JPP Journal of Pharmacy and Pharmacology

JPP 2006, 58: 489–493

Received October 5, 2005
Accepted December 20, 2005
DOI 10.1211/jpp.58.4.0008
ISSN 0022-3573

Participation of the spinal TRPV1 receptors in formalin-evoked pain transduction: a study using a selective TRPV1 antagonist, iodo-resiniferatoxin

Yoshihito Kanai, Tomokazu Hara and Aki Imai

Abstract

The involvement of spinal transient receptor potential vanilloid 1 (TRPV1) in formalin-evoked pain has remained unclear, because investigation of this kind of pain with selective antagonists has not been conducted. The purpose of this study is to investigate the participation of spinal TRPV1 in formalin-evoked pain with iodo-resiniferatoxin (I-RTX), a potent TRPV1-selective antagonist. I-RTX given intrathecally dose-dependently and significantly decreased the number of flinching responses in the formalin-evoked 1st and 2nd phase with ID50 values (drug dose producing 50% inhibition of response) of 1.0 and 3.8 μg, respectively, and concentration-dependently suppressed capsaicin-evoked calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) release from rat spinal cord slices with an IC50 value (drug concentration producing 50% inhibition of response) of 86 nM. Capsazepine, a classical non-selective TRPV1 antagonist, given intrathecally also inhibited formalin-evoked flinching in both the 1st and 2nd phase with ID50s of 420 and 200 μg, respectively, and CGRP-LI release from rat spinal cord slices with an IC50 of 7.8 μM. Ratios of in-vivo analgesic potencies of I-RTX and capsazepine well reflected their intrinsic in-vitro activity. These findings suggest that spinal TRPV1 participates in the transduction system of formalin-evoked pain.

Introduction

Transient receptor potential vanilloid 1 (TRPV1) is a ligand gated non-selective cation channel (Caterina et al 1997), and is recognized as a polymodal nociceptor that integrates multiple pain stimuli (e.g., noxious heat, protons and vanilloids) (Tominaga et al 1998). TRPV1 is localized on both Aδ- and C-fibres, which terminate in the superficial laminae of the dorsal horn of the spinal cord (Valtschanoff et al 2001). It has been reported that capsaicin, which is known as an exogenous stimulator of TRPV1, evokes depolarization (Dickenson & Dray 1991; Yang et al 1998) and enhances glutamate release (Tohda & Kuraishi 1996) in rat isolated spinal cord. These data suggest the possibility that activation of spinal TRPV1 is involved in the pain transduction system.

Previous reports showed that classical TRPV1 antagonists, such as ruthenium red (Ohkubo et al 1993) and capsazepine (Santos & Calixto 1997), inhibited formalin-evoked licking behaviour, indicating a possible role of TRPV1 in formalin-evoked pain. However, it has been difficult to reach a conclusion, because both ruthenium red and capsazepine inhibit not only TRPV1 but also voltage-gated calcium channels (VDCCs) (Hamilton & Lundy 1995; Docherty et al 1997), and VDCC antagonists are known to inhibit formalin-induced pain behaviour (Malmberg & Yaksh 1995). Therefore, the involvement of TRPV1 in formalin-evoked pain remains unclear.

Recently, iodo-resiniferatoxin (I-RTX) was reported to bind as a highly specific and potent antagonist at the TRPV1 (Wahl et al 2001). I-RTX blocks various TRPV1-related events (e.g., in-vitro capsaicin-induced currents in TRPV1-expressing cells (Wahl et al 2001), in-vivo capsaicin-induced pain responses (Wahl et al 2001) and plasma extravasation (Rigoni et al 2003)).

In this study, the role of spinal TRPV1 in formalin-evoked pain was examined by investigating the anti-nociceptive activity of I-RTX, a selective TRPV1

Discovery Biology Research, Nagoya Laboratories, Pfizer Global Research and Development, Pfizer Inc., 5-2 Taketoyo, Aichi, 470-2393, Japan

Yoshihito Kanai, Tomokazu Hara, Aki Imai

Correspondence: Y. Kanai, Discovery Biology Research, Nagoya Laboratories, Pfizer Global Research and Development, Pfizer Inc., 5-2 Taketoyo, Aichi, 470-2393, Japan. E-mail: Yoshihito.Kanai@pfizer.com

Acknowledgement: We gratefully acknowledge Drs Katsuo Toide and Yasuhiro Kita (Discovery Biology Research, Nagoya Laboratories, Pfizer Inc.) for their valuable advice and encouragement throughout work on this paper.

antagonist. Moreover, the mode of action of capsazepine, a well-known non-selective TRPV1 antagonist, in formalin-evoked pain was investigated by comparing the in-vitro and in-vivo activity of I-RTX with that of capsazepine.

