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PROTEINASE-ACTIVATED RECEPTOR 2 SENSITIZES TRANSIENT RECEPTOR POTENTIAL VANILLOID 1, TRANSIENT RECEPTOR POTENTIAL VANILLOID 4, AND TRANSIENT RECEPTOR POTENTIAL ANKYRIN 1 IN PACLITAXEL-INDUCED NEUROPATHIC PAIN

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Abstract—Paclitaxel chemotherapy is limited by a long-lasting painful neuropathy that lacks an effective therapy. In this study, we tested the hypothesis that paclitaxel may release mast cell tryptase, which activates protease-activated receptor 2 (PAR2) and, subsequently, protein kinases A and C, resulting in mechanical and thermal (both heat and cold) hypersensitivity. Correlating with the development of neuropathy after repeated administration of paclitaxel, mast cell tryptase activity was found to be increased in the spinal cord, dorsal root ganglia, and peripheral tissues in mice. FSLLRY-amide, a selective PAR2 antagonist, blocked paclitaxel-induced neuropathic pain behaviors in a dose- and time-dependent manner. In addition, blocking downstream signaling pathways of PAR2, including phospholipase C (PLC), protein kinase A (PKA), and protein kinase Cε (PKC), effectively attenuated paclitaxel-induced mechanical, heat, or cold hypersensitivity. Furthermore, sensitized pain response was selectively inhibited by antagonists of transient receptor potential (TRP) V1, TRPV4, or TRPA1. These results revealed specific cellular signaling pathways leading to paclitaxel-induced neuropathy, including the activation of PAR2 and downstream enzymes PLC, PKCε, and PKA and resultant sensitization of TRPV1, TRPV4, and TRPA1. Targeting one or more of these signaling molecules may present new opportunities for the treatment of paclitaxel-induced neuropathy.

Key words: paclitaxel-induced pain, proteinase-activated receptor 2, mast cell tryptase, TRPV1, TRPV4, TRPA1.

Paclitaxel, originally isolated from Pacific Yew tree *Taxus brevifolia* Nutt. (Wani et al., 1971), is used to treat breast, lung, ovarian, head and neck carcinomas (Rowinsky et al., 1993), by promoting microtubule dysfunction (Schiff and Horwitz, 1980; Jordan et al., 1993). Clinical use of paclitaxel, however, is significantly limited by the development of a dose-limiting painful neuropathy consisting of tingling, numbness, burning sensations, and other symptoms (Lipton et al., 1989; Rowinsky et al., 1993; Forsyth et al., 1997). Investigations into the mechanism of paclitaxel -induced neuropathy have been made possible by the development of several rodent models, which produce long-lasting, bilateral pain behaviors including mechanical allodynia and thermal hyperalgesia (Dina et al., 2001; Polomano et al., 2001; Smith et al., 2004). Here, we employed a mouse model of paclitaxel-induced neuropathy (Smith et al., 2004) to investigate cellular mechanisms underling paclitaxel-induced neuropathic pain.

Proteinase-activated receptors (PAR) belong to a family of G-protein-coupled receptors that are activated by a proteolytic mechanism (Cottrell et al., 2003). Specific serine proteases cleave the extracellular N-terminal domains of PAR to expose a tethered ligand that binds to and activates the receptors. Among the four members of PAR, PAR1, PAR3, and PAR4 are preferentially cleaved by thrombin, while PAR2 is activated by mast cell tryptase, trypsin, coagulation protease FVIIa, and FXa (Ossovskaya and Bunnett, 2004). PAR2 is widely distributed in various tissues, including skin, gastrointestinal, cardiovascular, and respiratory systems. Moreover, about 60% of neurons in L4–L6 dorsal root ganglia (DRG) express PAR2 (D'Andrea et al., 1998; Steinhoff et al., 2000). PAR2, as well as its ligand mast cell tryptase, have been shown to be important for inflammation (Noorbakhsh et al., 2006; Barbara et al., 2007; Cenac et al., 2007). However, peripheral or central administration of non-inflammatory doses of PAR2 agonists induced thermal and mechanical hyperalgesia in rodents through the release of neuropeptides calcitonin gene-related peptide (CGRP) and substance P (Vergnolle et al., 2001; Alier et al., 2008), suggesting its role in nociception. Indeed, PAR2 agonists induced thermal hyperalgesia in control, but not PAR2 deficient mice (Vergnolle et al., 2001).

To understand the molecular mechanism by which PAR2 promotes sensory hypersensitivity, it is important to study the downstream signaling of PAR2. In primary afferent neurons, PAR2 is co-expressed with transient receptor potential (TRP) vanilloid 1 (TRPV1), TRP vanilloid 4 (TRPV4), and TRP ankyrin 1 (TRPA1) (Amadesi et al., 2006; Dai et al., 2007; Grant et al., 2007). These TRP channels mediate sensory processes including thermo-, mechano-/osmo-, and cold-sensations (Dai et al., 2007; Vriens et al., 2009). The possibility that PAR2 mediates paclitaxel-induced neuropathic pain remains to be examined. In the present study, we tested the hypothesis that PAR2 activation sensitizes TRPV1, TRPV4, and TRPA1, via phospholipase C (PLC)-, protein kinase C

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Abbreviations: DRG, dorsal root ganglia; PAR, proteinase-activated receptors; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; TRP, transient receptor potential; TRPA1, TRP ankyrin 1; TRPV1, TRP vanilloid 1; TRPV4, TRP vanilloid 4.

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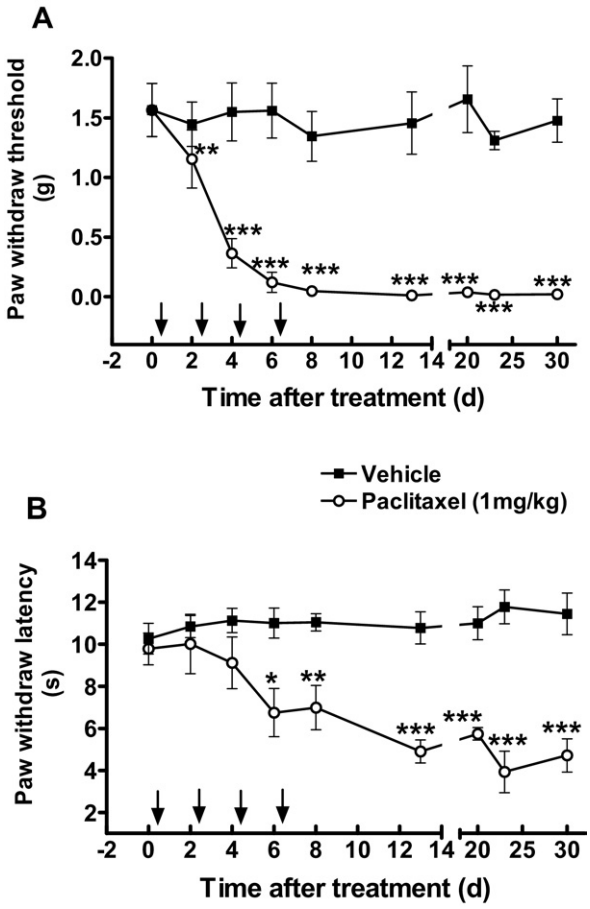

Fig. 1. Paclitaxel-induced mechanical allodynia (A) and thermal hyperalgesia (B). Mice received (i.p.) paclitaxel (1.0 mg/kg) or vehicle on days 0, 2, 4, and 6. Paw withdrawal threshold to von Frey filament probing and withdrawal latency to radiant heat were determined daily for 30 d starting from day 0 (pretreatment baseline). Paclitaxel induced persistent hypersensitivity to mechanical and heat stimuli. Data are expressed in mean±SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with the vehicle-treated group; $n=6$ for each group.

(PKC)-, and PKA-dependent mechanisms, leading to paclitaxel-induced mechanical, heat, and cold hypersensitivity.

EXPERIMENTAL PROCEDURES

Animals

Male ICR mice (20–30 g; Harlan, Indianapolis, IN, USA) were maintained on a 14/10 h light/dark cycle (5:00 AM on/7:00 PM off) with food and water provided *ad libitum* before experimental procedures. All animal experiments were carried out in accordance with the International Association for the Study of Pain (IASP, Pain, 1983, 16:109–110) and the NIH Guide for the Care and Use of Laboratory Animals after approval by the University of Illinois Institutional Animal Care and Use Committee.

