Cellular/Molecular

Transient Receptor Potential Vanilloid 4 Is Essential in Chemotherapy-Induced Neuropathic Pain in the Rat

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The development of treatments for neuropathic pain has been hindered by our limited understanding of the basic mechanisms underlying abnormalities in nociceptor hyperexcitability. We recently showed that the polymodal receptor transient receptor potential vanilloid 4 (TRPV4), a member of the transient receptor potential (TRP) family of ion channels, may play a role in inflammatory pain (Alessandri-Haber et al., 2003). The present study tested whether TRVP4 also contributes to neuropathic pain, using a rat model of Taxol-induced painful peripheral neuropathy. Taxol is the most widely used drug for the treatment of a variety of tumor types, but the dose of Taxol that can be tolerated is limited by the development of a small-fiber painful peripheral neuropathy.

We found that Taxol treatment enhanced the nociceptive behavioral responses to both mechanical and hypotonic stimulation of the hind paw. Spinal administration of antisense oligodeoxynucleotides to TRPV4, which reduced the expression of TRPV4 in sensory nerve, abolished Taxol-induced mechanical hyperalgesia and attenuated hypotonic hyperalgesia by 42%. The enhancement of osmotic nociception involves sensitization of osmotransduction in primary afferents because osmotransduction was enhanced in cultured sensory neurons isolated from Taxol-treated rats. Taxol-induced TRPV4-mediated hyperalgesia and the enhanced osmotransduction in cultured nociceptors were dependent on integrin/Src tyrosine kinase signaling. These results suggest that TRPV4 plays a crucial role in a painful peripheral neuropathy, making it a very promising target for the development of a novel class of analgesics.

Key words: **TRPV4; DRG nociceptors; Taxol; osmotransduction; mechanical hyperalgesia; neuropathy**

Introduction

Transient receptor potential vanilloid 4 (TRPV4), first identified as a mammalian osmotransducer (Liedtke et al., 2000; Strotmann et al., 2000), now has been shown to be a polymodal receptor also activated by heat (Guler et al., 2002; Watanabe et al., 2002b; Gao et al., 2003), phorbol esters (Watanabe et al., 2002a; F. Xu et al., 2003), low pH and citrate (Suzuki et al., 2003a), endocannabinoids, and arachidonic acid metabolites (Watanabe et al., 2003). TRPV4 distribution in cochlear hair cells, vibrissal Merkel cells, sensory ganglia (Liedtke et al., 2000; Guler et al., 2002; Alessandri-Haber et al., 2003; Suzuki et al., 2003a), and cutaneous mechanosensory terminals (Suzuki et al., 2003c) suggests a role of TRPV4 in mechanotransduction beyond osmosensation. Mice lacking the TRPV4 gene show impaired sensitivity to acid and an increase in mechanical nociceptive threshold but normal heat and touch sensation (Liedtke and Friedman, 2003; Suzuki et al., 2003a). Recently, we demonstrated that TRPV4 can function as a transducer of hypo-osmotic stimuli in primary afferent nociceptors in the setting of inflammation. Specifically, intradermal injection of hypotonic solution, which had no effect in control rats, induced pain-related behaviors after local injection of prostaglandin E_2 (Alessandri-Haber et al., 2003), and this effect was eliminated by treatment with TRPV4 antisense oligodeoxynucleotides.

Because TRPV4 appears to be more important in pathological pain conditions than in normal mechanical nociception, we investigated whether TRPV4 also is involved in mechanical hyperalgesia associated with painful peripheral neuropathy. Taxol, a widely used anti-cancer drug, is used to treat several types of malignant tumors. Its major dose-limiting side effect is a peripheral sensory neuropathy characterized by painful paresthesias of the hands and feet (Quasthoff and Hartung, 2002). In rats, chronic treatment with Taxol induces a painful peripheral neuropathy accompanied by mechanical hyperalgesia (Cliffer et al., 1998; Dina et al., 2001; Polomano et al., 2001).

Integrins play a role in Taxol-induced mechanical hyperalgesia (Dina et al., 2004) and also have been proposed to be involved in osmotransduction in hepatocytes as part of an integrin/actin/Src/MAPK/microtubule pathway (vom Dahl et al., 2003). Src tyrosine kinase phosphorylation directly activates TRPV4 *in vitro* (H. Xu et al., 2003). In the present study, we demonstrate that TRPV4 plays an essential role in Taxol-induced nociceptive behavioral responses to mechanical and hypotonic stimulation of the hind paw. We also demonstrate that Taxol-induced TRPV4-mediated hyperalgesia is essentially dependent on integrin/Src tyrosine kinase signaling.

Materials and Methods

Experiments were performed on 180–200 gm adult male Sprague Dawley rats (Charles River, Wilmington, MA). The experimental protocols were approved by the University of California San Francisco Committee

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on Animal Research and conformed to NIH guidelines for the use of animals in research. Taxol was administered as previously described (Dina et al., 2001). Briefly, Taxol was formulated at a concentration of 1 mg/ml in a vehicle composed of absolute ethanol and Cremophore EL; final Taxol concentration of 1 μg/2.5 μl was made in sterile NaCl, *United States Pharmacopeia*, at the time of injection. Taxol was injected intraperitoneally once a day for 10 doses.

Dorsal root ganglia cell culture

L2–L6 dorsal root ganglia (DRG) were harvested from rats and dissociated as described previously (Reichling and Levine, 1997). Cells were maintained in culture for 3 d in DMEM supplemented with 50–100 ng/ml nerve growth factor, 100 U/ml penicillin/streptomycin, MEM vitamins, and 10% heat-inactivated fetal calf serum (all culture components from Invitrogen Life Technologies, Carlsbad, CA). Dissociated cells were plated on coverslips treated with poly-DL-ornithine (0.1 mg/ml; Sigma, St. Louis, MO) and laminin (5 μg/ml; Invitrogen Life Technologies) and were incubated at 37°C in 96.5% air/3.5% CO_2.

Calcium imaging

Calcium imaging was performed by using the fluorescent calcium indicator fura-2 acetoxymethyl ester (fura-2 AM) between 24 and 72 hr after dissociation as previously described (Alessandri-Haber et al., 2003). Briefly, neurons were loaded with 5 μM fura-2 AM for 20 min in isotonic solution (312 mOsm). Experiments were performed at 20–23°C with the perfusion at a flow rate of 1–2 ml/min. Cells were perfused with isotonic solution for 10 min before the beginning of the recording to allow for complete de-esterification of fura-2 AM.

Measurement of the concentration of free calcium ions ($[Ca^{2+}]_i$) was performed by ratiometric imaging with an intensified charge-coupled device camera (ICCD; Stanford Photonics, Palo Alto, CA). Fluorescence was excited at 340 and 380 nm, and the emitted light was long filtered at 520 nm. The fluorescence ratio, F340/F380, was calculated with Metafluor software (Universal Imaging, Downingtown, PA).

In view of the absence of a specific blocker of TRPV4 and given the ionic complexity of DRG neurons, we performed recordings in conditions to minimize the activation of other ionic channels (i.e., low sodium concentration, room temperature, HEPES buffer, and variation of osmolarity only by modifying D-mannitol concentration). The standard isotonic solution (312 mOsm) contained (in mM): 88 NaCl, 5 KCl, 1 $MgCl_2$, 2.4 $CaCl_2$, 110 D-mannitol, 10 HEPES and was buffered at pH 7.38 with NaOH. The hypotonic solution was adjusted to 212 mOsm (30% hypotonic) by lowering the amount of D-mannitol to 10 mM. The vehicle for the fura-2 AM, dimethylsulfoxide (DMSO), was tested alone on DRG neurons to verify that it did not induce any response.