Materials and Methods

Reagents

I-RTX and capsazepine were purchased from Tocris Cookson (Bristol, UK). Formalin was obtained from Wako Chemical Industries (Osaka, Japan). The rat calcitonin gene-related peptide (CGRP) EIA kit was obtained from Cayman (Ann Arbor, MI, USA).

Animal experiments

The experiments were carried out according to a protocol approved by the animal ethics committee at the Nagoya Laboratories of Pfizer Global Research and Development (registry number: 2001-43).

Formalin-evoked flinch

Intrathecally catheterized male SD rats, 260–300 g, were obtained from Japan SLC (Shizuoka, Japan), and rats were used at 2 weeks after the implantation surgery. The formalin test was performed as described previously (Malmberg & Yaksh 1992). In brief, 10 μL of drug dissolved in 100% dimethyl sulfoxide (DMSO) was administered via an intrathecal catheter. For control rats, 10 μL of 100% DMSO was injected, also via the catheter. After 10 min, 50 μL of 5% formalin was injected subcutaneously into the dorsal skin of the left hind paw with a 29-gauge needle. Immediately after formalin injection, the rat was placed in an open plastic chamber ($9 \times 18 \times 32$ cm) and videotaped for 60 min. Pain-related behaviour was quantified by periodically counting the spontaneous flinching movements of the injected paw. To assess the 1st phase, flinching movements were counted from 1 to 2 min and from 5 to 6 min after formalin injection and the total flinching movement time was used for analysis. To assess the 2nd phase, flinching movements from 10 to 61 min post formalin were counted for 1 min each at 5-min intervals and the total flinching movement time was used for analysis. Data from each phase were examined separately.

Capsaicin-induced CGRP-LI release from rat spinal cord slices

Measurement of the capsaicin-induced release of CGRP-LI from rat spinal cord slices was performed essentially as described by Wardle et al (1997). The lumbar spinal cord of male SD rats (Japan SLC), 250–300 g, was removed and placed in cold oxygenated Krebs solution (composition in mM: NaCl 121.5; $CaCl_2$ 2.5; KH_2PO_4 1.2; KCl 4.7; $MgSO_4$ 1.2; $NaHCO_3$ 25.0 and glucose 5.6 at pH 7.4). The cord was sliced transversely (300 μm) on a McIlwain tissue chopper (Brinkmann, Westbury, NY, USA), and the slices were immersed in Krebs solution (assay buffer). Each slice was placed in a MultiScreen-DV plate (Millipore, Billerica, MA, USA) and incubated in 180 μL of assay buffer for 20 min at 37 °C with or without drugs, followed by an addition of 20 μL of capsaicin solution (final concn 3 μM). Then the tissue slices were incubated for a further 10 min. Portions of the medium were collected into 96-well plates under a vacuum and the released CGRP-LI amount was determined using a rat CGRP EIA kit.

Data analysis

Data from the 1st and 2nd phases of the formalin-evoked flinching test were examined separately. Drug concentrations or doses producing 50% inhibition of the responses (IC50 and ID50 for in-vitro and in-vivo study, respectively) were calculated by non-linear regression analysis (GraphPad Prism; GraphPad software Inc., San Diego, CA, USA). Significant data was determined using Student's *t*-test for comparing two groups and by one-way analysis of variance followed by Fisher's LSD test for multiple comparisons.

Results

Inhibitory effects of I-RTX and capsazepine on formalin-evoked pain

Subcutaneous injection of 50 μL of 5% formalin into the paw elicited a typical biphasic nociceptive response consisting of an initial and rapidly decaying acute phase (1st phase) of paw flinching, followed by a longer and slowly progressive tonic phase (2nd phase) (Figures 1A and 2A). The intrathecal injections of I-RTX and capsazepine decreased the number of flinching responses in a dose-dependent manner. The effects were statistically significant at 1.0 and 1000 μg in the 1st phase, respectively, and 3.0 and 100 μg in the 2nd phase, respectively (Figures 1B and 2B). The maximum inhibition was 91% and 76% for I-RTX, and 87% and 76% for capsazepine, in the 1st and 2nd phase, respectively. The ID50s (95% confidence limit) of I-RTX and capsazepine in the 1st phase were 1.0 (0.45–2.3) and 420 (160–1100) μg intrathecally, respectively, and in the 2nd phase they were 3.8 (1.8–8.0) and 200 (87–440) μg intrathecally, respectively (Table 1). Rats treated with only the vehicle (DMSO) showed pain-related behaviour (e.g., vocalization) in rare cases. These responses were completed within 30 s; after that, behavior of rats was normal. No abnormal behaviour was observed through the entire 60-min observation period of formalin-evoked pain.