Materials

Paclitaxel, KT5720 (PKA inhibitor), U73122 (PLC inhibitor), and capsazepine and SB366791 (TRPV1 antagonists) were purchased from Sigma-Aldrich (St. Louis, MO, USA). FSLLRY-amide (PAR2 antagonist) and its negative control peptide YRLLSF-amide were from Peptides International (Louisville, KY, USA). Myristoylated PKCε v1-2 (myristoylated EAVSLKPT, PKCε inhibitor) was custom-synthesized by AnaSpec (Fremont, CA, USA). RN1734 (TRPV4 antagonist) and HC030031 (TRPA1 antagonist) were purchased from Tocris Bioscience (Ellisville, MO, USA). The selectivity of these inhibitors has been demonstrated in previous studies (Amadesi et al., 2006; Dai et al., 2007; Grant et al., 2007; Wang et al., 2008; Vincent et al., 2009).

Paclitaxel-induced painful neuropathy and drug administration

Paclitaxel-induced painful neuropathy was produced according to a previously published method with some modifications (Smith et al., 2004). Mice received paclitaxel (1.0 mg/kg in 40% dimethyl sulfoxide (DMSO)/300 μl, i.p.) or vehicle (40% DMSO/300 μl, i.p.) every 2 days (days 0, 2, 4, and 6). Mechanical and thermal sensitivity was tested before and after paclitaxel administration. Vehicle itself was not toxic at this dose (Worthley and Schott, 1969), nor did it alter mechanical or thermal sensitivity when compared with pretreatment baseline or saline-injected (300 μl, i.p.) animals.

After mechanical allodynia and thermal hyperalgesia were fully induced by paclitaxel, PAR2 antagonist FSLLRY-amide and its negative control peptide YRLLSF-amide, PKCε inhibitor, or PKA inhibitor KT5720 was administered intrathecally (i.t.), in a volume of 5 μl, by percutaneous puncture through the L5–L6 intervertebral space (Hylden and Wilcox, 1980; Chen et al., 2010). TRP antagonists capsazepine, SB366791, RN1734, and HC030031 were given by i.p. Mechanical and thermal sensitivity tests were performed at 0, 0.5, 2, 4, and 8 h after the drug administration or until the effect of drug is diminished.

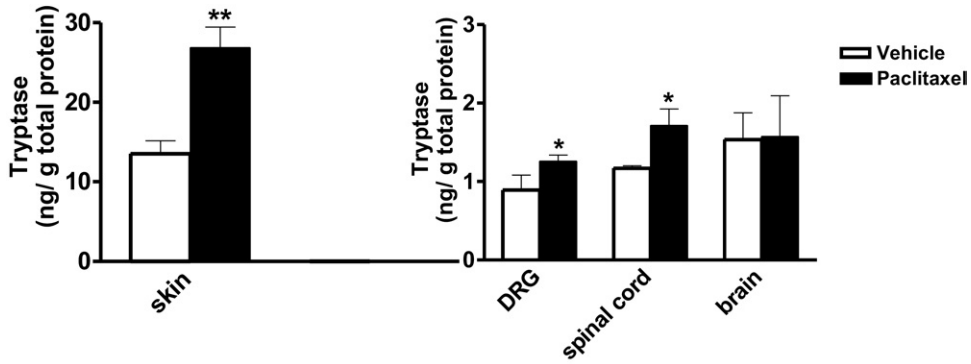

Fig. 2. Release of mast cell tryptase by paclitaxel. Mice received (i.p.) paclitaxel (1.0 mg/kg) or vehicle on days 0, 2, 4, and 6. On day 8, mice were sacrificed and the hindpaw skin, L4-L6 dorsal root ganglia, lumbar spinal cord, and forebrain tissue were taken for the analysis of mast cell tryptase activity. Data are expressed in mean±SEM. *$P<0.05$, **$P<0.01$, compared with the vehicle treated group.

Assessment of mechanical, heat, and cold sensitivity

For consistency, these tests were performed on the left hindpaw of animals. Mechanical sensitivity was assessed by von Frey filaments (Chen et al., 2010). Mice were placed in individual Plexiglas containers with wire mesh platform and a 30 min of acclimation was allowed before the test. Calibrated von Frey filaments (Stoelting, Wooddale, IL, USA) were used to press upward to the midplantar surface of the left hindpaw for 5 s or until a withdrawal response occurred. Using the "up–down" algorithm, 50% probability of paw withdrawal threshold was determined.

Sensitivity to heat stimulus was determined by paw withdrawal latency to radiant heat using a plantar tester (Model 7372, UGO BASILE, Stoelting, Wood Dale, IL, USA) (Hargreaves et al., 1988; Chen et al., 2010). Mice were placed in clear plastic chambers with a glass floor. Radiant heat stimulation was applied to the center of the planter surface of the left hindpaw and the latency to paw withdrawal was recorded. Each mouse was test once for each time or dose point and a cutoff time of 20 s was applied to avoid tissue damage.

Sensitivity to cold stimulus was examined according to a published method (da Costa et al., 2010) with some modification. Mice were placed in individual Plexiglas containers to adapt to the environment for 30 min. A cold stimulus was applied by a brief (1 s) spray of tetrafluoroethane to the ventral surface of left hindpaw. Mice were observed for 5 min and the number of licks and the duration of lifting of the sprayed paw were recorded.

Tryptase activity assay

Mice that received paclitaxel or saline were sacrificed and the brain, spinal cord, DRG, and hindpaw skin were quickly dissected, frozen in liquid nitrogen, and stored at −80 °C until the assay. Tryptase activity was determined by the hydrolysis of substrate tosyl-gly-pro-lys-p-nitroanilide (Millipore, Billerica, MA, USA) as described (Reed et al., 2003). Briefly, tissue lysate was incubated at 37 °C in buffer containing 0.25 mM substrate for 2 h. p-nitroanilide generated in the assay was measured at 405 nm by a microplate reader (Perkin Elmer, Downers Grove, IL, USA).

Immunoblotting

Mice were sacrificed and the lumbar segments of the spinal cord were quickly dissected and frozen on dry ice for Western blotting analysis as described before (Chen et al., 2010). Tissues were homogenized and centrifuged. Supernatant (containing 60 μg of

