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The Glutamatergic Nature of TRPV1-Expressing Neurons in the Spinal Dorsal Horn

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Abstract

The transient receptor potential vanilloid receptor 1 (TRPV1) is expressed on primary afferent terminals and spinal dorsal horn neurons. However, the neurochemical phenotypes and functions of TRPV1-expressing postsynaptic neurons in the spinal cord are not clear. In this study, we tested the hypothesis that TRPV1-expressing dorsal horn neurons are glutamatergic. Immunocytochemical labeling revealed that TRPV1 and vesicular glutamate transporter-2 were colocalized in dorsal horn neurons and their terminals in the rat spinal cord. Resiniferatoxin (RTX) treatment or dorsal rhizotomy ablated TRPV1-expressing primary afferents but did not affect TRPV1- and vesicular glutamate transporter-2-expressing dorsal horn neurons. Capsaicin significantly increased the frequency of glutamatergic sEPSCs and mEPSCs in almost all the lamina II neurons tested in control rats. In RTX-treated or dorsal rhizotomized rats, capsaicin still increased the frequency of sEPSCs and mEPSCs in the majority of neurons examined, and this effect was abolished by a TRPV1 blocker or by non-NMDA receptor antagonist. In RTX-treated or in dorsal rhizotomized rats, capsaicin also produced an inward current in a subpopulation of lamina II neurons. However, capsaicin had no effect on GABAergic and glycinergic sIPSCs of lamina II neurons in RTX-treated or dorsal rhizotomized rats. Collectively, our study provides new histological and functional evidence that TRPV1-expressing dorsal horn neurons in the spinal cord are glutamatergic and that they mediate excitatory synaptic transmission. This finding is important to our understanding of the circuitry and phenotypes of intrinsic dorsal horn neurons in the spinal cord.

Introduction

The capsaicin receptor transient receptor potential vanilloid type 1 (TRPV1) is a nonselective cation channel that can be activated by many physical and chemical stimuli, including heat, protons, capsaicin, and anandamide (Caterina et al. 1997; Tominaga et al. 1998; Szallasi and Blumberg 1999). TRPV1 is highly expressed in small- and medium-sized dorsal root ganglion (DRG) neurons and is located along the entire length of these sensory neurons from the peripheral nerve endings to the central terminals in the spinal cord. Stimulation of TRPV1 elicits the release of glutamate (Yang et al. 1998; Pan and Pan 2004) and neuropeptides (Gamse et al. 1979; Huang et al. 2002) from primary afferent terminals. TRPV1 plays an essential role in detecting noxious thermal stimuli. For example, thermal sensitivity is impaired in TRPV1-knockout mice (Caterina et al. 2000) and in rats in which

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TRPV1-expressing DRG neurons have been ablated by the administration of resiniferatoxin (RTX) (Pan et al. 2003; Chen and Pan 2006). TRPV1-mediated thermal sensitivity is enhanced by inflammatory mediators, such as bradykinin, nerve growth factor, and prostaglandins (Premkumar and Ahern 2000; Chuang et al. 2001; Schnitzler et al. 2008).

In addition to its strong expression on primary afferent terminals in the superficial dorsal horn, TRPV1 immunoreactivity is also present in neurons in the central nervous system, including the spinal dorsal horn (Valtschanoff et al. 2001; Chen and Pan 2006), hippocampus (Gibson et al. 2008), and hypothalamus (Li et al. 2004). However, the neurochemical phenotype and functional role of TRPV1-expressing dorsal horn neurons have not been studied previously. Dorsal horn neurons that express TRPV1 may play a role in pain transmission at the spinal level. For example, oral administration of a TRPV1 antagonist with greater CNS penetration is much more effective in blocking tissue inflammation-induced pain than a TRPV1 blocker with poor CNS penetration (Cui et al. 2006). Interestingly, the same two compounds effectively block nociception with comparable potency when administered intrathecally to the spinal cord (Cui et al. 2006). Therefore, TRPV1-expressing neurons may be involved in the relay and transmission of nociceptive input in the spinal dorsal horn. In this study, we tested the hypothesis that dorsal horn neurons that express TRPV1 are glutamatergic in nature and mediate excitatory synaptic transmission. Our findings are essential for understanding the phenotypes of dorsal horn neurons and spinal dorsal horn circuitry involved in nociceptive transmission.

Materials and Methods

Animals

All surgical preparation and experimental protocols were approved by the Animal Care and Use Committee of the University of Texas M. D. Anderson Cancer Center and conformed to the National Institutes of Health's guidelines for the ethical use of animals. Male Sprague-Dawley rats (4 weeks old; Harlan, Indianapolis, IN) were used in this study. Rats received a single intraperitoneal injection of RTX (200 µg/kg; LC Laboratories, Woburn, MA) while under 2–3% isoflurane anesthesia (Chen and Pan 2006). RTX is an ultrapotent TRPV1 agonist that can selectively ablate TRPV1-expressing primary afferent neurons in adult rats upon systemic administration (Pan et al. 2003; Chen and Pan 2006). RTX was dissolved in a mixture of 10% Tween 80 and 10% ethanol in normal saline (vehicle). Rats in the control group received intraperitoneal injection of vehicle alone. The final electrophysiological experiments were conducted 4 weeks after RTX and vehicle injections.

