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TRPV1 antagonist, SB-366791, inhibits glutamatergic synaptic transmission in rat spinal dorsal horn following peripheral inflammation

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Abstract

The anti-hyperalgesic effects of TRPV1 receptor antagonists are well documented in animal models of pain, however, the precise site of their action is not known. Here we have examined the effects of the selective TRPV1 antagonist SB-366791 on glutamatergic synaptic transmission in substantia gelatinosa using spinal cord slices from either control rats or animals that had undergone a peripheral inflammation induced by intraplantar injection of Freund's complete adjuvant (FCA). In control animals, SB-366791 (30 μM) had no effect on spontaneous excitatory post-synaptic currents (sEPSC) or evoked EPSCs. In slices from FCA-inflamed animals, SB-366791 decreased sEPSC frequency to 66±8% of control in 5/10 neurones, and decreased miniature glutamatergic EPSCs (mEPSC) frequency to 63±4% of control, in 6/7 neurones; with no significant effect on sEPSC or mEPSC amplitude. Dorsal root evoked EPSCs at C-fibre intensity were reduced to 72±6% of control by SB-366791 (30 μM) in 3/4 neurones from FCA-treated animals. In conclusion, SB-366791 inhibited glutamatergic transmission in a subset of neurones via a pre-synaptic mechanism following peripheral inflammation. We hypothesise that during peripheral inflammation spinal TRPV1 becomes tonically active, promoting the synaptic release of glutamate. These results provide evidence for a mechanism by which TRPV1 contributes to inflammatory pain and provides a basis for the understanding of the efficacy of TRPV1 antagonists.

Keywords: TRPV1; Spinal cord; Inflammation; Glutamate; Synaptic transmission; Substantia gelatinosa

1. Introduction

Transient receptor potential vanilloid 1 (TRPV1) is a ligand-gated cation channel that is primarily found on small-diameter primary afferents, particularly unmyelinated C-fibres, and is activated by heat (>42 °C), protons and a range of both endogenous and exogenous ligands (Caterina et al., 1997; Tominaga et al., 1998; Smart et al., 2000; Van Der Stelt and Di Marzo, 2004; Calixto et al., 2005). The polymodal activation exhibited by TRPV1 makes it of particular interest in pain transduction, because it creates the potential for a single protein to integrate a range of varied noxious stimuli (Caterina et al., 1997; Hayes et al., 2000). TRPV1 null mice are devoid of the hypersensitivity to thermal stimuli which accompanies an inflammatory insult (Caterina et al., 2000; Davis et al., 2000), providing key evidence for the role of TRPV1 in inflammatory pain processing and indicating that TRPV1 function may well be upregulated by inflammatory cascades. Mediators of inflammation, such as ATP, bradykinin, acid and prostaglandins, are known to activate, modulate and sensitise ion channels and receptors causing hypersensitivity, and there is evidence that not only is the expression of TRPV1 increased by peripheral inflammation (Amaya et al., 2003; Tohda et al., 2001), but that TRPV1 function is enhanced in the presence of inflammatory mediators (Tominaga et al., 1998, 2001; Sugiura et al., 2002; Moriyama et al., 2005).

Clinically, TRPV1 agonists have been used as topical analgesics (Mason et al., 2004 for review) and have shown promise in the treatment of incontinence (Cruz, 2004 for review). The clinical efficacy of TRPV1 agonists is thought to be through desensitisation and/or downregulation of the receptor (Simone et al., 1998; Nolano et al., 1999) or a physical or functional "ablation" of nociceptive terminals. A number of TRPV1 antagonists are reported to be in clinical development for pain and related conditions. There may be potential benefits of an antagonistic approach, including a more rapid onset of action and