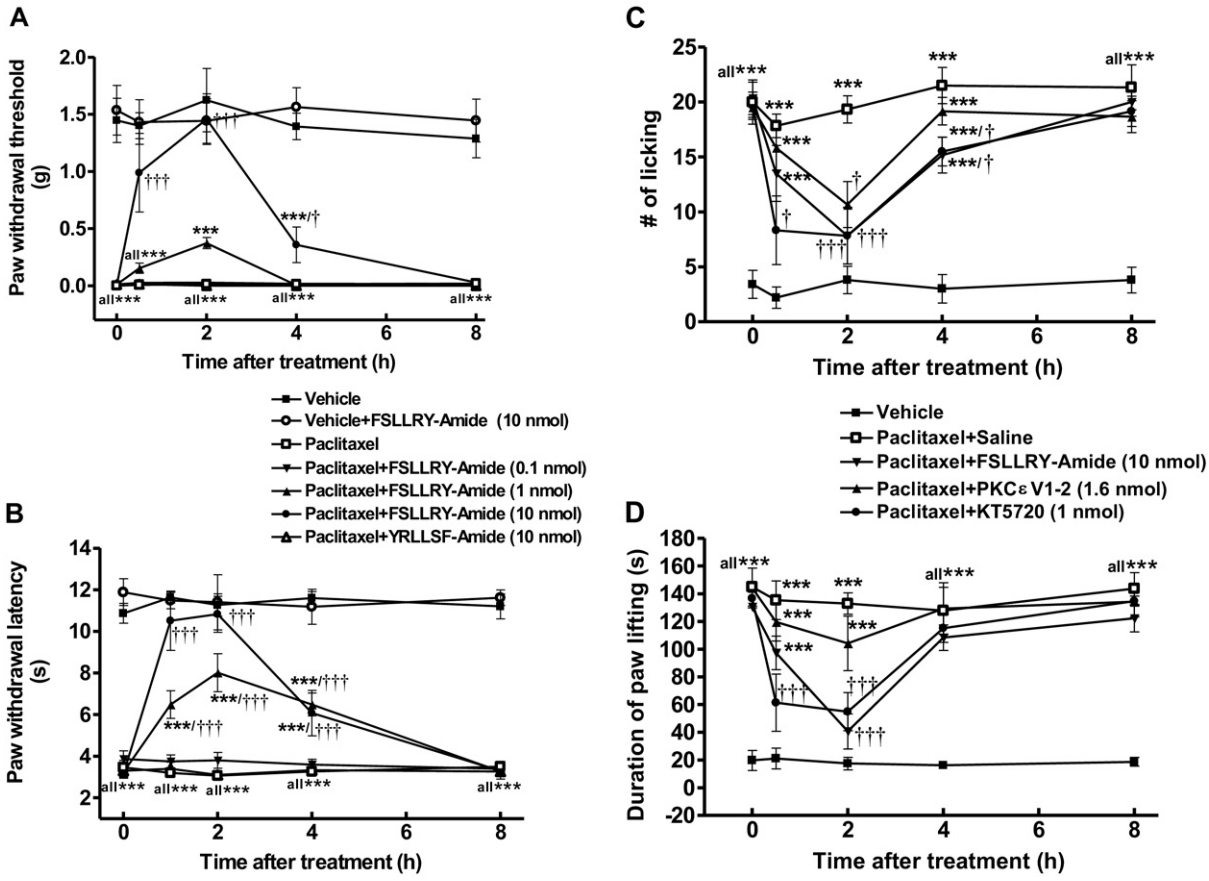

Fig. 3. Reversal of paclitaxel-induced mechanical allodynia (A), heat hyperalgesia (B), and cold hyperalgesia (C, D) by a PAR2 antagonist. After mechanical allodynia and thermal hyperalgesia have been well established by paclitaxel administration, mice were treated (i.t.) with PAR2 antagonist FSLLRY-amide (0.1–10 nmol), its negative control peptide YRLLSF-amide (10 nmol), or saline. Mechanical allodynia and thermal hyperalgesia were tested at the different time points as indicated. FSLLRY-amide, but not YRLLSF-amide, reversed the established paclitaxel-induced mechanical allodynia (A) and heat hyperalgesia (B) in a dose- and time-dependent manner. Values of group Paclitaxel+YRLLSF-Amide (10 nmol) (Δ), Paclitaxel+FSLLRY-Amide (0.1 nmol) (▼), and Paclitaxel (□) in figure (A) are close to 0.1 g. FSLLRY-amide and KT5720 (PKA inhibitor) also significantly attenuate cold hyperalgesia. PKCε v1-2 (PKCε inhibitor) reduced the number, but not duration, of cold stimulus-induced licking response. Data are expressed in mean±SEM. ***$P<0.001$, compared with the vehicle-treated group; $^{\dagger}P<0.05$, $^{\dagger\dagger\dagger}P<0.001$, compared with the paclitaxel-treated group; $n=6$ for each group.

total protein) was separated by 12% SDS-PAGE and electrotransferred onto PVDF membrane. The membrane was probed with phospho-PKC (pan) (1:1000, Cell signaling, Danvers, MA, USA) or phosphor-PKA (1:1000, Cell signaling) at room temperature for 3 h, followed by incubation with HRP-conjugated donkey anti-rabbit secondary antibody (1:1000, GE Healthcare). An enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL, USA) was applied for development. The membrane was then stripped and re-probed with a mouse anti-β-actin antibody (1:10,000, Santa Cruz Biotechnology) followed by an HRP-conjugated anti-mouse secondary antibody (1:1000, GE Healthcare) and developed as above. ECL signals were detected by Chemi-Doc and analyzed using the Quantity One program (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All data are presented as mean±SEM. Differences between groups were analyzed using Student's *t*-test (two groups) or two-way ANOVA followed by post hoc analyses using Dunnett's *t*-test (multiple groups). Statistical significance was established at the 95% confidence limit.

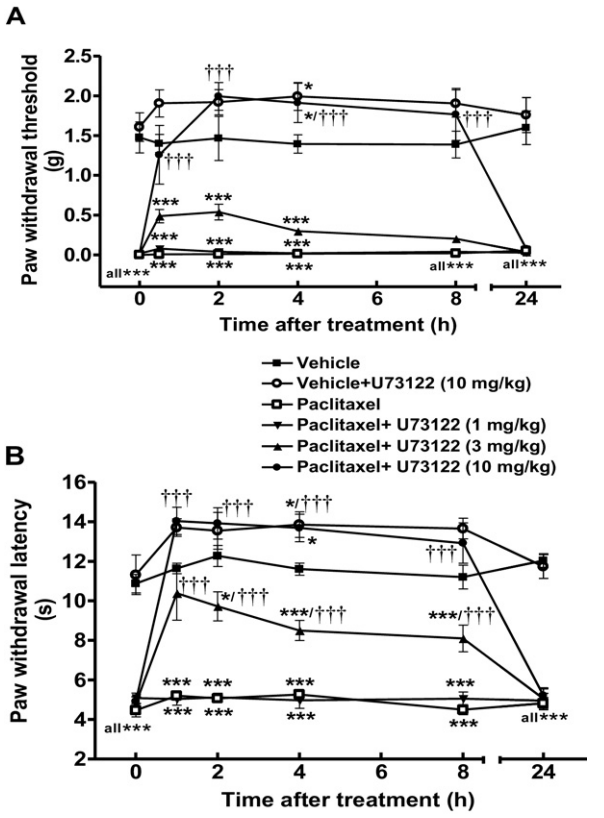

Fig. 4. Reversal of paclitaxel-induced mechanical allodynia (A) and thermal hyperalgesia (B) by a PLC inhibitor. After mechanical allodynia and thermal hyperalgesia have been well established post paclitaxel administration, mice were treated (i.p.) with PLC inhibitor U73122 (1–10 mg/kg) or saline. Mechanical allodynia and thermal hyperalgesia were tested at the different time points as indicated. U73122 reversed the established paclitaxel-induced mechanical allodynia and heat hyperalgesia in a dose- and time-dependent manner. Values of group Paclitaxel+U73122(1 mg/kg) (▼), and Paclitaxel (□) in figure (A) are close to 0.1 g. Data are expressed in mean±SEM. *$P<0.05$, ***$P<0.001$, compared with the vehicle-treated group; $^{\dagger\dagger\dagger}P<0.001$, compared with the paclitaxel-treated group; $n=6$ for each group.

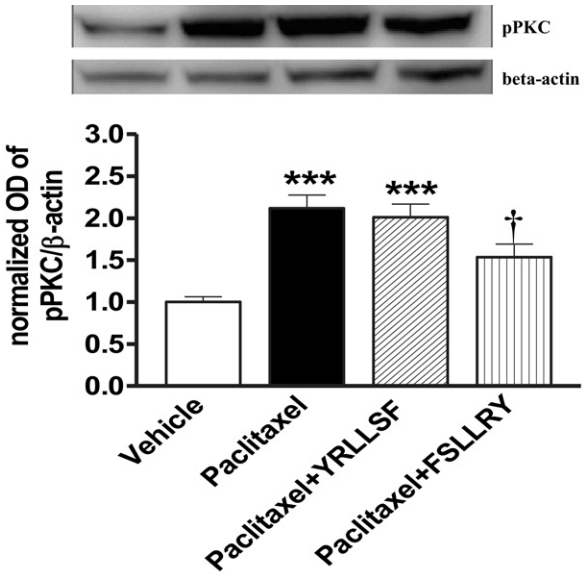

Fig. 5. Suppression of paclitaxel-induced PKC activation by a PAR2 antagonist. After mechanical allodynia and thermal hyperalgesia have been well established by paclitaxel administration, mice were treated (i.t.) with PAR2 antagonist FSLLRY-amide (0.1–10 nmol), its negative control peptide YRLLSF-amide (10 nmol), or saline. Two hours later, mice were sacrificed and lumbar segments of the spinal cords were taken and pooled for the analysis of PKC activation, by determining the amount of phosphorylated PKC (pPKC). Upper panels show a representative image of triplicate Western blotting assays. Paclitaxel-enhanced pPKC was suppressed by FSLLRY-amide, but not YRLLSF-amide. Data are expressed in mean±SEM. ***$P<0.001$, compared with the vehicle-treated group; $^{\dagger}P<0.05$, compared with the paclitaxel treated group; $n=3$ for each group.