Antibody generation and purification

A synthetic peptide (CDGHQQGYAPKWRAEDAPL) corresponding to the C terminus of rat TRPV4 was synthesized and conjugated to keyhole limpet hemocyanin via its N terminus by Invitrogen Life Technologies. Rabbits were immunized with the KLH-conjugated peptide (240 μg of peptide for the first immunization and 120 μg thereafter) according to standard procedures at Lampire Biological (Pipersville, PA). The resulting immune serum was purified by using affinity chromatography and made from the synthetic peptide and Sulfolink coupling gel (Pierce Biotechnology, Rockford, IL). Purification was performed by incubating 1 ml of antiserum/ml of affinity resin for 2 hr at room temperature with constant rotation, followed by washing with 20 bed volumes of binding buffer (100 mM sodium borate and 0.02% sodium azide, pH 8.0). The purified antibody was eluted with 10 bed volumes of ImmunoPure Gentle Ag/Ab elution buffer (Pierce Biotechnology). Approximately 0.8 mg of purified IgG was obtained from 1 ml of crude rabbit antiserum as determined by Coomassie blue staining of SDS-PAGE. The specificity of the antibody was confirmed by testing it on tissues and cell lines not expressing TRPV4, using Western blot, immunochemistry, and flow cytometry.

Western blot

Saphenous nerves from anesthetized rats were ligated with silk surgical sutures (4–0) 1 cm above the knee-level bifurcation. A 5 mm section of saphenous nerve proximal to the ligation was removed 3 d after the ligation. Protein membrane preparation and Western blot analyses were performed as described previously (Alessandri-Haber et al., 2003; Parada et al., 2003). The membrane was probed with affinity-purified anti-TRPV4 antibody (1:750), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated with enhanced chemiluminescence reagents (Pierce Biotechnology) and exposed to films. To normalize the loaded samples, we used affinity-purified goat polyclonal α-tubulin (1:2000; Sigma) to reprobe the membrane. HRP-conjugated goat anti-mouse IgG (1:10,000; Sigma) was used as the secondary antibody.

Oligodeoxynucleotides preparation

The TRPV4 antisense oligodeoxynucleotide (ODN) sequence 5′-CATCACCAGGATCTGCCATACTG-3′ (Invitrogen Life Technologies) was directed against a unique region of the rat TRPV4 channel (GenBank accession number AF263521). The mismatch ODN sequence was designed by mismatching seven bases (denoted by bold face) of the TRPV4 antisense sequence, 5′-CA**A**CA**GG**AGG**T**TC**A**G**G**CA**A**ACTG-3′. ODNs were reconstituted in nuclease-free 0.9% NaCl (10 μg/μl) and were administrated intrathecally once daily at a dose of 40 μg for 3 d.

Intrathecal ODN treatment

As described previously (Alessandri-Haber et al., 2003; Parada et al., 2003), the rats were anesthetized with 2.5% isoflurane inhalation anesthetic (97.5% O_2); a 30 gauge needle was inserted into the subarachnoid space on the midline between the L4 and L5 vertebrae, and 20 μl of ODN was injected at 1 μl/sec, using a micro-syringe.

Nociceptive behavior

Flinching test. As described previously (Taylor et al., 1995; Alessandri-Haber et al., 2003), the animals were acclimated in a transparent observation chamber for 30 min. Rats then were restrained while 10 μl of hypotonic (deionized water) or isotonic (0.9% NaCl) solution was administered intradermally into the dorsum of the hind paw, using a 30 gauge needle connected to a 100 μl Hamilton syringe by PE-10 polyethylene tubing. Rats were observed immediately after the injection for a 5 min period. For the experiments involving TRPV4 antisense treatment, the flinching tests were run on day 4 after the initiation of ODN treatment (12 hr after injection). For experiments involving acute effects of pharmacological agents, the drugs were injected intradermally at the site of hypotonic solution injection 45 min before the behavioral testing.

Mechanical nociceptive threshold. Mechanical nociceptive threshold was evaluated by the Randall–Sellito paw withdrawal test with a Ugo-Basile analgesimeter (Stoelting, Chicago, IL) as described previously (Aley et al., 2001). Baseline mechanical thresholds were calculated as the mean of three measurements performed 5 min apart before the injection of hypotonic solution or pharmacological agents. Each paw was treated as an independent observation, and each experiment was performed on a different group of rats. For experiments involving pharmacological antagonists, these agents were injected intradermally 30 min before behavioral testing. Experiments involving recovery from either TRPV4 antisense or mismatch ODN were performed 4 d after the last injection of ODN, whereas recovery experiments from either the tyrosine kinase inhibitors or RGD peptide injection were performed 24 hr after the injection.

Drugs

Stock solutions of the tyrosine kinase inhibitors PP_1, piceatannol, and genistein (Biomol, Plymouth Meeting, PA) and the phorbol ester 4 α-phorbol 12,12-didecanoate (4 α-PDD, Sigma) were made in 10% DMSO. Stock solutions of the integrin antagonist hexapeptide GRGDTP, its inactive analog GRADSP (Calbiochem, La Jolla, CA), and the blocker of swelling-activated chloride channels diisothiocyanatostilbene 2,2′-disulphonic acid (DIDS; Sigma) were made in water. Experimental concentrations were made daily from stock solutions.

Single-cell RT-PCR

Single-cell experiments were conducted as described previously (Alessandri-Haber et al., 2003). At the end of calcium imaging recordings

cytoplasm was harvested, and reverse transcription was performed immediately with reverse transcriptase Superscript II (Invitrogen Life Technologies). Two successive PCR amplifications that used Hotstar (Qiagen, Valencia, CA) were performed for 28 and 38 cycles (initial *Taq* activation step at 94°C for 15 min; then 94°C for 30 sec, 60°C for 1 min, 72°C for 1 min, and 72°C for 7 min). The primer pair used for TRPV4 (accession number AF263521) amplified nucleotides 523–950, and the primer pair for the housekeeping gene GAPDH (accession number AF106860) amplified nucleotides 320–556. Gels were analyzed with AlphaEaseFC software (Alpha Innotech, San Leandro, CA)

Data analysis

All calcium imaging results are presented as the mean ± SEM of normalized fluorescence ratios. To measure the response of a cell to hypotonicity or 4 α-PDD, we averaged the value of the fluorescence ratio when the stimulus-induced increase in $[Ca^{2+}]_i$ reached a plateau. Then the fluorescence ratio at the plateau was normalized: fluorescence ratio during stimulation (i.e., hypotonic or 4 α-PDD)/fluorescence ratio during isotonic. For the experiments in which the effect of an inhibitor was measured, the normalization was calculated as the fluorescence ratio during 30% hypotonic plus inhibitor/fluorescence ratio during isotonic plus inhibitor.

Calcium imaging and behavioral data are presented as the mean ± SEM, and comparisons between groups were done by using Student's *t* test, Tukey's multiple comparison test, or ANOVA. A probability of $p < 0.05$ was considered significant. To determine whether the percentage of DRG neurons responding to hypotonic stimulation in Taxol-treated rats differed significantly from that in control rats, we performed a χ^2 test with Yates correction.