Inhibitory effects of I-RTX and capsazepine on capsaicin-evoked CGRP-LI release

Capsaicin (300 nM) induced a significant, approximately 4-fold increase in CGRP-LI release from the rat spinal

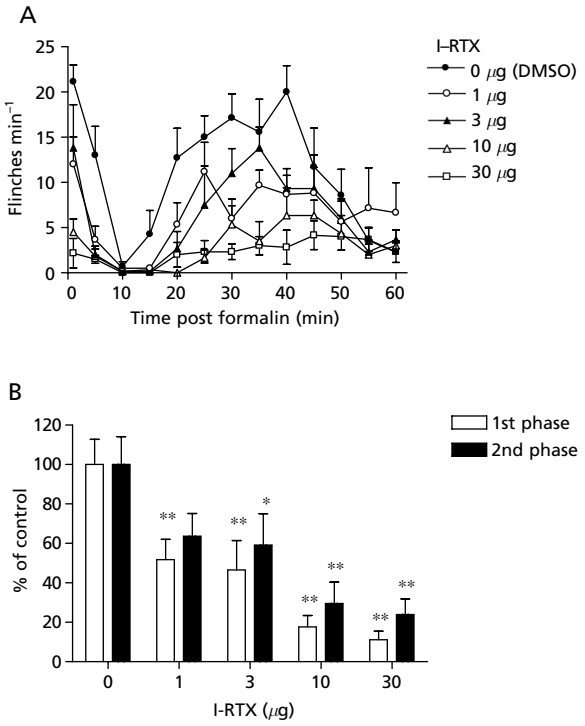

Figure 1 Effect of intrathecally administered I-RTX on the formalin-evoked flinching response in rats. A. Time course of formalin-evoked flinching responses. B. Cumulative scores are represented as a percentage of the control number of flinching responses in the 1st phase and the 2nd phase. Data are shown as the mean ± s.e.m., n = 6. $*P < 0.05$, $**P < 0.01$ compared with the control (0 μg) group (one-way analysis of variance followed by Fisher's LSD test).

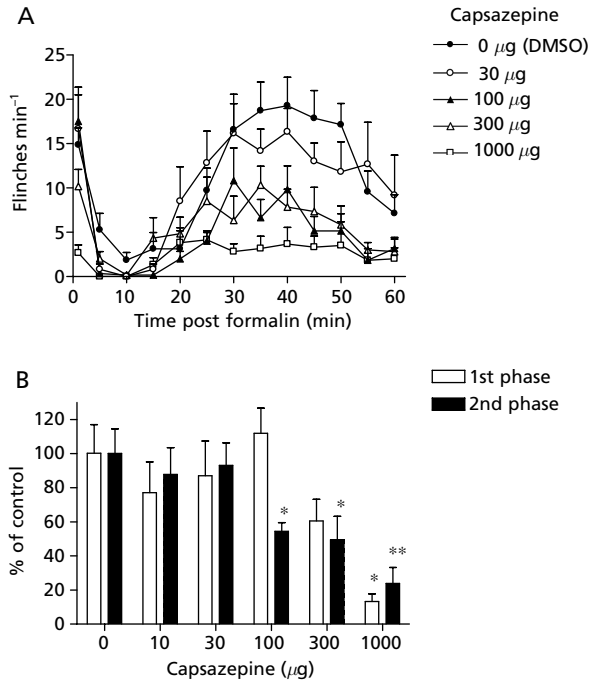

Figure 2 Effect of intrathecally administered capsazepine on the formalin-evoked flinching response in rats. A. Time course of formalin-evoked flinching responses. B. Cumulative scores are represented as a percentage of the control number of flinching responses in the 1st phase and the 2nd phase. Data are shown as the mean ± s.e.m., n = 6. $*P < 0.05$, $**P < 0.01$ compared with the control (0 μg) group (one-way analysis of variance followed by Fisher's LSD test).

cord slices. I-RTX and capsazepine inhibited the capsaicin-evoked CGRP-LI release concentration-dependently, and significantly at 300 nM and 10 μM, respectively (Figure 3). The IC50s (95% confidence limit) of I-RTX and capsazepine were 86 (26–300) nM and 7800 (2800–22000) nM, respectively (Table 1).