RESULTS

PAR2 antagonist reversed paclitaxel-induced sensory hypersensitivity

Paclitaxel induced long-lasting pain behaviors in ICR mice. Repeated administration of paclitaxel (1 mg/kg, i.p., on days 0, 2, 4, and 6) significantly increased mechanical and thermal sensitivity (Fig. 1). Mechanical allodynia was detectable on day 2 and lasted for at least 28 days ($P<0.001$ vs. vehicle, $n=6$). Heat hyperalgesia had an onset around day 6 and was detectable for at least 24 more days ($P<0.001$ vs. vehicle, $n=6$). We did not perform daily tests of cold hyperalgesia; however, it was detectable on day 10 (Fig. 3C, D) and lasted for at least 20 days.

Mast cell tryptase, a major component of mast cell granules, has been implicated in several inflammatory and neuropathic pain states via PAR2 activation. Because it is stored almost exclusively in mast cells, release of tryptase is considered as a common indicator of mast cell activation. We asked whether mast cell tryptase was released by paclitaxel, so we directly determined the tryptase activity in tissues taken from the paclitaxel-treated mice. Mast cell tryptase activity was significantly increased in the DRG, the spinal cord, and the hindpaw skin (where thermal and mechanical sensitivity was determined) in paclitaxel-treated mice. No significant change was detected in the

brain cortex (Fig. 2). These data indicated that paclitaxel increased the release of mast cell tryptase.

Whether PAR2 was activated by tryptase and responsible for paclitaxel-induced pain behavior was then tested by directly blocking PAR2 using FSLLRY-amide, a selective PAR2 antagonist. After mechanical and thermal hypersensitivity was well established as a result of repeated paclitaxel administration, mice were treated with FSLLRY-amide (0.1–10 nmol, i.t.), and mechanical and thermal (both cold and heat) sensitivity was determined 0.5, 2, 4, and 8 h later. FSLLRY-amide reversed paclitaxel-induced mechanical allodynia (Fig. 3A) and heat hyperalgesia (Fig. 3B) in a dose- and time-dependent manner. At the highest dose, FSLLRY-amide (10 nmol, i.t.) completely reversed mechanical allodynia and heat hyperalgesia. Its anti-allodynic/anti-hyperalgesic effect appeared 30 min after FSLLRY-amide administration, peaked at 2 h, and lasted for at least 4 h (Fig. 3). At lower doses, FSLLRY-amide (1 nmol, i.t.) partially suppressed thermal hyperalgesia, while FSLLRY-amide (0.1 nmol, i.t.) did not affect either thermal or mechanical sensitivity. In contrast, YRLLSF-amide (10 nmol, i.t.), a negative control peptide that lacks receptor affinity, did not alter the pain sensitivity at any time point tested. FSLLRY-amide (10 nmol, i.t.) was also effective reversing paclitaxel-induced hypersensitivity to noxious cold stimulus (Fig. 3C, D). When given to control mice, however, FSLLRY-amide (10 nmol, i.t.) did not change basal nociception threshold.

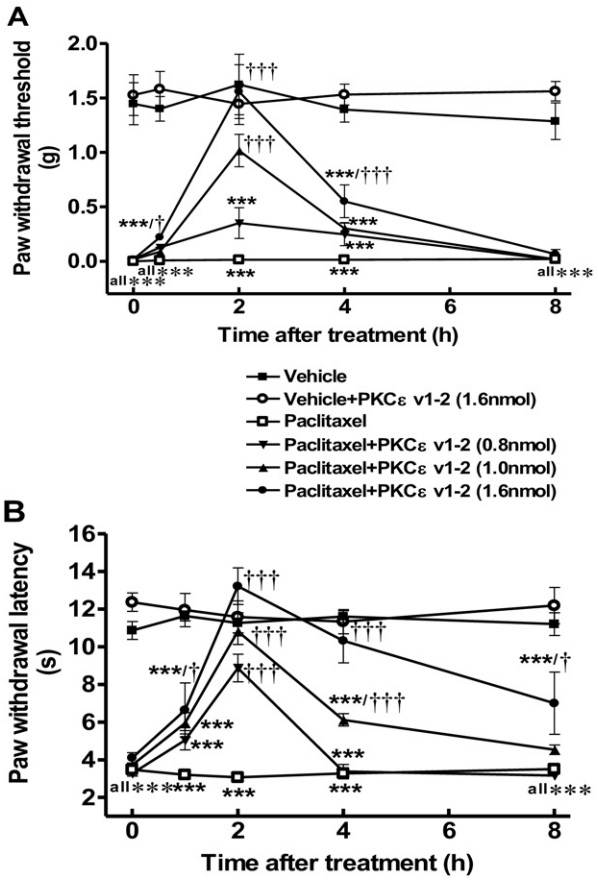

Fig. 6. Reversal of paclitaxel-induced mechanical allodynia (A) and heat hyperalgesia (B) by a PKCε inhibitor. After mechanical allodynia and thermal hyperalgesia have been well established by paclitaxel administration, mice were treated (i.t.) with PKCε inhibitor PKCε v1-2 (0.8–1.6 nmol) or saline. Mechanical allodynia and heat hyperalgesia were tested at the different time points as indicated. PKCε v1-2 reversed the established paclitaxel-induced mechanical allodynia and heat hyperalgesia in a dose- and time-dependent manner. Moreover, PKCε v1-2 (1.6 nmol i.t.) did not change nociception in control vehicle-treated mice. Data are expressed in mean±SEM. ***$P<0.001$, compared with the vehicle-treated group; †$P<0.05$, †††$P<0.001$, compared with the paclitaxel-treated group; $n=6$ for each group.

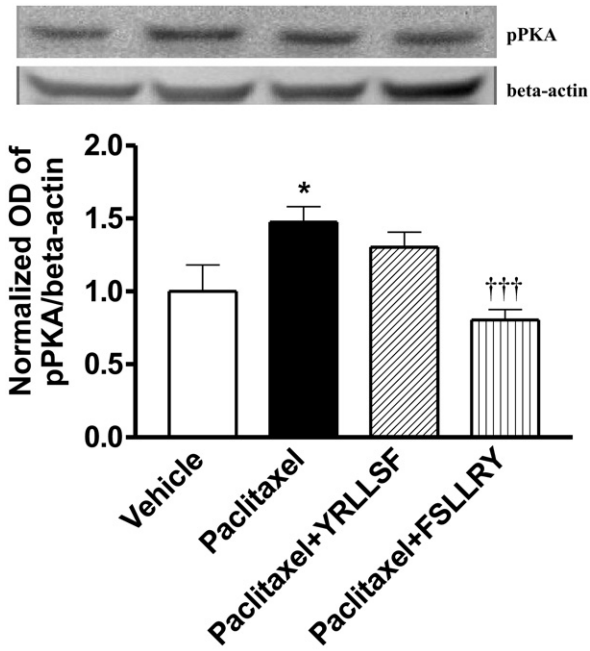

Fig. 7. Suppression of paclitaxel-induced PKA activation by a PAR2 antagonist. After mechanical allodynia and thermal hyperalgesia have been well established by paclitaxel administration, mice were treated (i.t.) with PAR2 antagonist FSLLRY-amide (0.1–10 nmol), its negative control peptide YRLLSF-amide (10 nmol), or saline. Two h later, mice were sacrificed and pooled samples of spinal cords lumbar segments were taken for the analysis of PKA activation, by determining the amount of pPKA. A representative image from triplicate western assay was shown in upper panels. Paclitaxel-enhanced pPKA was suppressed by FSLLRY-amide, but not YRLLSF-amide. Data are expressed in mean±SEM. *$P<0.05$, compared with the vehicle-treated group; †††$P<0.001$, compared with the paclitaxel-treated group; $n=3$ for each group.

PLCβ and PKCε mediate paclitaxel-induced pain behavior

PAR2 is known to be coupled to Gq/11 and its activation leads to the activation of PLCβ and PKC. We next examined this signaling pathway in paclitaxel-induced pain behaviors. In paclitaxel-treated mice that have developed mechanical and thermal sensitivity, U73122 (1–10 mg/kg, i.p.), a PLC inhibitor, dose- and time-dependently reversed the pain behaviors. At the highest dose used, U73122 (10 mg/kg, i.p.) showed a quick onset (30 min) of anti-allodynic and anti-heat hyperalgesic effect that lasted for over 8 h (Fig. 4). U73122 at this high dose exhibited some analgesic action in either control or paclitaxel-treated mice. At a

lower dose, U73122 (3 mg/kg, i.p.) was still very effective in attenuating heat hyperalgesia, although it did not significantly reduce mechanical allodynia. Similar to blocking PAR2 by the receptor antagonist, inhibiting PLC was more effective in attenuating thermal, than mechanical, hypersensitivity.