Results

Taxol-induced mechanical hyperalgesia and hypotonicity-induced nociception are TRPV4-dependent

After chronic treatment with Taxol, rats develop persisting mechanical hyperalgesia (Dina et al., 2001). To assess whether TRPV4 plays a role in Taxol-induced mechanical hyperalgesia, we treated the rats with TRPV4 antisense or mismatch ODN for 3 d. As shown in Figure 1*A*, spinal intrathecal administration of TRPV4 antisense ODN totally reversed the Taxol-induced mechanical hyperalgesia (−0.8 ± 1.3 gm, $n = 8$ for antisense-treated rats vs 31.5 ± 1.1 gm, $n = 16$ for baseline; $p < 0.05$, unpaired Student's *t* test). In contrast, mechanical hyperalgesia in mismatch-treated rats was not statistically different from the baseline (33.5 ± 1.4 gm, $n = 8$ for mismatch-treated rats vs 31.5 ± 1.1 gm, $n = 16$ for baseline; $p > 0.05$, unpaired Student's *t* test). The effect of TRPV4 antisense was reversible; 4 d after the last ODN treatment Taxol-induced mechanical hyperalgesia was not significantly different between the two ODN-treated groups and the baseline ($p > 0.05$, Tukey's multiple comparison test).

To determine whether osmotransduction mediated by TRPV4 could contribute to enhanced nociception in Taxol-treated rats, we injected 10 μl of hypotonic or isotonic solution in the hind paw of Taxol-treated and control rats (Fig. 1*B*). In Taxol-treated rats the injection of 10 μl of hypotonic solution induced flinches during the first 5 min after the injection (11.6 ± 1.1; $n = 16$), whereas 10 μl of isotonic solution did not (0.4 ± 0.2; $n = 8$). In control rats neither the isotonic nor hypotonic injection induced a significant number of flinches (0.2 ± 0.1 for isotonic vs 3.2 ± 0.3 for hypotonic injection; $n = 18$). To assess whether TRPV4 is responsible for the hypotonicity-induced nociception in Taxol-treated animals, we treated the rats with TRPV4 antisense or mismatch ODN (Fig. 1*C*). TRPV4 antisense ODN decreased the number of flinches by 42% compared with mismatch-treated rats (6 ± 1, $n = 6$ for the antisense-treated vs 10.5 ± 1.1, $n = 8$ for the mismatch-treated rats; $p = 0.01$, unpaired Student's *t* test). At 4 d after the last ODN treatment there were no significant differences in the number of hypotonicity-induced flinches between the two ODN groups (mean ± SEM; 8.5 ± 1.2, $n = 6$ for the antisense-treated vs 9.1 ± 1.3, $n = 8$ for the mismatch-treated rats; $p > 0.05$, unpaired Student's *t* test).

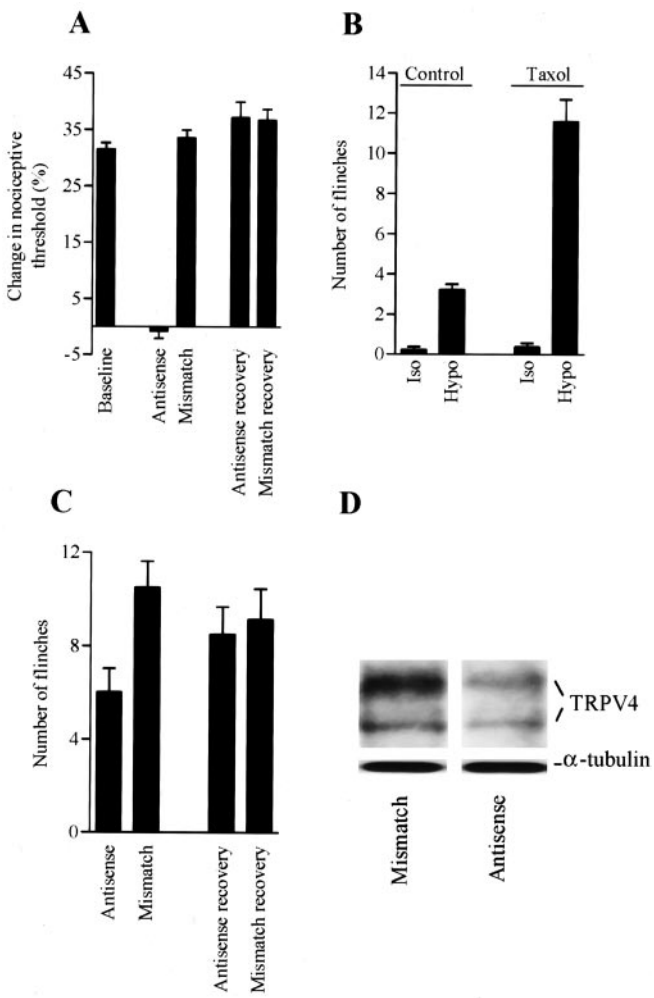

Figure 1. Taxol-induced mechanical hyperalgesia and hypotonicity-induced nociception are mediated by TRPV4. *A*, Taxol-induced mechanical hyperalgesia was abolished by TRPV4 antisense ODN treatment, whereas in mismatch-treated rats the mechanical hyperalgesia was not statistically different from the baseline (mean ± SEM; −0.8 ± 1.3 gm, $n = 8$ for antisense-treated vs 33.5 ± 1.4 gm, $n = 8$ for mismatch-treated rats; $p < 0.05$, unpaired Student's *t* test). At 4 d after the last antisense ODN treatment the Taxol-induced mechanical hyperalgesia was not statistically different from the pre-ODN baseline ($p > 0.05$, Tukey's multiple comparison test; $n = 8$). *B*, Injection of 10 μl of hypotonic solution induced a significant number of flinches in Taxol-treated rats as compared with isotonic solution (11.6 ± 1.1, $n = 16$ for hypotonic vs 0.4 ± 0.2, $n = 8$ for isotonic solution; $p < 0.05$, unpaired Student's *t* test). The hypotonicity-induced number of flinches was significantly higher in Taxol-treated rats versus control rats (11.6 ± 1.1, $n = 16$ for Taxol-treated vs 3.2 ± 0.3, $n = 18$ for control rats; $p < 0.05$, unpaired Student's *t* test). *C*, Effect of TRPV4 antisense on the number of flinches induced by the injection of 10 μl of hypotonic solution in the hind paw of Taxol-treated rats. Spinal intrathecal administration of TRPV4 antisense decreased the number of hypotonicity-induced flinches by 42% as compared with mismatch-treated rats (6 ± 1, $n = 6$ for the antisense-treated vs 10.5 ± 1.1, $n = 8$ for the mismatch-treated rats; $p = 0.01$, unpaired Student's *t* test). At 4 d after the last ODN treatment there were no significant differences in the number of hypotonicity-induced flinches between the two ODN groups (mean ± SEM; 8.5 ± 1.2, $n = 6$ for the antisense-treated vs 9.1 ± 1.3, $n = 8$ for the mismatch-treated rats; $p > 0.05$, unpaired Student's *t* test). *D*, A doublet band (98 and 107 kDa) was detected by Western blot in the saphenous nerve of Taxol-treated rats. There was a 55% ± 17 decrease in the level of TRPV4 protein in saphenous nerve from antisense-treated versus mismatch-treated rats ($p < 0.05$, unpaired Student's *t* test; $n = 5$ for antisense and $n = 6$ for mismatch). The amount of protein in both lanes was confirmed to be comparable (16.8 μg/lane) by reprobing the membrane with an α-tubulin antibody.