Discussion

A previous report showed that the intrathecal injection of ruthenium red inhibited formalin-evoked licking behaviour (Ohkubo et al 1993). Recently, ruthenium red has been reported to be a non-selective TRPV1 antagonist and the involvement of spinal TRPV1 in formalin-evoked pain has remained unclear. In this study, the participation of spinal TRPV1 in formalin-evoked pain was investigated with I-RTX, which is reported to be a selective TRPV1 antagonist.

Intrathecal injection of I-RTX dose-dependently decreased the number of formalin-evoked flinching responses in the 1st and 2nd phase with ID50s of 1.0 and 3.8 μg, respectively. Additionally I-RTX concentration-dependently suppressed capsaicin-evoked CGRP-LI release from rat spinal cord slices with an IC50 of 84 nM. These findings indicate that spinal TRPV1 participates in the transduction system of formalin-evoked pain.

Furthermore, we evaluated the effects of capsazepine, a non-selective TRPV1 antagonist, in comparison with I-RTX. Capsazepine also inhibited formalin-evoked flinching in both the 1st and 2nd phases with ID50s of 420 and 200 μg, respectively. Due to the poor selectivity of capsazepine, it is difficult to neglect the involvement of its non-selective effects (e.g., blocking of VDCC

Table 1 Comparison of in-vitro and in-vivo potency of I-RTX and capsazepine

Compound	In-vitro CGRP release IC50 (nM)[a]	In-vivo formalin-evoked flinching ID50 (nmol)[b]	
		1st phase	2nd phase
I-RTX	86 (1.0)[c]	1.9 (1.0)	5.0 (1.0)
Capsazepine	7800 (1/91)	1100 (1/580)	520 (1/104)

[a]Concentration required to inhibit a half of the capsaicin (300 nM)-evoked CGRP-LI release. [b]Mean inhibitory dose resulting in a 50% reduction of the control formalin-evoked flinching behaviour. [c]Each number in parentheses shows the ratio of activity to that of I-RTX.

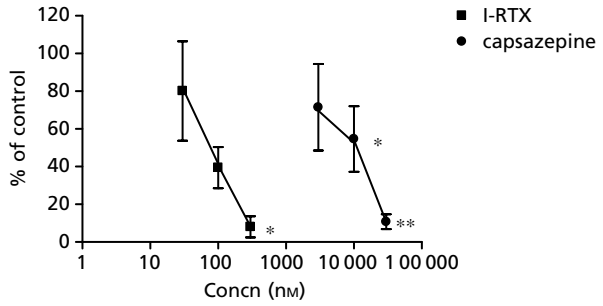

Figure 3 Effect of I-RTX and capsazepine on CGRP-LI release induced by 300 nM capsaicin in rat lumbar spinal cord slices. Each symbol represents the mean ± s.e.m., n = 8. $*P < 0.05$, $**P < 0.01$ compared with the capsaicin control (one-way analysis of variance followed by Fisher's LSD test).

(Docherty et al 1997)), since, VDCC antagonists are also known to inhibit formalin-induced pain behaviour (Malmberg & Yaksh 1995). However, this study demonstrated that the ratio (580- and 104-times in 1st and 2nd phases, respectively) of the analgesic potency of capsazepine to that of I-RTX was well correlated with that (91-times) of the intrinsic in-vitro activity (inhibitory activity of capsaicin-evoked CGRP-LI release from the rat spinal cord slices) of the compounds. These findings suggest that the analgesic effect of capsazepine would also be derived from TRPV1 antagonism.

Previous investigations that used neonatal capsaicin treatment are useful in considering the mechanism that underlies the analgesic effects of the TRPV1 antagonist. Neonatal capsaicin treatment in rats causes less pain behaviour in the formalin test (Peterson et al 1997; Andre et al 2004), and neonatal treatment with capsaicin significantly decreased [^{3}H]-resiniferatoxin binding sites in the rat spinal cord (Andre et al 2004). These findings indicate that TRPV1-positive neurons participate in formalin-evoked pain sensation, and correlated with our results. Recently, it has been reported that TRPV1 antagonists have analgesic effects in inflammatory pain models (Kwak et al 1998; Pomonis et al 2003; Walker et al 2003; Gavva et al 2005). These studies demonstrated the analgesic activity of systemically or intradermally administered TRPV1 antagonists. However, the site of participation of TRPV1 in pain is not fully understood. At the same time, the role of spinal TRPV1 in the pain transduction system is not known well. In this study, we demonstrated that spinal TRPV1 antagonists suppressed formalin-evoked pain. These findings suggest that the spinal cord is a possible site of action of TRPV1 antagonists in formalin-evoked pain transduction. Furthermore, electrophysiological studies demonstrated that spinal administration of capsazepine and I-RTX suppressed electrically evoked C- and Aδ-fibre responses of wide dynamic range neurons (Jhaveri et al 2005; Kelly & Chapman 2002). Those findings support the results from our study.