A separate group of rats was subjected to dorsal rhizotomy to remove primary afferent terminals in the spinal cord. Under 2–3% isoflurane anesthesia, the left lumbar spinal cord was exposed by laminectomy, and the L3–L5 dorsal roots on the left side were lifted and cut. After root transection, the wound was sutured in two layers (muscle and skin). In sham-operated rats, we performed a similar procedure but did not transect the dorsal roots. The final electrophysiological experiments were conducted 5–7 days after surgery.

Double immunofluorescence labeling of TRPV1 and VGluT₂

To determine the neurochemical phenotype of TRPV1-expressing neurons in the spinal dorsal horn, we used vesicular glutamate transporter-2 (VGluT₂) as the cellular marker for glutamatergic neurons (Ponzio et al. 2006; Brumovsky et al. 2007). Doubling immunofluorescence labeling of TRPV1 and VGluT₂ in the spinal cord was performed on samples from three vehicle-, three RTX-treated-rats (4 weeks after the treatment), and two dorsal rhizotomized rats (one week after the surgery). Under deep anesthesia with sodium pentobarbital (60 mg/kg, administered intraperitoneally), rats were perfused intracardially

with 250 ml of saline and 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS) and 10% sucrose in PBS (pH 7.4). The lumbar segment of the spinal cord was removed quickly and postfixed in the same fixative solution. The spinal cord sections were cut to 25 μ m and collected free floating in 0.1 M PBS. Sections were rinsed in Tris-HCl buffer (TBS, pH 7.4) and incubated with 1% H₂O₂ in TBS for 30 min to quench the endogenous peroxidase. Sections were blocked with 5% blocking reagent (PerkinElmer Life Sciences, Inc., Boston, MA) in 0.1 M Tris-HCl (TNB) for 1 hour at room temperature. Then the sections were incubated with the primary antibody mixture: guinea pig anti-TRPV1 (dilution 1:1000; Neuromics, Edina, MN) and mouse anti-VGluT₂ (dilution 10 μ g/ml, NeuroMab, Davis, CA) diluted in TNB for 2 hours at room temperature for 48 hours at 4EC. Subsequently, sections were rinsed in TBS and incubated with the secondary antibody mixture: Alexa Fluor-594-conjugated goat anti-guinea pig IgG (dilution 1:200; Molecular Probes, Eugene, OR) and peroxidase-conjugated goat anti-mouse IgG (dilution: 1:100; Jackson ImmunoResearch Laboratories, Inc.) for 2 hours at room temperature. Then the sections were rinsed and incubated with fluorescein tyramide (dilution: 1:100; PerkinElmer Life Sciences, Inc.) for 10 min.

Double immunofluorescence labeling of TRPV1 and IB₄

For double fluorescence labeling of TRPV1 and isolectin B₄ (IB₄), the sections were cut to 25 μ m for the spinal cord and to 30 μ m for the DRG. Sections were blocked with 4% normal goat serum and incubated with the primary antibody (dilution 1:1000; guinea pig anti-TRPV1, Neuromics, Edina, MN) for 2 hours at room temperature and overnight at 4EC. Subsequently, sections were rinsed in TBS and incubated with the secondary antibody (Alexa Fluor-488-conjugated goat anti-guinea pig IgG, dilution 1:200, Molecular Probes, Eugene, OR) for 2 hours at room temperature. The sections were rinsed and incubated with IB₄-Alexa Fluor-594 for 2 hours at room temperature. Finally the sections were rinsed, mounted on slides, dried, and coverslipped. The negative controls were prepared by omitting the primary antibody. The sections were examined on a confocal microscope (Carl Zeiss, Jena, Germany) and the areas of interest were photodocumented.

Spinal cord slice preparation

Under 2–3% isoflurane anesthesia, the lumbar segment of the spinal cord was removed through laminectomy. The spinal tissue was immediately placed in ice-cold sucrose artificial cerebrospinal fluid (aCSF) presaturated with 95% O₂ and 5% CO₂. The sucrose aCSF contained (in mM) 234 sucrose, 3.6 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 1.2 NaH₂PO₄, 12.0 glucose, and 25.0 NaHCO₃. The tissue was then placed in a shallow groove formed in a gelatin block and glued onto the stage of a vibratome (Technical Products International, St. Louis, MO). Transverse spinal cord slices (400 μ m) were cut in the ice-cold sucrose aCSF and preincubated in Krebs solution oxygenated with 95% O₂ and 5% CO₂ at 34EC for at least 1 hour before they were transferred to the recording chamber. The Krebs solution contained (in mM) 117.0 NaCl, 3.6 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 1.2 NaH₂PO₄, 11.0 glucose, and 25.0 NaHCO₃.

Electrophysiological recordings

Recordings of postsynaptic currents were performed using the whole-cell voltage-clamp method, as we described previously (Pan and Pan 2004; Zhou et al. 2007). The spinal cord slice was placed in a glass-bottomed chamber (Warner Instruments, Hamden, CT) and fixed with parallel nylon threads supported by a U-shaped stainless steel weight. The slice was continuously perfused with Krebs solution at 5.0 ml/min at 34EC maintained by an inline solution heater and a temperature controller (TC-324; Warner Instruments). The lamina II is identified as a distinct translucent band across the superficial dorsal horn under a microscope with transmitted illumination. Lamina II neurons in the spinal slice were identified using a

fixed-stage microscope (BX50WI; Olympus, Tokyo, Japan) with differential interference contrast/infrared illumination. The electrode for the whole-cell recordings was pulled from borosilicate glass capillaries with a puller (P-97; Sutter Instruments, Novato, CA). Excitatory postsynaptic currents (EPSCs) were recorded at a holding potential of -60 mV with an electrode (impedance was $5\text{--}8$ M Ω) filled with the following internal solution (in mM): 135.0 potassium gluconate, 5.0 TEA, 2.0 MgCl₂, 0.5 CaCl₂, 5.0 HEPES, 5.0 EGTA, 5.0 ATP-Mg, 0.5 Na-GTP and 10 lidocaine N-ethyl bromide (QX314); the solution was adjusted to pH 7.2 to 7.4 with 1 M KOH (290–300 m Ω sm). To record miniature EPSCs (mEPSCs), 1 μ M tetrodotoxin (TTX) was added to the bath perfusion solution.