To confirm the correlation between the action of the TRPV4 antisense and the decrease in the nociceptive behavioral responses, we performed Western blot analyses with an affinity-purified polyclonal anti-TRPV4 antibody, using a synthetic peptide corresponding to the C terminus of rat TRPV4 (Alessandri-Haber et al., 2003). In agreement with other reports that used the same epitope (Delany et al., 2001; Guler et al., 2002), we have reported that the level of TRPV4 expression in DRG neurons was below the detection limit of our antibody. However, we have demonstrated that, if the saphenous nerve is ligated to dam the transport of proteins from the cell body toward the periphery, TRPV4 protein can be detected in the saphenous nerve. Thus TRPV4 presumably is transported from the cell bodies toward the peripheral endings (Alessandri-Haber et al., 2003). To confirm that the time frame used for the ODN experiment was compatible with the recovery of TRPV4 protein expression level by day 4, we treated control rats with TRPV4 antisense or mismatch ODN for 3 d, and saphenous nerves were harvested either 10 hr after the last ODN injection or on day 4 and processed for Western blotting. TRPV4 protein level in the saphenous nerve was diminished significantly in the antisense- vs the mismatch-treated rats 10 hr after the last ODN injection (data not shown; $p < 0.05$, unpaired Student's t test; $n = 8$), whereas on day 4 the level of TRPV4 protein was not significantly different between antisense- vs mismatch-treated groups (data not shown; $p > 0.05$, unpaired Student's t test; $n = 5$). Western blots also were performed on saphenous nerves of Taxol-treated rats 10 hr after the last TRPV4 ODN treatment. As shown previously (Delany et al., 2001; Alessandri-Haber et al., 2003; Liedtke and Friedman, 2003; H. Xu et al., 2003), a doublet (98 and 107 kDa) was detected, suggesting the existence of N-glycosylated variants of TRPV4 protein, with a specific 55 ± 17% diminution of TRPV4 protein expression level in the saphenous nerve of antisense- vs mismatch-treated rats (Fig. 1*D*; $p < 0.05$, unpaired Student's t test; $n = 5$ for antisense and $n = 6$ for mismatch).

Taxol treatment enhances hypotonicity-induced increase in intracellular calcium in cultured nociceptors

Calcium imaging recordings were performed to support the suggestion that enhancement of osmotic nociception in Taxol-treated rats was attributable to an increase in osmotransduction specifically in sensory neurons. The experiments were performed in conditions that were less likely to activate other ions channels (see Materials and Methods), and only small-diameter (<40 μm) putative DRG nociceptors (responsive to capsaicin, 1 μM) were considered (Alessandri-Haber et al., 2003). We challenged 137 DRG nociceptors from Taxol-treated rats with 30% hypotonic solution (219 mOsm) for 3 min, followed by perfusion of isotonic solution (312 mOsm) until the concentration of free calcium ions ($[Ca^{2+}]_i$) had recovered to control level; a short exposure to KCl (20 mM) then was performed to confirm excitability of the neurons at the end of the experiment (Fig. 2*A*). As shown in Figure 2*B*, the percentage of nociceptors responsive to hypotonicity was significantly higher in Taxol-treated rats (55%, 76 of 137 neurons) than in control rats (39%, 33 of 84 neurons; $p < 0.025$, χ^2 test with Yates correction). The mean increase in fluorescence ratio in nociceptors from Taxol-treated rats was not, however, significantly different when compared with nociceptors from control rats (data not shown; $p > 0.05$, unpaired Student's t test; $n = 24$).

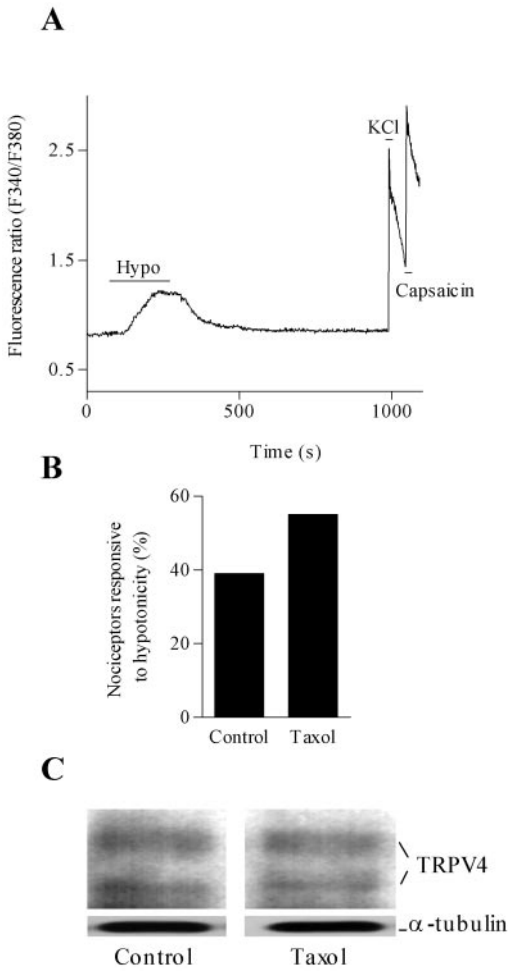

Figure 2. The enhanced osmotransduction in cultured nociceptors of Taxol-treated rats does not depend on an increase in the expression level of TRPV4 protein. *A*, Example of the hypotonicity-induced increase in fluorescence ratio in a neuron challenged with a 30% hypotonic solution (212 mOsm). The excitability of the neuron was confirmed by a short exposure to KCl (20 mM), and the neuron was identified as a putative nociceptor when capsaicin (1 μM) induced a significant increase in the fluorescence ratio. *B*, The percentage of nociceptors responding to a 30% hypotonic challenge with an increase in the fluorescence ratio in Taxol-treated rats is significantly higher than that in controls ($p < 0.025$, χ^2 test with Yates correction). *C*, There was no significant difference in the level of TRPV4 protein in saphenous nerve from control and Taxol-treated rats ($p > 0.05$, unpaired Student's t test; $n = 7$ for control and $n = 12$ for Taxol-treated rats). The amount of protein in both lanes was confirmed to be comparable by reprobing the membrane with an α-tubulin antibody.

Taxol treatment does not increase the expression level of TRPV4 in saphenous nerve

Because decreasing the level of expression of TRPV4 protein by spinal intrathecal injection of ODN reduces Taxol-induced hyperalgesia, we hypothesized that the hyperalgesia could be related to an increase in TRPV4 protein expression in Taxol-treated rats. To test that possibility, we performed Western blots on saphenous nerves of Taxol-treated and control rats (Fig. 2*C*). The level of TRPV4 protein in Taxol-treated rats was not significantly different from that in normal rats ($p > 0.05$, unpaired Student's t test; $n = 7$ for control and $n = 12$ for Taxol-treated rats). Because no change in TRPV4 expression could be detected, we hypothesized instead that TRPV4-mediated hyperalgesia could involve interactions of TRPV4 with intracellular signaling pathways.

Nociceptive behaviors in Taxol-treated rats are integrin-dependent

Many ligands that bind to integrins (e.g., laminin and fibronectin) contain the Arg-Gly-Asp (RGD) consensus sequence, and most of the known integrins recognize the RGD sequence in their adhesion protein ligands (Ruoslahti, 1996). Therefore, injection of an excess of RGD peptide can prevent the binding of integrins to their endogenous ligands. To assess whether integrins participate in the activation pathway of TRPV4-mediated nociceptive behaviors, we tested the effect of the hexapeptide GRGDTP, which contains the RGD sequence; its inactive analog GRADSP (1 μg/2.5 μl) was used as a control.

