC-067047 (TRPV4 RA; 10 mg/kg, i.p.) does not affect the cold allodynia induced by PXL (**c**). The development of cold allodynia observed in *Trpa1*$^{+/+}$ mice after PXL (6 mg/kg, i.p.) treatment is completely absent in *Trpa1*$^{-/-}$ mice (**d**). Values are mean ± SEM of n=8–10 mice. #p<0.05 vs. Veh PXL in **a**; Student's *t* test; *p<0.05 vs. VehPXL-VehTRPA1RA and VehPXL-TRPA1RA in **b**, or VehPXL-Veh TRPV4RA and VehPXL-TRPV4RA in **c** or VehPXL-*Trpa1*$^{+/+}$ in **d**; $^{§}p$<0.05 vs. PXL-VehTRPA1RA in **b** or PXL-*Trpa1*$^{-/-}$ in **e**; one-way ANOVA and Bonferroni's test. *BL* baseline withdrawal threshold

mechanisms proposed as causing the paclitaxel sensory neuropathy, recent evidence proposed a role for the TRPV4 channel in mechanical allodynia in mouse and rat models [1, 2]. Here, we confirm in a mouse model that TRPV4 contributes to mechanical allodynia induced by paclitaxel. We also show that TRPA1 accounts for the remaining TRPV4-

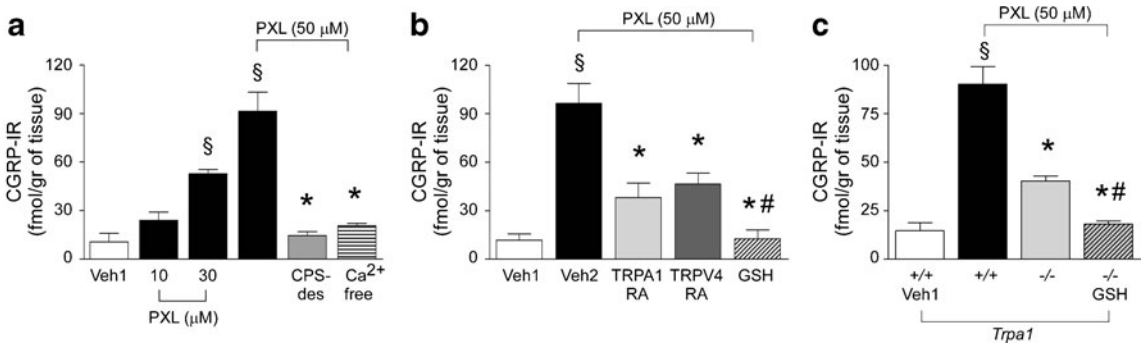

Fig. 3 Paclitaxel releases calcitonin gene-related peptide (CGRP) from mouse esophagus peripheral nerve endings. **a** Paclitaxel (*PXL*) increases the outflow of CGRP immunoreactivity (*CGRP-IR*) from slices of C57BL/6 mice esophagus in a concentration-dependent manner. CGRP-IR release evoked by PXL is abolished by capsaicin desensitization (*CPS-des*) or calcium removal (*Ca^{2+}-free*). **b** CGRP-IR evoked by PXL in peripheral tissues is significantly reduced by pretreatment with TRPA1, HC-030031 (*TRPA1 RA*, 30 μM), or TRPV4, HC-067047 (*TRPV4 RA*, 3 μM) selective antagonists and by glutathione (*GSH*, 1 mM). **c** Paclitaxel increases the release of CGRP-IR from esophageal slices obtained from *Trpa1*$^{+/+}$ mice, an effect significantly reduced in preparations taken from *Trpa1*$^{-/-}$ mice. Pretreatment of the esophageal slices taken from *Trpa1*$^{-/-}$ mice with GSH (1 mM) abated the CGRP-IR release induced by paclitaxel. Veh1 is the vehicle of PXL and Veh2 is a combination of vehicles of the various treatments. Values are mean ± SEM of n=5 experiments. $^{§}p$<0.05 vs. Veh1; *p<0.05 vs. Veh2 or PXL-*Trpa1*$^{+/+}$, $^{\#}p$<0.05 vs. TRPA1 RA and TRPV4 RA or PXL-*Trpa1*$^{-/-}$

resistant component of the mechanical hypersensitivity produced by the anticancer drug. This conclusion is derived from either pharmacological study, using selective TRPA1 and TRPV4 antagonists, or genetic study, using TRPA1-deficient mice. The TRPV4 antagonist, HC-067047, abated completely the component of the paclitaxel-evoked mechanical allodynia that was resistant to TRPA1 pharmacological blockade or genetic deletion.

Paclitaxel administration to rodents evokes a typical cold hypersensitivity, reminiscent of the clinical condition caused by the drug in treated patients [19]. In contrast, with mechanical allodynia, either pharmacological or genetic studies indicate a primary and unique role of TRPA1 in the present mouse model of cold hypersensitivity evoked by paclitaxel. This conclusion is derived from the observation that, either after treatment with HC-030031, or in TRPA1-deficient mice, paclitaxel-induced cold allodynia was completely abated, and that HC-067047 failed to affect the increased response to acetone after paclitaxel treatment. Thus, under the present circumstances, cold hypersensitivity is completely mediated by TRPA1, whereas both TRPA1 and TRPV4 contribute to mechanical allodynia.