Inhibitory postsynaptic currents (IPSCs) were recorded at a holding potential of 0 mV using a pipette electrode (the impedance was $3\text{--}5$ M Ω) filled with internal solution containing (in mM) 110 Cs₂SO₄, 5 TEA, 2.0 MgCl₂, 0.5 CaCl₂, 5.0 HEPES, 5.0 EGTA, 5.0 ATP-Mg, 0.5 Na-GTP and 10 lidocaine N-ethyl bromide; adjusted to pH 7.2–7.4 with 1 M CsOH (290–300 10 mOsm). IPSCs were recorded in the presence of 3 mM kynurenic acid, an ionotropic glutamate receptor antagonist (Hori et al. 1992). Recordings of EPSCs or IPSCs began about 6 min after whole-cell access was established and the current reached a steady state. The access resistance was monitored, and the recording was abandoned if it changed by more than 15%. Signals were recorded using an amplifier (MultiClamp 700B; Axon Instruments, Union City, CA), filtered at 1–2 kHz, digitized at 10 kHz, and stored in a computer.

Capsaicin, capsazepine, kynurenic acid, and CNQX were obtained from Sigma-Aldrich (St. Louis, MO). TTX and lidocaine N-ethyl bromide were obtained from Alomone Labs (Jerusalem, Israel). Drugs were dissolved in the Krebs solution and perfused into the slice chamber by using syringe pumps.

Data analysis

Data are presented as means $\pm$ S.E.M. The sEPSCs, mEPSCs, and sIPSCs were analyzed off-line using a peak detection program (MiniAnalysis; Synaptosoft, Decatur, GA). Measurements of the amplitude and frequency of sEPSCs, mEPSCs, and sIPSCs were performed over a period of at least 2 min during the control and capsaicin application. The sEPSCs, mEPSCs, and sIPSCs were detected by the fast rise time of the signal over an amplitude threshold (typically 6–8 pA) above the background noise. We manually excluded the event when the noise was erroneously identified as the EPSCs or IPSCs by the software program. In each of the neurons tested, the cumulative probability of the amplitude and the inter-event interval of EPSCs or IPSCs were compared using the Kolmogorov-Smirnov test, which estimates the probability if the two cumulative distributions are statistically different. This test was used first to determine whether the capsaicin effect on EPSCs and IPSCs was significantly different in individual neurons. To compare the difference between groups, the peak effect of capsaicin on the frequency and amplitude of sEPSCs, mEPSCs, and sIPSCs was analyzed over a period of 2 min and used for the comparison of the capsaicin effect with the baseline value. Statistical differences were determined by using Wilcoxon paired test or Kruskal-Wallis test (between 3 groups). $P < 0.05$ was considered statistically significant.

Results

Thermal nociception was measured by a radiant heat stimulus to the hindpaw in vehicle- and RTX-treated rats (Pan et al. 2003; Chen and Pan 2006). The nociceptive withdrawal latency was not significantly altered by vehicle treatment (9.46 ± 1.42 s vs. 9.52 ± 1.36 s of pretreatment) 4 weeks after injection. By contrast, profound impairment in thermal nociception was detected in RTX-treated rats (26.36 ± 2.31 s vs. 9.53 ± 1.39 s of pretreatment; $P < 0.05$). Furthermore, all rats subjected to unilateral dorsal rhizotomy failed to respond to noxious pinch and heat stimulation of the ipsilateral hindpaw.

Effects of RTX treatment or dorsal rhizotomy on TRPV1- and IB₄-labeling in the DRG and spinal dorsal horn

In vehicle-treated rats, TRPV1 immunoreactivity and IB₄ labeling were present in small- and medium-sized DRG neurons. IB₄ was colocalized with TRPV1 in the majority of DRG neurons (Fig. 1A). In RTX-treated rats, TRPV1-immunoreactive cell bodies were largely absent in all the DRG sections examined. Numbers of IB₄-positive neurons were also substantially reduced in DRG sections from RTX-treated rats (Fig. 1A). Quantitative analysis of the effect of RTX treatment on TRPV1- and IB₄-positive DRG neurons was reported in our previous study (Chen and Pan 2006).

In vehicle-treated and sham-operated rats, TRPV1-immunoreactive and IB₄-positive terminals were present in the superficial dorsal horn of the spinal cord (Fig. 1B). TRPV1 immunoreactivity was especially dense at the dorsal root entry zone and in the superficial laminae. TRPV1 immunoreactivity appeared to be largely colocalized on the same terminals labeled with IB₄ in the laminae I and II of these control rats. Notably, there was a subpopulation of neurons that were TRPV1 immunoreactive in the spinal dorsal horn of vehicle-treated and sham-operated control rats (Fig. 1B). In RTX-treated or dorsal rhizotomized rats, the TRPV1 immunoreactive terminals were largely depleted in the superficial dorsal horn. IB₄-positive terminals were also substantially reduced in the superficial dorsal horn of RTX-treated rats or those subjected to dorsal rhizotomy (Fig. 1B). Similar to the results of our previous study (Chen and Pan 2006), the presence of TRPV1-immunoreactive dorsal horn neurons in the spinal cord was not affected by RTX treatment or dorsal rhizotomy.