We first tested whether the injection of GRGDTP had an effect on Taxol-induced mechanical hyperalgesia. Taxol-treated rats received an injection of either GRGDTP or GRADSP in the hind paw, and nociceptive mechanical threshold was measured 30 min later (Fig. 3*A*). Injection of GRGDTP totally reversed Taxol-induced mechanical hyperalgesia (0.45 $\pm$ 2.1, $n = 6$ after vs 35.6 $\pm$ 1.7, $n = 12$ before GRGDTP; $p < 0.05$, unpaired Student's *t* test), whereas GRADSP had no significant effect on mechanical threshold (34 $\pm$ 2.1, $n = 6$ after vs 35.6 $\pm$ 1.7, $n = 12$ before GRADSP; $p > 0.05$, unpaired Student's *t* test). At 24 hr after peptide treatment there were no significant differences among control, GRGDTP-, and GRADSP-treated rats ($p > 0.05$, Tukey's multiple comparison test). So that the specificity of action of the GRGDTP peptide could be verified, the injection of GRADSP or GRGDTP also has been performed in control rats; neither had an effect on paw withdrawal threshold (Dina et al., 2004).

We then investigated whether the GRGDTP peptide had an effect on hypotonicity-induced nociceptive behavior. The flinching test was performed 45 min after the injection of either GRGDTP or GRADSP (Fig. 3*B*). GRGDTP inhibited by 50% the number of hypotonicity-induced flinches (11.6 $\pm$ 1.6, $n = 6$ before vs 5.7 $\pm$ 0.9 after GRGDTP, $n = 5$; $p < 0.05$, unpaired Student's *t* test), whereas GRADSP had no significant effect (12.6 $\pm$ 1.6 after vs 11.6 $\pm$ 1.6, $n = 6$ before GRADSP; $p > 0.05$, unpaired Student's *t* test). At 24 hr after the peptide injection there were no significant differences in the number of hypotonicity-induced flinches among the baseline, GRADSP-, or GRGDTP-treated rats ($p > 0.05$, Tukey's multiple comparison test).

Integrins contribute to the $[Ca^{2+}]_i$ response to hypotonic stimuli in sensory neurons

To test whether enhanced osmotransduction in nociceptors depends on an integrin pathway, we performed calcium imaging experiments. Nociceptors from Taxol-treated rats were challenged with a 30% hypotonic solution for 3 min and perfused with isotonic solution until $[Ca^{2+}]_i$ had recovered fully. Then the neurons were perfused for 20 min with an isotonic solution containing either GRGDTP or GRADSP peptide (50 μM) and challenged with a 30% hypotonic solution containing either GRGDTP or GRADSP for 3 min. As shown in Figure 3*C*, GRGDTP inhibited the hypotonicity-induced increase in fluorescence ratio by 88% (1.19 $\pm$ 0.05 for hypotonic alone vs 1.05 $\pm$ 0.02 in presence of GRGDTP; $p < 0.05$, paired Student's *t* test; $n = 8$), whereas the mean of fluorescence ratio during hypotonic stimulation was not significantly different in the presence or absence of GRADSP (1.08 $\pm$ 0.01 hypotonic alone vs 1.11 $\pm$ 0.02 in presence of GRADSP; $p > 0.05$, paired Student's *t* test; $n = 9$). Of note, in a control experiment we confirmed that nociceptors challenged with the same protocol but with two hypotonic challenges in-

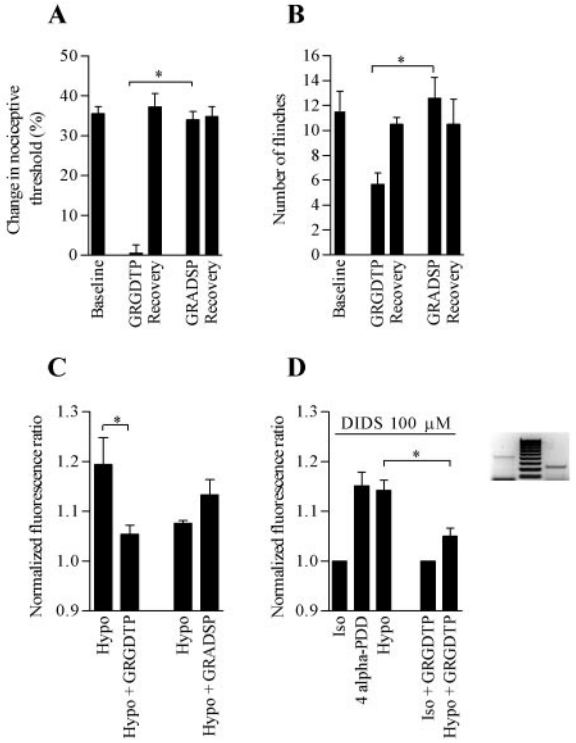

Figure 3. TRPV4-mediated enhanced nociception is integrin-dependent. *A*, Effect of integrin antagonist peptide GRGDTP or its inactive analog GRADSP (1 μg/2.5 μl) on mechanical hyperalgesia in Taxol-treated rats. Injection of GRGDTP 30 min before the evaluation of mechanical nociceptive threshold reversed Taxol-induced mechanical hyperalgesia (mean $\pm$ SEM; 0.45 $\pm$ 2.1 gm, $n = 6$ after vs 35.6 $\pm$ 1.7 gm, $n = 12$ before GRGDTP; $^*p < 0.05$, unpaired Student's *t* test), whereas GRADSP had no significant effect (mean $\pm$ SEM; 34 $\pm$ 2.1 gm, $n = 6$ after vs 35.6 $\pm$ 1.7 gm, $n = 12$ before GRADSP; $p > 0.05$, unpaired Student's *t* test). At 4 d after treatment with either GRGDTP or GRADSP peptide the mechanical threshold was not significantly different from the baseline ($p > 0.05$, Tukey's multiple comparison test). *B*, Effect of GRGDTP and its inactive analog GRADSP (1 μg/2.5 μl) on the number of hypotonicity-induced flinches in Taxol-treated rats. Injection of GRGDTP peptide 45 min before the hypotonic stimulation inhibited by 50% the number of hypotonicity-induced flinches (mean $\pm$ SEM; 5.7 $\pm$ 0.9, $n = 6$ after vs 11.6 $\pm$ 1.6, $n = 12$ before GRGDTP; $^*p < 0.05$, unpaired Student's *t* test), whereas GRADSP had no significant effect (mean $\pm$ SEM; 12.6 $\pm$ 1.6, $n = 5$ after vs 11.6 $\pm$ 1.6, $n = 12$ before GRADSP; $p > 0.05$, unpaired Student's *t* test). At 4 d after the peptide treatment there were no significant differences between the baseline number of flinches induced by hypotonicity and the number of flinches in GRGDTP- and GRADSP-treated rats ($p > 0.05$, Tukey's multiple comparison test). *C*, Mean of normalized fluorescence ratio $\pm$ SEM when nociceptors are challenged with hypotonic solution in the absence or presence of GRGDTP or GRADSP peptide (10 μM). Presence of GRGDTP during the hypotonic stimulus inhibited the hypotonicity-induced increase in fluorescence ratio by 88% (normalized fluorescence ratio was 1.19 $\pm$ 0.05 without vs 1.05 $\pm$ 0.02 in presence of GRGDTP; $n = 8$; $^*p < 0.05$, paired Student's *t* test), whereas GRADSP had no significant effect (1.08 $\pm$ 0.01 before vs 1.11 $\pm$ 0.02 after GRADSP; $n = 9$; $p > 0.05$, paired Student's *t* test). Data are reported as the fluorescence ratio amplitude of the effect of hypotonic in the absence or presence of RGD peptide normalized to the fluorescence ratio obtained in isotonic solution in the absence or presence of RGD peptide. *D*, Mean of normalized fluorescence ratio $\pm$ SEM for nociceptors stimulated by different extracellular solutions containing DIDS (100 μM). Nociceptors from control rats were challenged first with the direct TRPV4 activator 4 α-PDD, after full recovery of $[Ca^{2+}]_i$. Nociceptors were challenged with 30% hypotonic solution and perfused with isotonic solution until $[Ca^{2+}]_i$ recovery. Finally, nociceptors were perfused for 20 min with an isotonic solution containing GRGDTP (50 μM) and challenged with a 30% hypotonic solution containing GRGDTP for 3 min. Only nociceptors responding to both 30% hypotonic and 4 α-PDD stimuli were included in the graph ($n = 10$). The mean fluorescence ratio during 4 α-PDD challenge was not significantly different from the one during hypotonic (1.15 $\pm$ 0.03 during 4 α-PDD vs 1.14 $\pm$ 0.03 during hypotonic stimulation; $p > 0.05$, paired Student's *t* test; $n = 10$). The presence of GRGDTP during the hypotonic stimulation significantly inhibited the hypotonicity-induced increase in fluorescence ratio (1.14 $\pm$ 0.03 during hypotonic alone vs 1.05 $\pm$ 0.03 during hypotonic in the presence of GRGDTP; $^*p < 0.05$, paired Student's *t* test; $n = 10$). Inset, TRPV4 mRNA (428 bp) was detectable by single-cell RT-PCR in the responsive nociceptors.