Formalin-evoked pain is a commonly used and conventional model for studying analgesic activity, which consists of both acute (1st phase) and tonic (2nd phase) pain. The formalin-evoked 1st phase is caused by direct chemical stimulation of neurons, while the 2nd phase is caused by both inflammation of the injected paw and central sensitization of the spinal neurons. These results suggest that TRPV1 in the spinal cord is involved in the pain pathway of both phases.

In terms of maximum efficacy, I-RTX and capsazepine showed higher efficacies in the 1st phase than the 2nd phase in our study. Also in previous reports, capsazepine injection (intradermal or intracerebroventricular) or VR1 depletion by capsaicin treatment exhibited higher efficacies in the 1st phase than in 2nd phase of the formalin test (Santos & Calixto 1997; Andre et al 2004).

Formalin-evoked pain was attenuated by intrathecally administered NK1 and NMDA receptor antagonists (Chapman & Dickenson 1993; Nishiyama 2000), and capsaicin causes release of substance P and glutamate from the rat spinal cord slices (Gamse et al 1979; Tohda & Kuraishi 1996). Taken together, it could be speculated that TRPV1 is involved in pain transmission via Substance P and glutamate release from the primary afferent endings in the spinal cord.

Conclusions

In this study, we showed that intrathecal treatment with a specific TRPV1 receptor antagonist I-RTX inhibited the formalin-evoked flinching responses of both the 1st and 2nd phases in rats. In addition, capsazepine, a non-selective antagonist, also inhibited these responses. In-vivo potencies of these two antagonists were well correlated with their in-vitro TRPV1 antagonism, suggesting that spinal TRPV1 participates in the transduction of formalin-evoked pain.

References

Andre, E., Ferreira, J., Malheiros, A., Yunes, R. A., Calixto, J. B. (2004) Evidence for the involvement of vanilloid receptor in the antinociception produced by the dialdehydes unsaturated sesquiterpenes polygodial and drimanial in rats. *Neuropharmacology* **46**: 590–597

Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., Julius, D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**: 816–824

Chapman, V., Dickenson, A. H. (1993) The effect of intrathecal administration of RP67580, a potent neurokinin 1 antagonist on nociceptive transmission in the rat spinal cord. *Neurosci. Lett*. **157**: 149–152

Dickenson, A. H., Dray, A. (1991) Selective antagonism of capsaicin by capsazepine: evidence for a spinal receptor site in capsaicin-induced antinociception. *Br. J. Pharmacol*. **104**: 1045–1049

Docherty, R. J., Yeats, J. C., Piper, A. S. (1997) Capsazepine block of voltage-activated calcium channels in adult rat dorsal root ganglion neurones in culture. *Br. J. Pharmacol*. **121**: 1461–1467

Gamse, R., Molnar, A., Lembeck, F. (1979) Substance P release from spinal cord slices by capsaicin. *Life Sci*. **25**: 629–636

Gavva, N. R., Tamir, R., Qu, Y., Klionsky, L., Zhang, T. J., Immke, D., Wang, J., Zhu, D., Vanderah, T. W., Porreca, F., Doherty, E. M., Norman, M. H., Wild, K. D., Bannon, A. W., Louis, J. C., Treanor, J. J. (2005) AMG 9810 [(E)-3-(4-t-Butylphenyl)-N-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)acrylamide], a novel vanilloid receptor 1, (TRPV1) antagonist with antihyperalgesic properties. *J. Pharmacol. Exp. Ther.* **313**: 474–484

Hamilton, M. G., Lundy, P. M. (1995) Effect of ruthenium red on voltage-sensitive Ca++ channels. *J. Pharmacol. Exp. Ther.* **273**: 940–947

Jhaveri, M. D., Elmes, S. J., Kendall, D. A., Chapman, V. (2005) Inhibition of peripheral vanilloid TRPV1 receptors reduces noxious heat-evoked responses of dorsal horn neurons in naïve, carrageenan-inflamed and neuropathic rats. *Eur. J. Neurosci.* **22**: 361–370