Effect of RTX or dorsal rhizotomy on TRPV1- and VGluT₂-labeling in the spinal cord

VGluT₂ is a specific marker for the glutamatergic neurons in the spinal cord, which are not reduced by peripheral nerve transection and dorsal rhizotomy (Alvarez et al. 2004; Brumovsky et al. 2007). Negative controls omitting the primary antibody did not result in detectable labeling. In vehicle-treated or sham-operated rats, TRPV1- and VGluT₂-immunoreactive neurons and the terminals were present in the spinal dorsal horn (Fig. 2A). All TRPV1-immunoreactive neurons were colocalized with VGluT₂ in the laminae I and II of control rats.

In RTX-treated or dorsal rhizotomized rats, TRPV1-immunoreactive terminals located in the dorsal root entry zone were eliminated. However, the TRPV1- and VGluT₂-immunoreactive neurons in the spinal dorsal horn were still present in RTX-treated or dorsal rhizotomized rats (Fig. 2B). Notably, VGluT₂-expressing neurons became more readily visible in RTX-treated and dorsal rhizotomized rats after chemical or surgical removal of primary afferent terminals in the superficial dorsal horn. These data suggest that TRPV1-expressing postsynaptic dorsal horn neurons are glutamatergic neurons.

Effect of capsaicin on glutamatergic sEPSCs in the spinal cord of RTX-treated rats

We then conducted electrophysiological studies to determine whether TRPV1-expressing dorsal horn neurons are glutamatergic in the spinal cord. We first examined the effect of the TRPV1 agonist capsaicin on glutamatergic sEPSCs of lamina II neurons in spinal cord slices from vehicle- and RTX-treated rats. In vehicle-treated rats, bath perfusion of 2 μ M capsaicin for 3 min significantly increased the frequency of sEPSCs in 12 of 13 (92.3%) neurons (Fig. 3). The cumulative probability analysis of sEPSCs revealed that the distribution pattern of the inter-event interval of sEPSCs was shifted toward the left in response to capsaicin (Fig. 3). In 11 of these 12 (91.7%) neurons, capsaicin also significantly increased the amplitude of sEPSCs and shifted the distribution pattern of the amplitude of sEPSCs toward the right (Fig. 3).

In RTX-treated rats, bath perfusion of 2 μ M capsaicin significantly increased the frequency of sEPSCs from 5.9 ± 0.7 to 12.3 ± 0.7 Hz in 17 of 23 (73.9%) neurons (Fig. 4). However, capsaicin significantly increased the amplitude of sEPSCs in only 6 of these 17 (35.3%) neurons (Fig. 4B). In the remaining 6 neurons, capsaicin had no significant effect on the frequency (4.7 ± 1.1 vs. 4.5 ± 1.3 Hz) or amplitude (19.6 ± 3.5 vs. 20.2 ± 3.6 pA) of sEPSCs. Bath perfusion of the non-NMDA receptor antagonist CNQX (10 μ M) abolished sEPSCs and the effect of capsaicin on sEPSCs of all 9 lamina II neurons tested in RTX-treated rats (Fig. 4A). Furthermore, capsaicin failed to significantly change sEPSCs in the presence of 20 μ M capsazepine, a selective TRPV1 receptor antagonist (Yang et al. 1998; Szallasi and Blumberg 1999), in any of 8 neurons tested from RTX-treated rats (Fig. 4C).

Effect of capsaicin on sEPSCs in the spinal cord of dorsal rhizotomized rats

We then examined the effect of capsaicin on glutamatergic sEPSCs of lamina II neurons in spinal cord slices from which the primary afferent terminals had been surgically removed by dorsal rhizotomy. We have shown that RTX treatment depletes ~97% of TRPV1-expressing DRG neurons (Chen and Pan 2006). Because TRPV1-expressing primary afferent neurons are not completely removed by RTX, we used dorsal rhizotomy as an alternative approach to remove primary afferent terminals in the spinal cord.

Bath application of 2 μ M capsaicin for 3 min significantly increased the frequency of sEPSCs in all 13 lamina II neurons in the spinal cord from sham-operated rats (Fig. 5A). In 12 of these 13 (92.3%) neurons, capsaicin significantly increased the amplitude of sEPSCs (Fig. 5A). In rats subjected to dorsal rhizotomy, bath perfusion of 2 μ M capsaicin for 3 min significantly increased the frequency of sEPSCs from 8.2 ± 1.0 to 16.2 ± 1.4 Hz in 23 of 36 neurons (63.9%, Fig. 5B). However, capsaicin significantly increased the amplitude of sEPSCs (from 19.7 ± 1.0 to 30.1 ± 1.9 pA) in only 12 of these 23 neurons (52.2%, Fig. 5B). In the remaining 13 neurons, capsaicin did not significantly alter the frequency (11.0 ± 1.5 vs. 10.4 ± 1.6 Hz) or amplitude (22.5 ± 1.8 vs. 21.9 ± 2.1 pA) of sEPSCs. Bath perfusion of capsaicin failed to significantly change sEPSCs in the presence of the non-NMDA receptor antagonist CNQX (10 μ M) in any of 9 neurons from dorsal rhizotomized rats (data not shown). Also, capsaicin failed to affect sEPSCs in the presence of the TRPV1 antagonist capsazepine (20 μ M) in another 9 neurons tested from dorsal rhizotomized rats (Fig. 5C). Because capsaicin can rapidly desensitize TRPV1, capsazepine was administered before capsaicin application in these experiments.