There is compelling evidence obtained both in vitro or in vivo, both in experimental animals and in humans, that paclitaxel treatment is associated with production of oxidative stress [3, 38]. Indeed, accumulation of hydrogen peroxide is an early and crucial step for paclitaxel-induced cancer cell death [3]. In general, induction of oxidative stress as a mechanism that may contribute to the antineoplastic effect of several chemotherapeutic agents has been gaining acceptance [39]. Antioxidants, such as *N*-acetylcysteine, have been shown to inhibit both paclitaxel-evoked decreases in cell viability and increases in intracellular levels of ROS and apoptosis, [30]. *N*-acetylcysteine has been reported to prevent completely paclitaxel-evoked mechanical hypersensitivity [20]. Thus, the proapototic effects on one side, and the establishment of the sensory PN on the other side, seem to be dependent on one single mechanism, e.g., the ability of paclitaxel to generate oxidative stress. We have recently identified the primary role of TRPA1 in mediating mechanical and cold hypersensitivity to oxaliplatin and its ability to target TRPA1, not directly, but rather via oxidative stress generation [34]. In fact, we showed that, in contrast with the selective TRPA1 agonist, AITC, oxaliplatin per se does not activate TRPA1 in cultured DRG neurons, as measured by the ability to evoke an early calcium response [34]. However, in a more complex preparation, such as the isolated guinea pig pulmonary artery, oxaliplatin caused a TRPA1- and CGRP-dependent relaxation that mechanistically was indistinguishable from the relaxation evoked by AITC [34]. This finding suggested that oxaliplatin, like AITC, targets TRPA1 on sensory nerve endings, thereby releasing the sensory neuropeptide CGRP, which eventually relaxes the artery [34]. Of interest for the present discussion is the finding that oxaliplatin-evoked, but not AITC-evoked, arterial relaxation was completely abated by GSH. These findings imply that oxaliplatin does not directly gate TRPA1, but rather probably exerts this action indirectly via the generation by neighboring cells of oxidative stress byproducts that eventually target the channel in sensory nerve terminals, through direct formation of disulfide bridges.

Following this hypothesis, we tested whether paclitaxel could target sensory nerve terminals in a manner similar to that of oxaliplatin by measuring the release of the sensory neuropeptide, CGRP. Previous papers [27, 40] reported that paclitaxel releases substance P (SP) from airway sensory nerves, another neuropeptide co-expressed with CGRP in a subset of primary sensory neurons [26]. The mechanism of action of paclitaxel on sensory neurons remained unknown, although inhibition of paclitaxel-evoked SP release from DRG neurons by ruthenium red [33], a nonspecific TRP channels inhibitor [35], suggests the involvement of this type of channels. Here, we confirm that paclitaxel releases neuropeptides from terminals of capsaicin-sensitive primary sensory neurons, and for the first time we show, by using both pharmacological and genetic data, that the action of paclitaxel is mediated in part by TRPA1 activation and in part by TRPV4 activation. In addition, the ROS and reactive aldehydes, scavenger, GSH, completely abolished paclitaxel-evoked CGRP release from esophageal slices of either wild type or TRPA1-deficient mice. These findings indicate that GSH-sensitive compounds are generated by paclitaxel and finally target TRPA1 and TRPV4.

Additional issues remain to be determined. Although release experiments indicate that paclitaxel is apparently able to acutely stimulate both TRPA1 and TRPV4, it is only after a significant time delays (days) that mechanical allodynia (mediated by both TRPA1 and TRPV4) and cold hypersensitivity (mediated by TRPA1) develop. The time-dependent mechanism(s), which from early stimulation leads to the delayed and enduring hypersensitivity, is unknown. Pathophysiological functions of TRPV4 and TRPA1 are not completely understood; although TRPV4 is considered to mediate osmomechanical stimuli [29], TRPA1 has been proposed as a sensor of chemical irritants [10], and both may play a role in hyperalgesia [17]. Our present data are in agreement with recent findings reporting a contribution of TRPA1 and TRPV4 in paclitaxel-evoked hypersensitivity [14]. However, in our study, pharmacological inhibition and, more importantly, TRPA1 genetic deletion, reduced mechanical allodynia only partially, and it was only after TRPV4 inhibition that paclitaxel-evoked response was completely abated. The difference may be due to the diverse protocols of paclitaxel administration used in the present study (one single administration) as compared to the other study (repeated administrations) [14]. In the latter paper,

antagonism in the central nervous system of the proteinase-activated receptor 2 (PAR2) completely inhibited heat, cold, and mechanical hypersensitivity, three sensory modalities that, at different degrees, were mediated by TRPV1, TRPV4, and TRPA1, respectively. TRPV4 has been reported to induce thermal and mechanical hyperalgesia [44]. There is evidence that TRPA1 and TRPV4 can be sensitized by PAR2 [15, 24]. Thus, it is possible that PAR2 orchestrates the mechanism that eventually results in TRP channel-mediated hypersensitivity. However, the mechanism of the interaction between PAR2 and TRP channels, and the anatomo-functional site where the interaction occurs, remain to be determined.

A number of studies reported that antioxidants protect against the sensory neuropathy induced by paclitaxel [21, 38]. Present evidence shows that GSH inhibits TRPA1 and TRPV4 targeting on sensory nerves induced by paclitaxel. However, whereas endogenous oxidative stress byproducts capable of activating TRPA1 are well identified, little information [7] is available regarding activation of TRPV4 by oxidative stress byproducts, and no evidence exists that oxidative stress may activate PAR2. Thus, further studies are required to define upstream (oxidative stress) or downstream (PAR2) mechanisms apparently associated to paclitaxel-induced and TRPA1/TRPV4-mediated hypersensitivity. Irrespective of the underlying mechanism, previous [1, 2, 14, 34] and present findings support the hypothesis of using TRPA1 and TRPV4 antagonists to treat patients with PN evoked by anticancer medicines, such as paclitaxel or oxaliplatin.

Acknowledgments This work was supported by grants from Istituto Italiano di Tecnologia (Grant SEED, P.G.), Regione Toscana (Regional Health Research Program 2009, P.G.), and Ente Cassa Risparmio di Firenze.

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