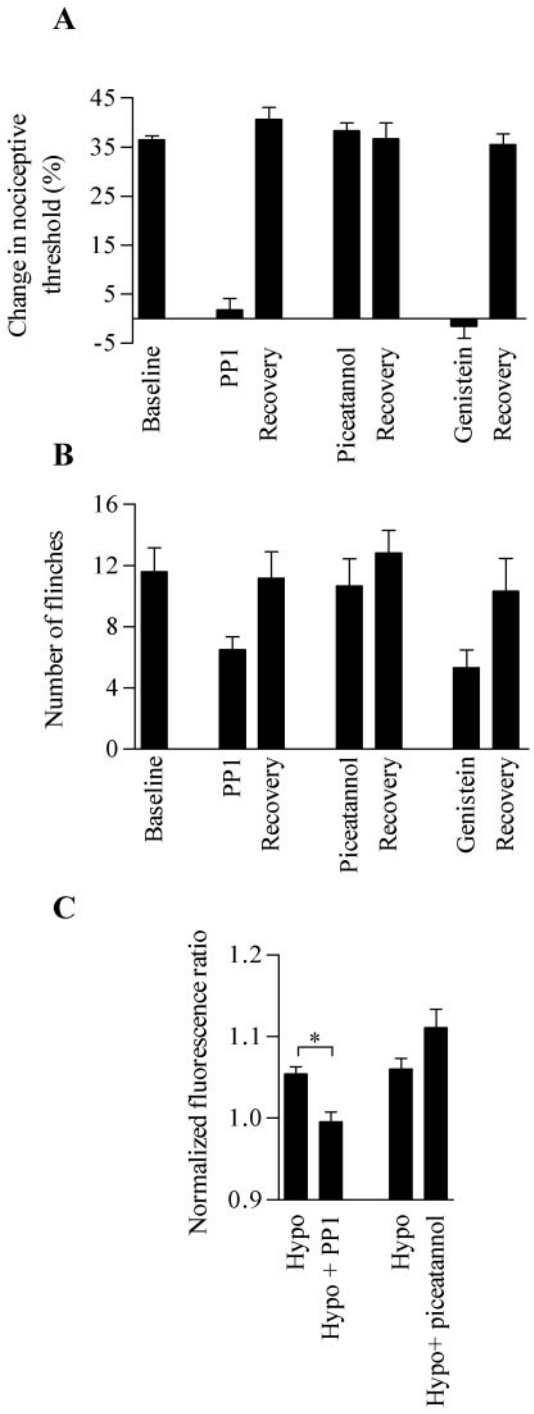

Figure 4. TRPV4-mediated enhanced nociception is Src tyrosine kinase-dependent. *A*, Effect of tyrosine kinase inhibitors on mechanical hyperalgesia in Taxol-treated rats. Injection of PP_1 (Src family kinase-specific inhibitor; 1 μg/2.5 μl) and genistein (general tyrosine kinase inhibitor; 1 μg/2.5 μl) 30 min before the evaluation of mechanical nociceptive threshold reversed Taxol-induced mechanical hyperalgesia (mean ± SEM; 1.75 ± 2.3, $n = 6$ for PP_1-treated rats; −1.61 ± 2.4, $n = 6$ for the genistein-treated rats; 36.5 ± 0.9, $n = 46$ for the baseline). Piceatannol (specific inhibitor of Syk, a related cytoplasmic tyrosine kinase; 1 μg/2.5 μl) did not have any significant effect (mean ± SEM; 38.4 ± 1.6, $n = 6$ for piceatannol-treated rats vs 36.5 ± 0.9, $n = 46$ for the baseline; $p > 0.05$, unpaired Student's *t* test). At 24 hr after inhibitor injection there were no significant differences among the three groups of rats (mean ± SEM; 11.2 ± 1.8, $n = 6$ after recovery from PP_1, 12.8 ± 1.4, $n = 6$ after piceatannol, and 10.3 ± 2.1 after recovery from genistein treatment; $p > 0.05$, Tukey's multiple comparison test). *B*, Effect of tyrosine kinase inhibitors on the number of hypotonicity-induced flinches. Injection of PP_1 or genistein in Taxol-treated rat hind paw 45 min before hypotonic stimulation inhibited the number of flinches by 44% (mean ± SEM; 6.5 ± 0.8, $n = 6$ for PP_1-treated vs 5.3 ± 1.1, $n = 6$ for the genistein-treated rats), whereas piceatannol did not significantly affect the number of flinches (mean ± SEM; 10.7 ± 1.8, $n = 6$ for piceatannol-treated vs 11.6 ± 1.6, $n = 18$ for the baseline number of flinches; $p > 0.05$, unpaired Student's *t* test). At 24 hr after the injection of inhibitors there were no significant differences in the number of flinches among the groups of rats ($p > 0.05$, Tukey's multiple comparison test). *C*, Mean of normalized fluorescence ratio for nociceptors, *in vitro*, first challenged with a 30% hypotonic solution and then challenged with a 30% hypotonic solution containing either PP_1 or piceatannol (10 μM). PP_1 prevented the hypotonicity-induced increase in fluorescence ratio (1.06 ± 0.01, $n = 5$ before vs 0.99 ± 0.01, $n = 5$ after PP_1; $^*p < 0.05$, paired Student's *t* test), whereas the addition of piceatannol did not have any effect (1.06 ± 0.01, $n = 8$ before vs 1.11 ± 0.02, $n = 8$ after piceatannol; $p > 0.05$, paired Student's *t* test).

duced two similar hypotonicity-induced increases in fluorescence ratio (data not shown).