Effect of capsaicin on mEPSCs in the spinal cord of RTX-treated rats

To determine whether TRPV1 is also expressed on the terminals of glutamatergic dorsal horn neurons, we examined the effect of capsaicin on the quantal (action potential-independent) glutamate release in the spinal cord of RTX-treated rats. Bath perfusion of 2 μ M capsaicin for 3 min significantly increased the frequency of recorded mEPSCs in all 16 neurons in the spinal cord of vehicle-treated rats (Fig. 6B). The cumulative probability analysis of mEPSCs revealed that the distribution pattern of the inter-event interval of mEPSCs was shifted toward the left in response to capsaicin (Fig. 6A). In 15 of these 16 (93.8%) neurons, capsaicin significantly increased the amplitude of mEPSCs (Fig. 6B).

In RTX-treated rats, bath perfusion of 2 μ M capsaicin for 3 min significantly increased the frequency of mEPSCs in 27 of 33 (81.8%) neurons (Fig. 6D). However, capsaicin significantly increased the amplitude of mEPSCs in only 13 of these 27 (48.1%) neurons studied (Fig. 6D). In the remaining 6 neurons, capsaicin did not significantly alter the frequency (3.2 ± 1.0 vs. 3.1 ± 0.9 Hz) or amplitude (16.3 ± 1.3 vs. 15.5 ± 1.2 pA) of mEPSCs.

Effect of capsaicin on mEPSCs in the spinal cord of dorsal rhizotomized rats

Next, we assessed the effect of capsaicin on the quantal glutamate release in the spinal cord of dorsal rhizotomized rats. In sham-operated rats, bath perfusion of 2 μ M capsaicin for 3 min significantly increased the frequency of mEPSCs in 17 of 18 (94.4%) neurons recorded in the spinal cord (Fig. 7A). In 16 of 17 (94.1%) neurons, capsaicin also significantly increased the amplitude of mEPSCs (Fig. 7A).

In rats subjected to dorsal rhizotomy, bath perfusion of 2 μ M capsaicin for 3 min significantly increased the frequency of mEPSCs in 12 of 17 (70.6%) neurons (Fig. 7B). However, capsaicin significantly increased the amplitude of mEPSCs in only 6 of 12 (50%) neurons (Fig. 7B). These results suggest that TRPV1 receptors are present at both terminals and somatodendritic sites of the glutamatergic dorsal horn neurons.

Capsaicin evoked inward currents in lamina II neurons of RTX-treated and dorsal rhizotomized rats

We examined whether capsaicin-evoked currents are present in any lamina II neurons in RTX-treated rats and rats subjected to dorsal rhizotomy. In 11 lamina II neurons in which capsaicin increased the amplitude of sEPSCs in RTX-treated rats, bath application of 2 μ M capsaicin also produced an evident inward current in 4 of these 11 (36.4%) neurons (Fig. 8). However, capsaicin did not produce any inward current in another 11 neurons that did not show an increase in the amplitude of sEPSCs in RTX-treated rats. In the presence of 20 μ M capsazepine, capsaicin also failed to elicit any inward current in 8 lamina II neurons in RTX-treated rats.

In dorsal rhizotomized rats, 20 lamina II neurons showed an increase in the amplitude of sEPSCs in response to 2 μ M capsaicin. Capsaicin produced an inward current in 7 of these 20 (35%) neurons (Fig. 8). In contrast, capsaicin did not evoke any inward current in another 13 neurons that did not show an increase in the amplitude of sEPSCs in rats subjected to dorsal rhizotomy. Capsaicin also failed to produce inward currents in 9 lamina II neurons pretreated with 20 μ M capsazepine in dorsal rhizotomized rats.

Effect of capsaicin on sIPSCs in the spinal cord of RTX-treated or dorsal rhizotomized rats

To determine whether TRPV1 is expressed on the inhibitory GABAergic and glycinergic neurons in the spinal dorsal horn, we examined the effect of capsaicin on GABAergic and glycinergic sIPSCs of lamina II neurons in the spinal cord of RTX-treated or dorsal rhizotomized rats. We have shown that sIPSCs are present in all lamina II neurons (Li et al. 2002; Wang et al. 2006; Zhou et al. 2007, 2008), suggesting that this population of dorsal horn neurons receives extensive input from GABAergic and glycinergic interneurons. In RTX-treated rats, bath perfusion of 2 μ M capsaicin for 3 min had no significant effect on the frequency and the amplitude of sIPSCs in any of 9 neurons tested (Fig. 9, A and B). Similarly, 2 μ M capsaicin did not significantly alter the frequency and amplitude of sIPSCs in all 11 neurons examined from dorsal rhizotomized rats (Fig. 9C). Bath perfusion of the GABA_A and glycine receptor antagonist picrotoxin (50 μ M) abolished sIPSCs in all neurons studied (Fig. 9A).