Kelly, S., Chapman, V. (2002) Spinal administration of capsazepine inhibits noxious evoked responses of dorsal horn neurons in non-inflamed and carageenan inflamed rats. *Brain Res.* **935**: 103–108

Kwak, J. Y., Jung, J. Y., Hwang, S. W., Lee, W. T., Oh, U. (1998) A capsaicin-receptor antagonist, capsazepine, reduces inflammation-induced hyperalgesic responses in the rat: evidence for an endogenous capsaicin-like substance. *Neuroscience* **86**: 619–626

Malmberg, A. B., Yaksh, T. (1992) Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat. *J. Pharmacol. Exp. Ther.* **263**: 136–146

Malmberg A. B., Yaksh, T. (1995) Effect of continuous intrathecal infusion of omega-conopeptides, N-type calcium-channel blockers, on behavior and antinociception in the formalin and hot-plate tests in rats. *Pain* **60**: 83–90

Nishiyama, T. (2000) Interaction between intrathecal morphine and glutamate receptor antagonists in formalin test. *Eur. J. Pharmacol.* **395**: 203–210

Ohkubo, T., Shibata, M., Takahashi, H. (1993) The analgesia induced by intrathecal injection of ruthenium red. *Pain* **54**: 219–221

Peterson, M. A., Basbaum, A. I., Abbadie, C., Rohde, D. S., McKay, W. R., Taylor, B. K. (1997) The differential contribution of capsaicin-sensitive afferents to behavioral and cardiovascular measures of brief and persistent nociception and to Fos expression in the formalin test. *Brain Res.* **755**: 9–16

Pomonis, J. D., Harrison, J. E., Mark, L., Bristol, D. R., Valenzano, K. J., Walker, K. (2003) N-(4-Tertiarybutylphenyl)-4-(3-cholorphyridin-2-yl)tetrahydropyrazine-1(2H)-carbox-amide (BCTC), a novel, orally effective vanilloid receptor 1 antagonist with analgesic properties: II. In vivo characterization in rat models of inflammatory and neuropathic pain. *J. Pharmacol. Exp. Ther.* **306**: 387–393

Rigoni, M., Trevisani, M., Gazzieri, D., Nadaletto, R., Tognetto, M., Creminon, C., Davis, J. B., Campi, B., Amadesi, S., Geppetti, P., Harrison, S. (2003) Neurogenic responses mediated by vanilloid receptor-1 (TRPV1) are blocked by the high affinity antagonist, iodo-resiniferatoxin. *Br. J. Pharmacol.* **138**: 977–985

Santos, A. R., Calixto, J. B. (1997) Ruthenium red and capsazepine antinociceptive effect in formalin and capsaicin models of pain in mice. *Neurosci Lett.* **235**: 73–76

Tohda, C., Kuraishi, Y. (1996) Visualization of glutamate release from rat spinal cord with a confocal laser scanning microscope. *Neurosci. Res.* **24**: 183–187

Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Gilbert, H., Skinner, K., Raumann, B. E., Basbaum, A. I., Julius, D. (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* **21**: 531–543

Valtschanoff, J. G., Rustioni, A., Guo, A., Hwang, S. J. (2001) Vanilloid receptor VR1 is both presynaptic and postsynaptic in the superficial laminae of the rat dorsal horn. *J. Comp. Neurol.* **436**: 225–235

Wahl, P., Foged, C., Tullin, S., Thomsen, C. (2001) Iodo-resiniferatoxin, a new potent vanilloid receptor antagonist. *Mol. Pharmacol.* **59**: 9–15

Walker, K. M., Urban, L., Medhurst, S., J., Patel, S., Panesar, M., Fox, A., J., McIntyre, P. (2003) The VR1 antagonist capsazepine reverses mechanical hyperalgesia in models of inflammatory and neuropathic pain. *J. Pharmacol. Exp. Ther.* **304**: 56–62

Wardle, K. A., Ranson, J., Sanger, G. J. (1997) Pharmacological characterization of the vanilloid receptor in the rat spinal cord. *Br. J. Pharmacol.* **121**: 1012–1016

Yang, K., Kumamoto, E., Furue, H., Yoshimura, M. (1998) Capsaicin facilitates excitatory but not inhibitory synaptic transmission in substantia gelatinosa of the rat spinal cord. *Neurosci. Lett.* **255**: 135–138