To investigate further a possible interaction between integrin and TRPV4 function, we studied nociceptors from control rats via calcium imaging. Recordings were performed in the presence of a blocker of swelling-activated chloride channels, DIDS (100 μM). Nociceptors were challenged first with a 30% hypotonic solution for 3 min and then perfused with isotonic solution until $[Ca^{2+}]_i$ had recovered fully. Then nociceptors were challenged with the direct TRPV4 activator 4 α-PDD and perfused with isotonic solution until $[Ca^{2+}]_i$ had recovered fully. Finally, nociceptors were perfused for 20 min with an isotonic solution containing GRGDTP (50 μM) and challenged with a 30% hypotonic solution containing GRGDTP for 3 min. Only nociceptors responding to both the 30% hypotonic and 4 α-PDD stimuli were selected (10 of 41 neurons). As shown in Figure 3*D*, the mean fluorescence ratio during 4 α-PDD was not significantly different from the one during hypotonic stimulation (1.15 ± 0.03 during 4 α-PDD vs 1.14 ± 0.03 during hypotonic stimulation; $p > 0.05$, paired Student's *t* test; $n = 10$). The presence of GRGDTP during the hypotonic stimulation significantly inhibited the hypotonicity-induced increase in $[Ca^{2+}]_i$; the fluorescence ratio was 1.14 ± 0.03 during hypotonic alone versus 1.05 ± 0.03 during hypotonic in presence of GRGDTP ($p < 0.05$, paired Student's *t* test; $n = 10$). After calcium imaging the cytoplasm of 12 cells was harvested (5 responsive and 7 nonresponsive to both stimuli), and RT-PCR was performed. As shown in Figure 3*D*, inset, TRPV4 expression was detectable in the responsive nociceptors (5 of 5), but not in the nonresponsive ones (TRPV4 mRNA was detected in only 1 of 7 neurons), whereas the housekeeping gene GAPDH mRNA was detectable in all 12 cells.

Nociceptive behaviors in Taxol-treated rats depend on Src tyrosine kinase

Interaction between the extracellular matrix and integrins directly activates protein kinase cascades. The best-characterized integrin-mediated signaling pathway involves cytoplasmic tyrosine kinase (FAK or Src) cascades. Interestingly, it has been shown that hypotonicity-induced activation of TRPV4 *in vitro* depends on Src tyrosine kinase phosphorylation (H. Xu et al., 2003). To investigate whether TRPV4-mediated enhanced nociception depends on Src tyrosine kinase phosphorylation, we administered Taxol-treated rats an injection in the hind paw of PP_1 (Src family kinase specific inhibitor; 1 μg/2.5 μl), piceatannol (specific inhibitor of Syk, a related protein tyrosine kinase not belonging to the Src family; 1 μg/2.5 μl), or genistein (nonspecific tyrosine kinase inhibitor; 1 μg/2.5 μl).

We first investigated the effect of the kinase inhibitors on the Taxol-induced mechanical hyperalgesia (Fig. 4*A*). Injection of PP_1 in the hind paw 30 min before mechanical stimulation inhib-

ited this mechanical hyperalgesia by 95% (1.75 ± 2.3 gm, $n = 6$ after PP_1 vs 36.5 ± 0.9 gm, $n = 46$ for baseline; $p < 0.05$, unpaired Student's t test). Similar results were obtained with genistein (−1.61 ± 2.4 gm, $n = 6$ for genistein-treated vs 1.75 ± 2.3 gm, $n = 6$ for PP_1-treated rats; $p > 0.05$, unpaired Student's t test). Injection of piceatannol, however, did not have a significant effect (38.4 ± 1.6 gm, $n = 6$ before vs 36.5 ± 0.9 gm, $n = 46$ after piceatannol; $p > 0.05$, unpaired Student's t test). At 24 hr after administration of the inhibitors there were no significant differences among the groups of rats ($p > 0.05$, Tukey's multiple comparison test). Of note, the injection of PP_1, piceatannol, or genistein alone did not affect the mechanical threshold in normal rats ($n = 6$ for each group; data not shown).

We then investigated the effect of these inhibitors on hypotonicity-induced nociceptive behavior (Fig. 4*B*). Hypotonic solution was injected in the hind paw 45 min after the injection of the inhibitor at the same site. PP_1 inhibited by 44% the number of hypotonicity-induced flinches (6.5 ± 0.8, $n = 6$ after vs 11.6 ± 1.6, $n = 18$ before PP_1 treatment; $p < 0.05$, unpaired Student's t test). Similar results were obtained with genistein (6.5 ± 0.8, $n = 6$ for the PP_1-treated vs 5.3 ± 1.1, $n = 6$ for the genistein-treated rats; $p > 0.05$, ANOVA), whereas piceatannol had no significant effect (10.7 ± 1.8, $n = 6$ after vs 11.6 ± 1.6, $n = 18$ before piceatannol; $p > 0.05$, unpaired Student's t test). At 24 hr after administration of the inhibitors there were no significant differences in the number of hypotonicity-induced flinches among the three groups of rats (11.6 ± 1.6, $n = 18$ for baseline vs 11.2 ± 1.8, $n = 6$ after recovery from PP_1; 12.8 ± 1.4, $n = 6$ from piceatannol; 10.3 ± 2.1, $n = 6$ from genistein treatment; $p > 0.05$, Tukey's multiple comparison test).

Response to hypotonic stimuli in sensory neurons depends on Src kinase phosphorylation

Finally, we investigated whether the enhanced response to hypotonic stimulation in cultured nociceptors from Taxol-treated rats also depends on Src tyrosine kinase phosphorylation (Fig. 4*C*). Nociceptors were challenged with a 30% hypotonic solution for 3 min and perfused with isotonic solution until $[Ca^{2+}]_i$ had recovered fully to basal level. An isotonic solution containing either PP_1 or piceatannol (10 μM) was perfused for 15 min, and nociceptors were challenged with a 30% hypotonic solution containing either PP_1 or piceatannol for 3 min. The presence of PP_1 prevented the hypotonicity-induced increase in fluorescence ratio (1.06 ± 0.01, $n = 5$ during hypotonic stimulation vs 0.99 ± 0.01, $n = 5$ during hypotonic in presence of PP1; $p < 0.05$, paired Student's t test), whereas the addition of piceatannol did not have any effect. The normalized fluorescence ratio during the hypotonic challenge in the presence of piceatannol (1.06 ± 0.01, $n = 8$) was not significantly different from the one during the hypotonic challenge alone (1.11 ± 0.02; $n = 8$ after piceatannol; $p > 0.05$, paired Student's t test).

Discussion

We recently demonstrated a contribution of TRPV4 in inflammatory pain (Alessandri-Haber et al., 2003); here we investigated whether TRPV4 also plays a role in neuropathic pain. We demonstrate that TRPV4 plays a major role in Taxol-induced mechanical hyperalgesia and that it also contributes to enhanced nociception to hypo-osmotic stimuli in Taxol-treated rats. The role of TRPV4 in Taxol-induced enhanced nociception is not attributable to an increase in its level of protein expression; therefore, we investigated whether its role was attributable to a specific interaction with second messenger pathways.