In additional RTX-treated rats, we compared the effect of the muscarinic acetylcholine receptor agonist oxotremorine and capsaicin on sIPSCs in the same lamina II neurons. The muscarinic acetylcholine receptors are present on the GABAergic and glycinergic interneurons, and stimulation of muscarinic acetylcholine receptors excites these inhibitory interneurons in the spinal dorsal horn (Zhang et al. 2005; Wang et al. 2006). In all 11 lamina II neurons tested, bath application of 3 μ M oxotremorine caused a significant increase in the frequency and amplitude of sIPSCs (Fig. 10). In contrast, 2 μ M capsaicin had no effect on

sIPSCs in any of these 11 neurons studied. These results suggest that TRPV1 receptors are not expressed on the inhibitory GABAergic and glycinergic dorsal horn neurons.

Discussion

The phenotype and function of TRPV1-expressing neurons in the spinal dorsal horn have not previously been defined. In the present study, we found that TRPV1-expressing dorsal horn neurons were colocalized with VGluT₂, a specific marker for glutamatergic neurons, in the spinal cord. We also found that, in spinal cord slices in which TRPV1-expressing primary afferents had been removed by RTX treatment or dorsal rhizotomy, capsaicin still increased the frequency of glutamatergic sEPSCs and mEPSCs in the majority of lamina II neurons examined and that this effect was blocked by the TRPV1 receptor antagonist. In RTX-treated and dorsal rhizotomized rats, capsaicin also evoked an inward current in a subset of lamina II neurons that showed an increase in the amplitude of sEPSCs. However, capsaicin had no significant effect on the frequency and amplitude of GABAergic and glycinergic sIPSCs in either RTX-treated or dorsal rhizotomized rats. Collectively, these findings suggest that TRPV1-expressing dorsal horn neurons and their terminals are glutamatergic in nature and are involved in excitatory synaptic transmission in the spinal cord.

TRPV1 receptors are primarily expressed on the primary sensory neurons and their terminals (Guo et al. 1999; Szallasi and Blumberg 1999; Chen and Pan 2006). Activation of TRPV1 receptors on primary afferents increases glutamate release to spinal dorsal horn neurons (Yang et al. 1998; Pan and Pan 2004) and can trigger pain in humans (Simone et al. 1987; LaMotte et al. 1991). Although it has been recognized that TRPV1 receptors are also expressed on spinal dorsal horn neurons (Valtschanoff et al. 2001; Chen and Pan 2006), the functional role of this subpopulation of neurons remains little known. In this study, we first used double immunofluorescence labeling of TRPV1 and VGluT₂ to determine whether TRPV1-expressing neurons are glutamatergic. VGluT₂ is a highly specific marker widely used to identify glutamatergic neurons in the brain and spinal cord (Alvarez et al. 2004; Ponzio et al. 2006; Brumovsky et al. 2007). We found that although RTX treatment and dorsal rhizotomy effectively removed TRPV1-expressing primary afferent terminals, these interventions did not alter the presence of TRPV1-expressing postsynaptic neurons in the spinal dorsal horn. Because TRPV1-expressing dorsal horn neurons were coexpressed with VGluT₂ in the spinal dorsal horn, these data suggest that dorsal horn neurons that express TRPV1 are glutamatergic.

To provide functional evidence that TRPV1-expressing dorsal horn neurons are glutamatergic, we used capsaicin to stimulate this group of neurons in the spinal cord slices in which TRPV1-expressing primary afferents had been ablated by RTX treatment or dorsal rhizotomy. Removal of TRPV1-expressing primary afferent terminals by RTX treatment or dorsal rhizotomy in the rat spinal cord was confirmed by profound impairment in thermal nociception and by immunocytochemical results. In vehicle-treated and sham-operated rats, capsaicin increased the frequency of glutamatergic sEPSCs and mEPSCs in more than 90% of lamina II neurons. We found that capsaicin still significantly increased the frequency of glutamatergic sEPSCs in the majority of neurons in RTX-treated or dorsal rhizotomized rats. The stimulating effect of capsaicin on sEPSCs was abolished by the selective TRPV1 antagonist capsazepine in these rats. Furthermore, in the majority of neurons recorded from RTX-treated rats or those subjected to dorsal rhizotomy, capsaicin significantly increased the frequency of glutamatergic mEPSCs. Combined with our immunocytochemical analysis, these electrophysiological results provide further support for our hypothesis that intrinsic dorsal horn neurons that express TRPV1 are glutamatergic neurons.