The protein kinase C is a downstream effector of PLC (Fig. 13). Although activation of PKCε has been demonstrated in paclitaxel-induced mechanical allodynia (Dina et al., 2001), it was not known whether PKC activation was mediated by PAR2. We first examined PKC activity in the spinal cord (lumber segments), and found that PKC activity was increased by repeated administration of paclitaxel (Fig. 5, lane 2 vs. 1). The enhanced PKC activation was reduced by the PAR2 antagonist FSLLRY-amide (10 nmol, i.t.), but not altered by the negative control peptide YRLLSF-amide (10 nmol, i.t.). A previous study demonstrated that local (intraplantar) administration of a PKCε inhibitor (PKCε v1-2) suppressed paclitaxel-induced pain behaviors measured at the ipsilateral hindpaw (Dina et al., 2001). We demonstrated here that centrally administered PKCε v1-2 (0.8–1.6 nmol, i.t.) inhibited the paclitaxel-induced mechanical allodynia and heat hyperalgesia in a time- and dose-dependent manner (Fig. 6). PKCε v1-2 alone, at the highest dose used, did not affect nociception baseline in control animals. These data suggested the importance of the signaling pathway from PLCβ to PKCε in paclitaxel-induced mechanical and heat pain. The pathway appeared to be initiated by the activation of PAR2, since the PAR2 antagonist suppressed PKC activation and pain behaviors induced by paclitaxel. Inhibiting PKCε by PKCε v1-2, however, was marginally effective in reducing cold hyperalgesia. Only the number of paw-licks, not total duration, was inhibited by PKCε v1-2 (Fig. 3C, D).

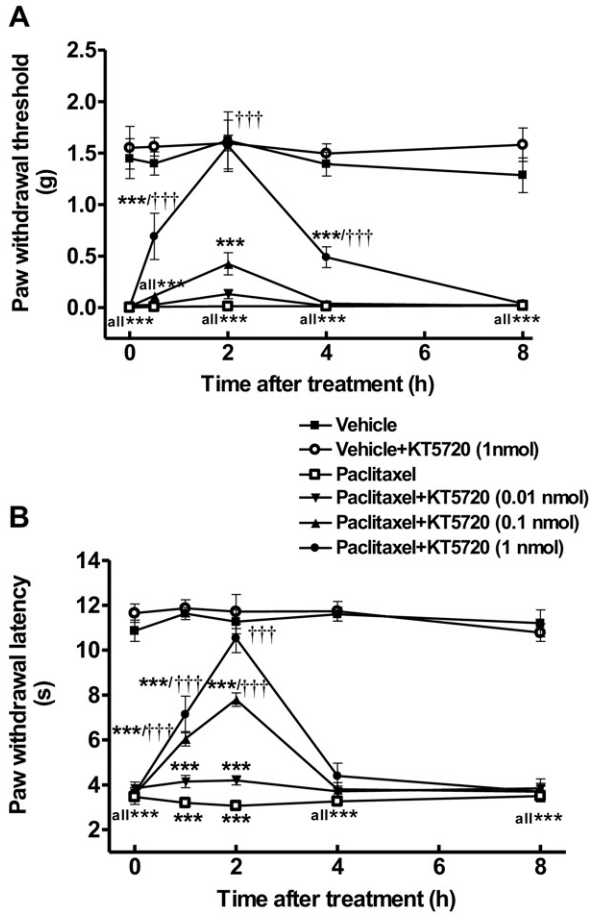

Fig. 8. Reversal of paclitaxel-induced mechanical allodynia (A) and heat hyperalgesia (B) by a PKA inhibitor. After mechanical allodynia and thermal hyperalgesia have been well established by paclitaxel administration, mice were treated (i.t.) with PKA inhibitor KT5720 (0.01–1 nmol) or saline. Mechanical and heat sensitivity was tested 0, 0.5, 2, 4, and 8 h later. Established paclitaxel-induced mechanical allodynia and heat hyperalgesia was reversed by KT5720 in a dose- and time-dependent manner. KT5720 (1 nmol i.t.) alone did not alter nociception baseline. Cold hyperalgesia data are presented in Fig. 3. Data are expressed in mean±SEM. ***$P<0.001$, compared with the vehicle-treated group; $^{\dagger\dagger\dagger}P<0.001$, compared with the paclitaxel-treated group; $n=6$ for each group.

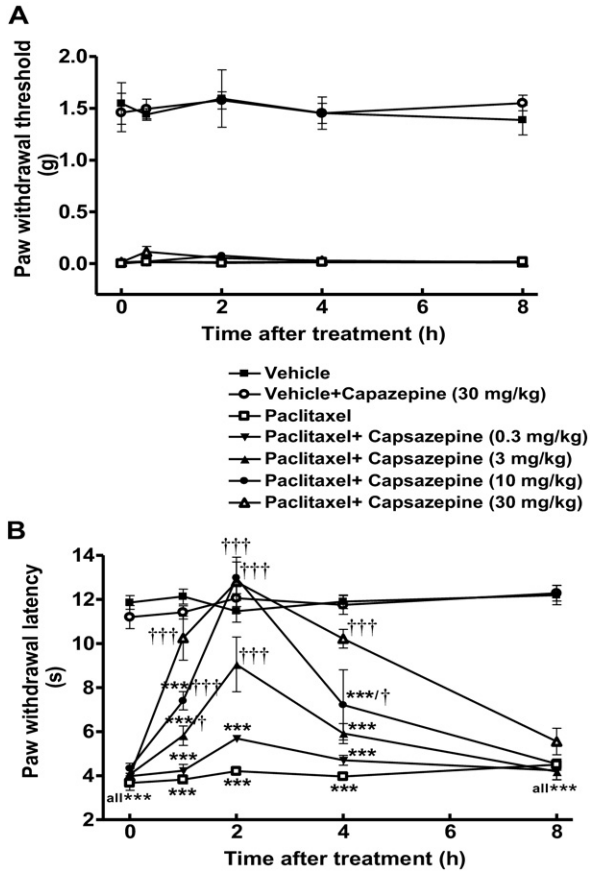

Fig. 9. TRPV1 antagonist capsazepine reversed paclitaxel-induced heat hyperalgesia. Mice treated with vehicle or paclitaxel received TRPV1 antagonist capsazepine (0.3–30 mg/kg, i.p.) or saline (i.p.). Mechanical and heat sensitivity was tested at time points indicated. Capsazepine reversed paclitaxel-induced heat hyperalgesia (B), but not mechanical allodynia (A). Data are expressed in mean±SEM. ***$P<0.001$, compared with the vehicle-treated group; $^{\dagger}P<0.05$, $^{\dagger\dagger\dagger}P<0.001$, compared with the paclitaxel-treated group; $n=6$ for each group.