The integrin antagonist hexapeptide GRGDTP inhibited Taxol-induced enhanced nociception to the same extent as TRPV4 antisense. Our results *in vitro* also support the suggestion that TRPV4 activation can depend on an integrin pathway not only in Taxol-treated but also in control rats. Thus our results are compatible with the suggestion that TRPV4 can be activated by mechanical or osmotic stimulation via an integrin-dependent pathway.

In other cell types the interaction between the extracellular matrix and integrins directly activates members of various downstream protein kinase cascades (Schwartz et al., 1995; Parsons, 1996), often the cytoplasmic tyrosine kinases (FAK or Src). Furthermore, integrins have been proposed as osmotransducers in hepatocytes as part of an integrin/actin/Src/MAPK/microtubule signaling pathway (vom Dahl et al., 2003), and TRPV4 is expressed in liver (Strotmann et al., 2000; Delany et al., 2001). Interestingly, *in vitro* hypotonic stimulation results in rapid tyrosine phosphorylation of TRPV4, and this phosphorylation is sensitive to the inhibitor PP1 in a dose-dependent manner, but not to piceatannol (H. Xu et al., 2003). Here we demonstrate that TRPV4-mediated nociceptive behaviors in Taxol-treated rats as well as the hypotonicity-induced increase in $[Ca^{2+}]_i$ in nociceptors *in vitro* were inhibited markedly by the Src-specific inhibitor PP_1 and unaffected by piceatannol.

We show here and in our previous study (Alessandri-Haber et al., 2003) that TRPV4 is one of the major candidates involved in the hypotonicity-induced nociceptive behavior, but we do not rule out the possible contribution of other conductances such as the swelling-activated chloride channels ($I_{Clswell}$) or the voltage-gated calcium channels. Such conductances also might interact with the Src tyrosine kinase pathway (Voets et al., 1998; Weiss and Burgoyne, 2001; Shi et al., 2002; Wijetunge et al., 2002). However, our data suggest that TRPV4 is the predominant mediator of the observed hyperalgesia. Thus the hypotonicity-induced increase in the fluorescence ratio in nociceptors in the presence or absence of DIDS ($I_{Clswell}$ blocker) is not significantly different (Fig. 3*C*,*D*). Also, injection of PP1 reduced the hypotonicity- and mechanical-induced nociceptive behaviors to the same extent as treatment with TRPV4 antisense. We conclude that TRPV4 contributes to enhanced nociception in Taxol-treated rats, and we suggest that one pathway for the activation of TRPV4 in primary afferent nociceptors depends on integrin/Src kinase phosphorylation in the rat.

Recently, it has been shown *in vitro* that hypotonic stimulation did not induce a Src tyrosine kinase phosphorylation of TRPV4 but required instead the activity of phospholipase A2 (Vriens et al., 2004). This does not contradict our findings or those of H. Xu et al. (2003) but, rather, suggests that TRPV4 as a polymodal receptor integrating a variety of different stimuli is versatile in its gating properties (Nilius et al., 2004). Different stimuli (i.e., hypotonicity, heat, and chemical) activating TRPV4 via distinct pathways (Vriens et al., 2004) do not exclude that a stimulus (i.e., hypotonicity) may activate TRPV4 via several pathways.

It was suggested recently that microtubule-associated protein 7 (MAP7) links TRPV4 to actin filaments and thus lowers its threshold to mechanical stimuli (Suzuki et al., 2003b). In addition, TRPV4 requires its N-terminal domain, which contains three ankyrin repeats, for its activation in response to hypotonicity (Liedtke et al., 2000) and heat (Watanabe et al., 2002b), and ankyrin repeats can anchor the channel to the cytoskeleton. These findings and our current results suggest a possible activation of

TRPV4 via interactions with extracellular matrix signaling pathways as well as with the actin–microtubule complex.

Whereas TRPV4 antisense ODN totally abolished Taxol-induced mechanical hyperalgesia, we have shown previously that TRPV4 antisense does not cause a significant change in either the baseline mechanical nociceptive threshold or the PGE_2-induced mechanical hyperalgesia in normal rats (Alessandri-Haber et al., 2003). We suggest four possible explanations for the differential role of TRPV4 in Taxol-induced versus PGE_2-induced mechanical hyperalgesia.

First, it could be attributable to differences in the mechanisms responsible for the induced mechanical hyperalgesia. PGE_2-induced mechanical hyperalgesia is independent of the integrity of the cytoskeleton (Dina et al., 2003), whereas Taxol-induced hyperalgesia is dependent on the cytoskeleton (Dina et al., 2001). Taxol binds to microtubules and suppresses microtubule dynamics (Manfredi and Horwitz, 1984; Jordan et al., 1993), supporting the idea of interaction between TRPV4 and the actin–microtubule complex (Suzuki et al., 2003b).

Second, the differential role of TRPV4 in Taxol-induced versus PGE_2-induced mechanical hyperalgesia could be attributable to the activation of different second messenger pathways. PGE_2-induced hyperalgesia depends in part on PKA and on 5- and 12-lipoxygenase products of arachidonic acid metabolism (Aley and Levine, 2003). In contrast, Taxol-induced mechanical hyperalgesia involves both PKCε and PKA second messenger pathways (Dina et al., 2001). The 5- and 12-lipoxygenase pathways do not activate TRPV4 *in vitro* (Watanabe et al., 2003), whereas integrin activation results in PKC translocation to cell membrane (Wrenn and Herman, 1995) and PKC can interact with the Src family kinases (Song et al., 1998; Chang et al., 2002). Furthermore, PKC is involved in TRPV4 activation *in vitro* (Gao et al., 2003; F. Xu et al., 2003).

Third, the differential role of TRPV4 in the Taxol-induced versus the PGE_2-induced mechanical hyperalgesia may be attributable to an indirect effect of Taxol to increase tyrosine phosphorylation of MAP kinase (Ding et al., 1993). More specifically, it has been reported that Lyn kinase is activated in Taxol-treated macrophages (Henricson et al., 1995). Interestingly, F. Xu et al. (2003) showed in HEK cells transfected with TRPV4 that Src family tyrosine kinases, especially Lyn, were activated by hypotonic stress and phosphorylated TRPV4 by direct interaction. Thus Taxol might sensitize TRPV4 via the integrin/Src/Lyn pathway, lowering its activation threshold to different stimuli.

Finally, the differential role of TRPV4 in the Taxol-induced versus the PGE_2-induced mechanical hyperalgesia also could be attributable to the interaction with other accessory proteins, as suggested for other TRP family members thought to be involved in mechanotransduction, such as *nan* (Kim et al., 2003), NOMPC (Walker et al., 2000), and *Osm-9* (Colbert et al., 1997; Tobin et al., 2002; Liedtke et al., 2003).

TRPV4 is a transducer of osmotic and mechanical stimuli that contributes to enhanced nociception in neuropathic pain. Its contribution to pathological pain rather than normal mechanical sensation can be compared with the role of another member of the TRPV family, TRPV1, which contributes to noxious heat detection and was demonstrated to be essential for inflammatory thermal hyperalgesia (Davis et al., 2000), but not for normal heat sensation (Caterina et al., 2000). Our understanding of TRPV4 function in nociception is limited still by the complexity of its polymodality, and crucial questions remain. The essential contribution of TRPV4 in chemotherapy-induced neuropathic and inflammatory pain (Alessandri-Haber et al., 2003) makes it a novel target for the development of a new class of analgesics.

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