TRPV1 has a high permeability for divalent cations such as Ca^{2+} (Caterina et al. 1997; Wu et al. 2005), and Ca^{2+} influx through TRPV1 can trigger a large amount of glutamate release from presynaptic terminals. The major effect of RTX treatment or dorsal rhizotomy on the capsaicin-induced responses was that the percentage of lamina II neurons with the increase in the amplitude of sEPSCs and mEPSCs by capsaicin was significantly reduced, compared with that in the vehicle-treated or sham-operated control group. This likely indicates that TRPV1 is expressed at a higher density on primary afferent terminals than on postsynaptic dorsal horn neurons. We found that in 35–50% of lamina II neurons in spinal cord slices from rats subjected to RTX treatment and dorsal rhizotomy, capsaicin still increased the amplitude of sEPSC and mEPSCs. This effect probably suggests that TRPV1 is present on the somatodendritic site of some TRPV1-expressing presynaptic neurons. Because TRPV1 is present in a subpopulation of dorsal horn neurons, the increased amplitude of sEPSCs or mEPSCs caused by capsaicin may be due to its direct postsynaptic effect at least in some neurons. In support of this possibility, we found that capsaicin did produce an inward current in some lamina II neurons that showed an increase in the amplitude of sEPSCs. These findings provide further evidence for the presence of TRPV1 receptors on intrinsic spinal dorsal horn neurons. Because capsaicin still caused a similar magnitude of increase in the frequency of sEPSCs and mEPSCs in the majority of lamina II neurons in RTX-treated and dorsal rhizotomized rats, it appears that a major proportion of glutamatergic synaptic input to lamina II neurons elicited by capsaicin was from TRPV1-expressing dorsal horn neurons in the superficial dorsal horn. Thus, TRPV1-expressing dorsal horn neurons may be involved in the amplification of nociceptive input from primary afferents. We have shown that this subpopulation of dorsal horn neurons is endowed with μ -opioid, but not δ -opioid, receptors (Chen and Pan 2006, 2008). Our finding is consistent with the previous study showing that μ -opioid receptors are expressed only on non-GABAergic and non-glycinergic neurons in the spinal cord (Kemp et al. 1996). It is possible that direct inhibition of TRPV1-expressing dorsal horn neurons could play an important role in the potent analgesic effect of spinally administered μ -opioid receptor agonists.

Because almost all lamina II neurons have functional GABA_A and glycine receptors and receive GABAergic and glycinergic inputs (Pan and Pan 2004; Zhou et al. 2007, 2008), lamina II neurons were used as a part of the “electrode” in our whole-cell recordings to detect the effect of capsaicin on the excitability of these inhibitory interneurons, reflected in GABA and glycine release, in the spinal cord. However, we observed that capsaicin had no significant effect on the frequency and amplitude of GABAergic and glycinergic sIPSCs in any of lamina II neurons tested from RTX-treated and dorsal rhizotomized rats. Furthermore, the muscarinic acetylcholine receptors are present on the GABAergic and glycinergic interneurons, and stimulation of muscarinic acetylcholine receptors excites these inhibitory interneurons in the spinal dorsal horn (Zhang et al. 2005; Wang et al. 2006). We found that in the same neurons in which capsaicin had no effect on sIPSCs, activation of muscarinic acetylcholine receptors with oxotremorine induced a substantial increase in the frequency and amplitude of sIPSCs of lamina II neurons in RTX-treated rats. Therefore, these data suggest that TRPV1 receptors are not expressed on inhibitory GABAergic and glycinergic interneurons in the spinal dorsal horn.

In summary, using immunocytochemical and electrophysiological approaches, we have demonstrated that TRPV1-expressing neurons are glutamatergic and that activation of these neurons enhances excitatory synaptic transmission in the spinal dorsal horn. This subpopulation of dorsal horn neurons is likely involved in the relay and modulation of nociceptive information from the primary afferents (Pan and Pan 2004; Cui et al. 2006; Zhou et al. 2007, 2008). Within the spinal cord, the endogenous ligands that activate TRPV1 on postsynaptic dorsal horn neurons remain unclear. Three classes of endovanilloids, including anandamide, certain lipoxygenase products, such as 12-(S)- and 15-(S)-

hydroperoxyeicosatetraenoic acid (12S- and 15S-HPETE), and N-arachidonoyl-dopamine, can activate TRPV1 (Hwang et al. 2000; Van Der Stelt and Di Marzo 2004). Activation of TRPV1 at the spinal level by these endovanilloids could produce pronociceptive responses. For example, intrathecal administration of 15S-HPETE or N-arachidonoyl-dopamine in rats induces hyperalgesia and allodynia (Trang et al. 2004; Pitcher et al. 2007). Furthermore, intrathecal injection of a high dose of anandamide in rats causes excitation and vocalization, suggestive of pain (Horvath et al. 2008). Our findings that TRPV1-expressing dorsal horn neurons are involved in excitatory synaptic transmission could explain why centrally acting TRPV1 antagonists are more efficacious in reducing inflammatory pain (Cui et al. 2006).

List of abbreviations

sEPSCs	spontaneous excitatory postsynaptic currents
DRG	dorsal root ganglion
mEPSCs	miniature excitatory postsynaptic currents
RTX	Resiniferatoxin
sIPSCs	spontaneous inhibitory postsynaptic currents
QX314	lidocaine N-ethyl bromide
NMDA	N-methyl-D-aspartate
VGLUT₂	vesicular glutamate transporter-2
TRPV1	transient receptor potential vanilloid receptor 1
TTX	tetrodotoxin

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Fig. 1.

Confocal images showing the effect of RTX on TRPV1- and IB₄-positive dorsal root ganglion (DRG) neurons and afferent terminals in the spinal cord. A, representative confocal images showing TRPV1-immunoreactive (green) and IB₄-positive (red) DRG neurons from one vehicle-treated and one RTX-treated rat. B, confocal images showing TRPV1-immunoreactive (green) and IB₄ (red)-positive afferent terminals in the spinal dorsal horn of one vehicle-treated and one RTX-treated rat. Colocalization of TRPV1 immunoreactivity and IB₄ labeling is indicated in yellow when two images are digitally merged. All images are single confocal optical sections.

Fig. 2.