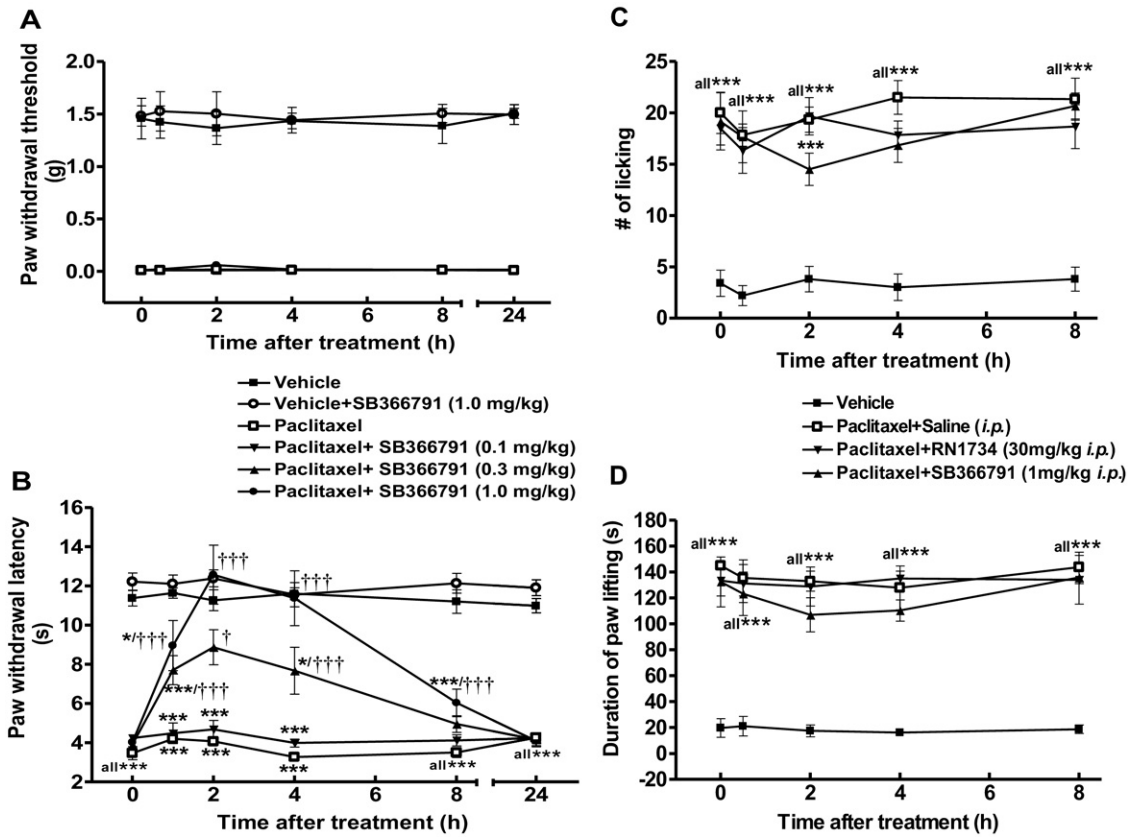

Fig. 10. TRPV1 antagonist SB366791 reversed paclitaxel-induced thermal hyperalgesia. Mice treated with vehicle or paclitaxel received TRPV1 antagonist SB366791 (0.1–1.0 mg/kg, i.p.) or saline (i.p.). Mechanical, heat, and cold sensitivity was tested at time points indicated. SB366791 reversed paclitaxel-induced heat hyperalgesia (B), but not mechanical allodynia (A) or cold hyperalgesia (C, D). RN1734, a TRPV4 inhibitor, also did not affect paclitaxel-induced cold hyperalgesia. Data are expressed in mean±SEM. *$P<0.05$, ***$P<0.001$, compared with the vehicle-treated group; $^{\dagger}P<0.05$, $^{\dagger\dagger\dagger}P<0.001$, compared with the paclitaxel-treated group; $n=6$ for each group.

PKA mediates paclitaxel-induced mechanical and thermal hypersensitivity

In addition to PKCε, PKA—another serine/threonine protein kinase—has been implicated in paclitaxel pain (Dina et al., 2001). We found that spinal PKA was activated by repeated administration of paclitaxel, which was suppressed by blocking PAR2 (Fig. 7). KT5720 (0.01–1 nmol, i.t.), a PKA inhibitor, dose- and time-dependently reversed paclitaxel-induced mechanical allodynia, heat hyperalgesia (Fig. 8), and cold hyperalgesia (Fig. 3C, D). In control mice, KT5720 itself (1 nmol) did not alter nociception baseline. These data confirmed a role of PKA activation in paclitaxel-induced pain behaviors.

Antagonists of TRPV1, TRPV4, and TRPA1 inhibited paclitaxel-induced persistent sensory hypersensitivity

What are the downstream effectors of PKCε and PKA, leading to paclitaxel-induced pain behaviors? Next, we focused on potential roles of TRP ion channels. TRPV1 is a non-selective cationic channel that is activated by noxious heat (>43 °C), protons, and ligands such as capsaicin (Tominaga et al., 1998). Capsazepine is the most commonly used antagonist of TRPV1. We found that capsazepine (0.3–30 mg/kg, i.p.) dose-dependently reversed heat hyperalgesia induced by paclitaxel (Fig. 9B), but did not alter the mechanical pain threshold (Fig. 9A). We also employed SB366791 (0.1–10 mg/kg, i.p.), a newer, more selective TRPV1 antagonist (Niiyama et al., 2009), and confirmed inhibiting TRPV1 attenuated paclitaxel-induced heat hyperalgesia (Fig. 10B), but not mechanical allodynia (Fig. 10A) or cold hyperalgesia (Fig. 10C, D). These results suggested a critical role of TRPV1 in sensing or transducing paclitaxel-induced heat hyperalgesia. Neither capsazepine nor SB 366791 altered nociception baseline in control mice (Figs. 9 and 10).

TRPV4 is gated by tonicity, innocuous warmth (>24 °C), endogenous ligands such as metabolites of arachidonic acid as well as exogenous chemicals such as bisandrographolide A (Güler et al., 2002; Alessandri-Haber et al., 2008; Vriens et al., 2009). A role of TRPV4 in paclitaxel-induced sensory hypersensitivity has been previously demonstrated by using antisense-mediated TRPV4 knockdown and TRPV4 knockout mice (Alessandri-Haber et al., 2008). Here, we applied RN1734, a TRPV4 antagonist (Vincent et al., 2009) that did not alter nociception baseline in control mice, and found RN1734 (30 mg/kg, i.p.) partially reversed the mechanical allodynia and heat

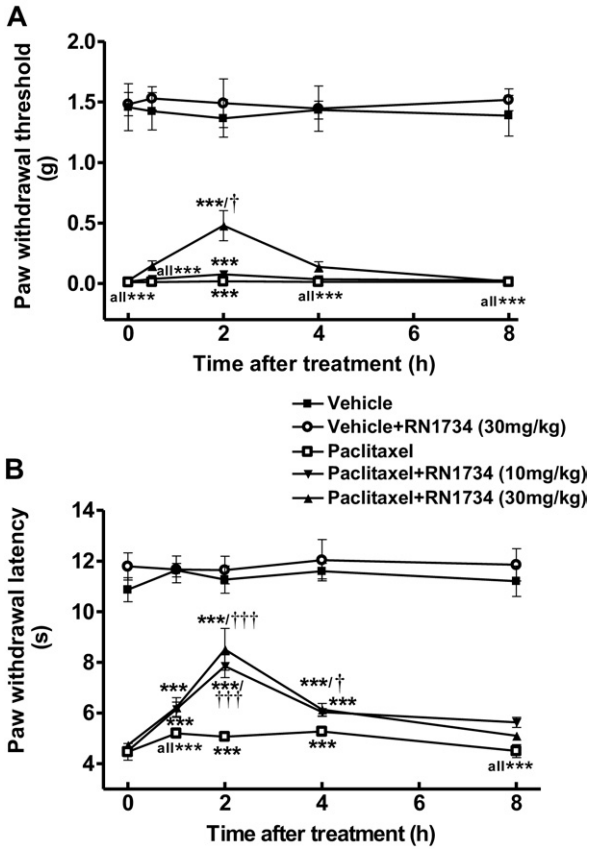

Fig. 11. Reversal of paclitaxel-induced mechanical allodynia and heat hyperalgesia by a TRPV4 antagonist. Mice treated with vehicle or paclitaxel received RN1734 (10–30 mg/kg, i.p.) or saline. The established mechanical allodynia (A) and heat hyperalgesia (B) were partially reversed by RN1734. RN1734 did not affect paclitaxel-induced cold hyperalgesia (Fig. 10). Data are expressed in mean±SEM. ***$P<0.001$, compared with the vehicle-treated group; †$P<0.05$, †††$P<0.001$, compared with the paclitaxel-treated group; $n=6$ for each group.

hyperalgesia (Fig. 11), but not cold hyperalgesia (Fig. 10C), induced by paclitaxel. Further increase of dosage was limited by its toxicity. On the other hand, RN1734 at 10 mg/kg was equally effective as that of 30 mg/kg in attenuating heat hyperalgesia, suggesting that the drug may have reached a ceiling effect at 10 mg/kg.

TRPA1 is activated by cold stimulus (<17 °C) (Story et al., 2003) and pungent chemicals such as cinnamaldehyde (Bandell et al., 2004). TRPA1 was initially proposed as a mechanosensor (Corey et al., 2004), and was later considered to detect cold in the noxious range, although conflicting data have been obtained from studies employing TRPA1 knockout mice (Basbaum et al., 2009). To directly test a role of TRPA1 in paclitaxel-induced mechanical allodynia and thermal hyperalgesia, we employed HC030031 (1–30 mg/kg, i.p.), an antagonist of TRPA1 channel, in the study. HC030031 (30 mg/kg, i.p.) completely reversed mechanical allodynia and heat hyperalgesia induced by paclitaxel, without affecting nociception baseline in control mice (Fig. 12A, B). We reduced the dose to 10 mg/kg, which was still able to achieve the maximum effects. A lower dose (3 mg/kg) achieved partial effects, whereas the lowest dose (1 mg/kg) was ineffective. Therefore, HC030031 dose- and time-dependently reversed mechanical allodynia, cold, and heat hyperalgesia produced by paclitaxel (Fig. 12A–D).

DISCUSSION

In the current study, we tested the hypothesis that PAR2 mediates paclitaxel-induced mechanical and thermal (heat and cold) hypersensitivity via the actions of TRPV1, TRPV4, and TRPA1. PAR2 was initially considered to be involved in inflammatory response (Vergnolle, 1999). The observation that a PAR2 agonist released neuropeptides substance P and CGRP from sensory neurons suggested a neurogenic mechanism by which PAR2 is involved in inflammation (Steinhoff et al., 2000). Since then, a rapidly accumulating body of evidence has reproducibly implicated the role of PAR2, as well as its ligand mast cell tryptase, in several inflammatory pain conditions (Noorbakhsh et al., 2006; Barbara et al., 2007; Cenac et al., 2007). Further studies showed that intraplantar injection of sub-inflammatory doses of PAR2 agonists was able to induce mechanical and thermal hyperalgesia in rodents and increased spinal c-fos immunoreactivity, suggesting a potentially direct action of PAR2 in nociception (Vergnolle et al., 2001). Whether PAR2 is important for chronic pain has yet to be established. Here, we investigated PAR2-mediated signaling pathways in paclitaxel-induced neuropathic pain. Increased mast cell tryptase activity was found in the hindpaw skin, DRG, and spinal cord of paclitaxel-treated mice. Brain tryptase activity was not altered. The latter does not necessarily suggest an absence of supraspinal modulation in paclitaxel pain behaviors. Rather, it may suggest that release of tryptase by paclitaxel is likely an early event in the pain initiation cascade. In addition, it has been found that the brain has the lowest concentration of paclitaxel in the nervous system (Cavaletti et al., 2000).

The role of PAR2 was directly determined by a PAR2 antagonist that abolished paclitaxel-induced mechanical allodynia and thermal hyperalgesia. To ascertain a PAR2-mediated mechanism and to understand the signaling network leading to paclitaxel-induced sensory hypersensitivity, we further investigated the downstream signaling pathways of PAR2 activation. PAR2 is coupled to Gq that signals to PLC (Macfarlane et al., 2001; Linley et al., 2008). We showed that U73122, a PLC inhibitor, reversed the paclitaxel-induced mechanical allodynia and thermal (cold and heat) hyperalgesia, confirming the signaling from PAR2 to PLC leading to paclitaxel-induced pain behaviors.

Activation of PKCε and PKA are known signaling events after the activation of PAR2 (Amadesi et al., 2006; Grant et al., 2007). We found that repeated treatment with paclitaxel activated PKA and PKC, which was blocked by a PAR2 antagonist. Furthermore, we applied (i.t.) selective inhibitors for PKCε and PKA, which showed significant anti-allodynic/anti-heat hyperalgesic effects. These data are consistent with a previous report that inhibited these kinases in the periphery (paw) (Dina et al., 2001). Although

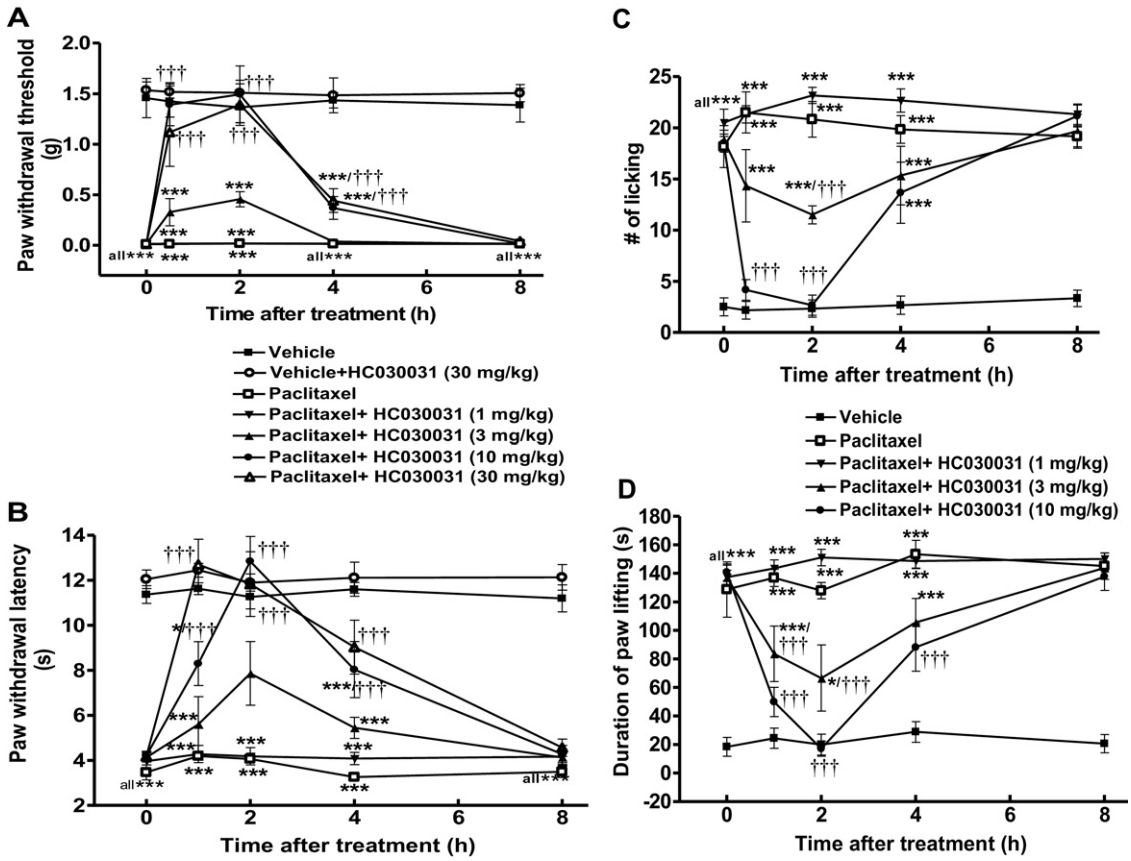

Fig. 12. Reversal of paclitaxel-induced mechanical allodynia (A), heat hyperalgesia (B), and cold hyperalgesia (C, D) by a TRPA1 antagonist. After mechanical allodynia and thermal hyperalgesia have been well established by paclitaxel, mice were treated (i.p.) with TRPA1 PLC antagonist HC030031 (1–30 mg/kg) or saline. Mechanical allodynia and thermal hyperalgesia were tested at the different time points as indicated. The established mechanical allodynia (A), heat hyperalgesia (B), and cold hyperalgesia (C, D) were reversed by HC030031 in a dose- and time-dependent manner. In control mice, neither mechanical (A) nor thermal (B) sensitivity was affected by HC030031 (30 mg/kg). Data are expressed in mean±SEM. *$P<0.05$, ***$P<0.001$, compared with the vehicle treated group; †††$P<0.001$, compared with the paclitaxel treated group; $n=6$ for each group.

signaling from PLC to PKC is well established, how PAR2 activates PKA is not exactly clear (Amadesi et al., 2006). Besides Gq, PAR2 is also coupled to Gi/o (Traynelis and Trejo, 2007; McCoy et al., 2010), which is expected to decrease adenylyl cyclase and PKA activity. However, many Gi/o-coupled receptors, such as the opioid receptors and the dopamine D2 receptor, are known to paradoxically upregulate adenylyl cyclase upon prolonged receptor activation, leading to increased intracellular cAMP levels and PKA activation (Sharma et al., 1975; Wang et al., 1994). It is possible that PAR2 activation may also trigger a direct activation of PKA, through a yet-to-be defined mechanism. Cyclic-AMP levels were increased in DRG neurons and HEK 293 cells expressing PAR2 upon acute (1–5 min) exposure to PAR2 agonists (Amadesi et al., 2006). It has been suggested that PKCε can be activated through the increased cAMP levels and activation of guanine exchange factor Epac (Hucho et al., 2005). Whether such a mechanism exists in paclitaxel-induced pain was not directly tested in this study, since it is still unclear if and how cAMP was elevated. Nonetheless, inhibiting PKA was highly effective in attenuating pain behaviors, suggesting that PKA is crucial and does not depend on cAMP/Epac/PKCε pathway for its action in paclitaxel-induced pain. Additional research is needed to elucidate relative contributions of PKA and PKC.