Confocal images showing the presence of TRPV1- and VGlut₂-immunoreactive neurons and terminals in the superficial spinal dorsal horn in RTX-treated and dorsal rhizotomized rats. A, representative low- and high-magnification confocal images showing VGlut₂ (green)- and TRPV1 (red)-immunoreactive neurons and terminals in laminae I and II from one vehicle-treated control rat. B, representative confocal images showing VGlut₂ (green)- and TRPV1 (red)-immunoreactive neurons and terminals in laminae I and II from one RTX-treated and one dorsal rhizotomized rat. Colocalization of TRPV1 and VGlut₂ is indicated in yellow when two images are digitally merged. All images are single confocal optical sections.

Fig. 3. Effect of capsaicin on the frequency and amplitude of sEPSCs of lamina II neurons in vehicle-treated rats. **A**, original traces show sEPSCs during control and application of 2 μ M capsaicin in an amplitude increase type neuron. Cumulative distributions of the inter-event interval and amplitude of sEPSCs of the same neuron during control and application of 2 μ M capsaicin. **B**, summary data show the effect of capsaicin on the frequency ($n = 12$) and amplitude ($n = 11$) of sEPSCs in lamina II neurons of vehicle-treated rats. * $P < 0.05$ compared with the control.

Fig. 4.

Effect of capsaicin and capsazepine on the frequency and amplitude of sEPSCs of lamina II neurons in RTX-treated rats. A, original traces show sEPSCs during control and application of 2 μ M capsaicin in an amplitude no-change type neuron. Note that 10 μ M CNQX abolished the sEPSCs in both amplitude no-change and amplitude increase type neurons. Cumulative distributions of the inter-event interval and amplitude of sEPSCs of the same neuron as that in panel A during control and application of capsaicin. B, summary data show the differential effect of capsaicin on the frequency and amplitude of sEPSCs of 23 lamina II neurons in RTX-treated rats. C, summary data show the effect of capsaicin on sEPSCs of 8 lamina II neurons in the presence of 20 μ M capsazepine in RTX-treated rats. * $P < 0.05$ compared with the control.

Fig. 5.

Effect of capsaicin on the frequency and amplitude of sEPSCs of lamina II neurons in sham-operated and dorsal rhizotomized rats. A, summary data show the effect of capsaicin on the frequency and amplitude of sEPSCs of lamina II neurons in sham-operated rats. B, group data show the differential effect of capsaicin on sEPSCs of 36 lamina II neurons in dorsal rhizotomized rats. C, summary data show results for 9 lamina II neurons in the presence of 20 μ M capsazepine in dorsal rhizotomized rats. * $P < 0.05$ compared with the control.

Fig. 6.

Effect of capsaicin on the frequency and amplitude of mEPSCs of lamina II neurons in vehicle- and RTX-treated rats. A, original traces show mEPSCs during control and application of 2 μ M capsaicin in an amplitude increase type neuron in one vehicle-treated rat. Cumulative distributions of the inter-event interval and amplitude of mEPSCs of the same neuron. B, summary data show the effect of capsaicin on the frequency (n=16) and amplitude (n=15) of mEPSCs of lamina II neurons in vehicle-treated rats. C, original traces show mEPSCs during control and application of 2 μ M capsaicin in an amplitude no-change type neuron. Cumulative distributions of the inter-event interval and amplitude of mEPSCs

of the same neuron. D, group data show the differential effect of capsaicin on mEPSCs of 33 lamina II neurons in RTX-treated rats. * $P < 0.05$ compared with the control.

Fig. 7. Effect of capsaicin on the frequency and amplitude of mEPSCs of lamina II neurons in sham-operated rats and those subjected to dorsal rhizotomy. A, group data show the effect of capsaicin on the frequency and amplitude of sEPSCs of lamina II neurons in sham-operated rats. B, summary data show the differential effect of capsaicin on the frequency and amplitude of mEPSCs of 17 lamina II neurons in dorsal rhizotomized rats. * $P < 0.05$ compared with the control.

Fig. 8.

Capsaicin-induced currents in a subpopulation of spinal lamina II neurons of RTX-treated or dorsal rhizotomized rats. A, original records show an inward current in response to application of 2 μ M capsaicin in an EPSC amplitude-increase type of lamina II neuron of one RTX-treated rat. B, original recordings show an inward current during capsaicin application in an EPSC amplitude-increase type of lamina II neuron of one dorsal rhizotomized rat. C, summary data show the peak amplitude of inward currents produced by capsaicin application in lamina II neurons of RTX-treated ($n = 4$ neurons) and dorsal rhizotomized ($n = 7$ neurons) rats.

Fig. 9.

Effect of capsaicin on the frequency and amplitude of sIPSCs of lamina II neurons in RTX-treated and dorsal rhizotomized rats. A: original traces show sIPSCs of a lamina II neuron during control and application of 2 μ M capsaicin in one dorsal rhizotomized rat. Note that bath application of 50 μ M picrotoxin abolished sIPSCs. B and C: summary data show the lack of effect of capsaicin on the sIPSCs of lamina II neurons in RTX-treated or dorsal rhizotomized rats.

Fig. 10.

Comparison of the effects of oxotremorine and capsaicin on the frequency and amplitude of sIPSCs of lamina II neurons in RTX-treated rats. A: original traces show sIPSCs of a lamina II neuron during control and during bath application of 3 μ M oxotremorine (Oxo) or 2 μ M capsaicin (Caps) in one neuron of an RTX-treated rat. B: summary data showing that oxotremorine, but not capsaicin, potentiated the frequency and amplitude of sIPSCs of 11 lamina II neurons in RTX-treated rats. * $P < 0.05$ compared with the control.