How does PAR2 signaling lead to paclitaxel-induced heightened state of mechanical and thermal sensitivity? The first clue comes from the findings that PAR2 is co-expressed with TRPV1, TRPV4, or TRPA1 in primary afferent neurons (Amadesi et al., 2006; Dai et al., 2007; Grant et al., 2007). Electrophysiological studies have suggested that PAR2 sensitized TRPV4 and TRPA1, which was blocked by a PLC inhibitor (Dai et al., 2007; Grant et al., 2007). Similarly, PAR2 sensitized TRPV1 and TRPV4 in a PKCε- and PKA-dependent manner (Amadesi et al., 2006; Grant et al., 2007). PKA and PLC were also found to be involved in TRPA1 sensitization by nociceptive signals (Wang et al., 2008; Schmidt et al., 2009). In many painful conditions, TRPV1 has been shown to mediate heat hyperalgesia (Caterina et al., 2000; Dai et al., 2004; Vriens et al., 2009). We found that capsazepine and SB366791, inhibitors of TRPV1, attenuated paclitaxel-induced heat hyperalgesia. In contrast, no significant effect was observed in mechanical or cold sensitivity.

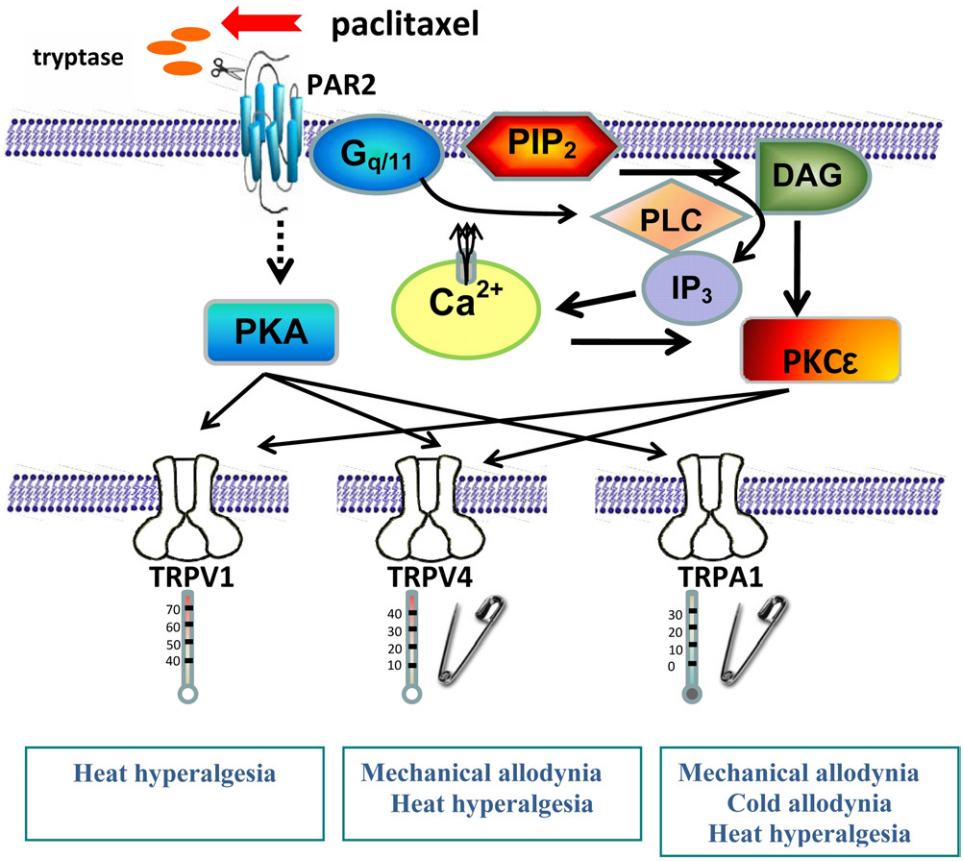

Fig. 13. Proposed model of PAR2-mediated signaling pathways in paclitaxel-induced neuropathic pain. Paclitaxel promotes the release of mast cell tryptase, which cleaves and activates PAR2. The latter directly activates PLCβ via $Gq_{/11}$, resulting in generation of DAG and IP3. IP3 releases Ca^{2+} from the intracellular storage, which, together with DAG, lead to the activation of PKC. PKA is likely activated by an indirect mechanism such as the compensatory upregulation of the adenylyl cyclase system. Activated second messenger kinases PKCε and PKA phosphorylate and sensitize TRPV1, TRPV4, or TRPA1, ultimately leading to mechanical, heat and cold hypersensitivity. Sensitization of TRPA1 by PKCε may be limited, as cold hyperalgesia is only marginally affected by inhibiting PKCε. Keys: PAR2, proteinase-activated receptor 2; PLC, phospholipase C; TRPV1, transient receptor potential channel V1; TRPV4, transient receptor potential channel V4; TRPA1, transient receptor potential channel A1; PIP2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate. Thermometers and needles used in figure indicate thermal/cold or mechanical stimuli that activate corresponding TRP channels. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

TRPV4, gated by innocuous warmth, hypotonicity, or other stimuli (Güler et al., 2002; Alessandri-Haber et al., 2008; Vriens et al., 2009), has also been reported to mediate mechanical and thermal sensitivity (Grant et al., 2007). TRPV4 knockout or antisense gene knockdown reduced the paclitaxel-induced mechanical allodynia (Alessandri-Haber et al., 2008), suggesting the contribution of TRPV4 in paclitaxel-induced neuropathy. In our study, this anti-dllodynic/anti-hyperalgesic effect was further confirmed by employing the TRPV4 antagonist RN1734.

TRPA1 has been proposed as a sensor for noxious cold (Story et al., 2003). We found that HC030031, an antagonist of TRPA1, attenuated paclitaxel-induced cold hyperalgesia. A role of TRPA1 in gating cold sensation is somewhat controversial. In one series of studies, TRPA1 knockout mice showed deficit response to noxious cold (Kwan et al., 2006; Karashima et al., 2009). However, cold sensitivity was not changed in another study employing a different TRPA1 null mouse strain (Bautista et al., 2006). Findings from cultured trigeminal neurons also unpaired TRPA1 with cold sensitization (Jordt et al., 2004). Since TRPA1 is also known to transduce mechanical stimuli (Hill and Schaefer, 2007), it is not surprising that HC030031 also reversed paclitaxel-induced mechanical allodynia (Fig. 12). Again, the role of TRPA1 as mechanosenser is still being debated. It has been suggested that TRPA1 may be better suited as a modulator of mechanosensitive afferent excitability, rather than a primary mechanosensor (Basbaum et al., 2009). Surprisingly, HC030031 also revered heat hyperalgesia induced by paclitaxel in our study. It is known, however, that TRPA1 agonists such as cinnamaldyhyde and mustard oil produce heat hyperalgesia and sensitize noxious heat, instead of cold, response (Dunham et al., 2010; Sawyer et al., 2009; Tsagareli et al., 2010). Since TRPA1 is expressed in a subset of TRPV1-positive nociceptive afferent neurons (Story et al., 2003), TRPA1-positive afferents may merge signals of noxious heat and cold (McKemy, 2005).

Our findings suggest a critical role of PAR2 signaling in sensitizing TRPV1, TRPV4, and TRPA1, leading to paclitaxel-induced mechanical allodynia and thermal hyperalgesia. For the first time, the network of neurocircuitry, involving PAR2, PLC, PKA, PKCε, and multiple TRP channels, was simultaneously investigated in a single study. Based on these findings, we like to propose a tryptase/PAR2-initiated signaling network that involves the aforementioned G proteins, kinases, and TRP channels in paclitaxel-induced neuropathic pain (Fig. 13). TRPV1, TRPV4, and TRPA1 appeared to contribute, at different levels, to the sensation of mechanical, noxious heat and cold stimuli. Since inhibiting several individual signaling molecules was highly effective in abolishing pain behavior, it suggests that there is a certain degree of signal integration in this neurocircuitry. Identifying members of this signaling circuitry presents new opportunities for the treatment of paclitaxel-induced neuropathy.

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