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a lack of the initial pain sensation that results from agonist activation of TRPV1 (Rami and Gunthorpe, 2004 for review). The analgesic potential of TRPV1 antagonists was initially studied using the tool compound capsazepine. Although efficacious, this molecule is far from an ideal TRPV1 antagonist on a number of fronts, it has well documented actions at a range of other channels (Docherty et al., 1997; Liu and Simon, 1997; Gill et al., 2004; Behrendt et al., 2004; Weil et al., 2005) and a poor pharmacokinetic profile (Lopez-Rodriguez et al., 2003). In recent years, a number of more selective TRPV1 antagonists have appeared, the first of which was Iodo-Resiniferatoxin (Wahl et al., 2001). Although Iodo-Resiniferatoxin is a potent and selective inhibitor of TRPV1 it has limited utility in vivo (Almasi et al., 2003; Rigoni et al., 2003; Seabrook et al., 2002), and has recently been shown to have partial agonist actions at TRPV1 in vitro (Shimizu et al., 2005). The effect of other antagonists of TRPV1 have been investigated using tools such as BCTC (Valenzano et al., 2003), AMG9810 (Gavva et al., 2005) and A-425619 (El Kouhen et al., 2005), as well as SB-366791 (Gunthorpe et al., 2004). All of these compounds are potent inhibitors of TRPV1 activation by heat, acid and capsaicin and a number of these have been shown to possess efficacy in in vivo animal models of pain, such as capsaicin-induced wiping, capsaicin- and FCA-induced hyperalgesia, and in neuropathic pain models including partial sciatic nerve injury and chronic constriction injury (Gavva et al., 2005; Honore et al., 2005; Kanai et al., 2005; Pomonis et al., 2003; Varga et al., 2005). Although this provides clear evidence for the utility of TRPV1 antagonists in pain, the specific site of their action is still to be determined.

The small-diameter primary afferent fibres, on which TRPV1 is located, carry nociceptive information from the periphery to the superficial dorsal horn of the spinal cord. It is here, in particular within substantia gelatinosa (lamina II of dorsal horn), where the initial modulation and integration of nociceptive information occurs (Sugiura et al., 1986; Willis and Coggeshall, 1991). To date, the post-inflammatory role of TRPV1 has yet to be extensively investigated at this first level of integration of peripheral transmission. Here we report that following peripheral inflammation, a tonic excitatory presynaptic activity appears in the spinal cord that is sensitive to the TRPV1 antagonist SB-366791. This provides further validation for the role of TRPV1 in inflammatory pain, and an insight into the potential site of action that may underlie the therapeutic potential of TRPV1 antagonists.

2. Materials and methods

2.1. Freund's complete adjuvant (FCA) model of inflammatory pain

All animal procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines. Lister-hooded rats (17–29 days old) were injected intraplantar with a 25 μl Freund's complete adjuvant (FCA, 1 mg/ml solution of Mycobacterium tuberculosis; Sigma, St. Louis, MO), in their left hind paw. This procedure produced a peripheral inflammation and pain hypersensitivity that was characterised by a reduction in paw withdrawal thresholds, peaking 24 h post-injection (Walker et al., 2001). 24 h after receiving the FCA treatment animals underwent euthanasia and removal of the spinal cords, as described below.

2.2. Slice preparation

These methods have been previously described in detail (Morisset and Nagy, 1989). Briefly, Lister-hooded rats, aged 17 to 29 days old, were anaesthetised with halothane and subsequently decapitated in accordance with UK Home Office guidelines. A dorsal laminectomy was performed to remove the spinal cord, and 400 μm transverse slices were obtained from the lumbar enlargement (L4–L6), using a Leica VT1000S automatic slicer. In animals that had undergone an FCA injection in the left hind paw, the spinal cord was orientated such that a small incision could be made through the tissue on the right ventral side of the cord, therefore allowing identification of the left dorsal horn and substantia gelatinosa to enable recording ipsilateral to the injected side. Spinal cord slices were transferred to a submerged recording chamber where they were superfused at 2–3 ml/min with an artificial cerebrospinal fluid (aCSF) equilibrated with 95% O_2–5% CO_2, at a temperature of 30 °C. The aCSF contained (in mM) 124 NaCl, 2.4 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 26 $NaHCO_3$, and 10 glucose. Small deviations in the pH of the solution were adjusted back to pH 7.4. Slices were allowed to recover for at least 1 h before recordings were initiated.

2.3. Electrophysiological recordings

Lamina II neurones were visually identified using infrared differential interference contrast microscopy (BX50WI, Olympus) and patch-clamp recordings were made in the whole cell configuration with pipettes (6–10 MΩ) containing (in mM) 120 K-Gluconate, 20 KCl, 0.1 $CaCl_2$, 1.3 $MgCl_2$, 1 EGTA, 10 HEPES, 0.1 GTP, 0.2 cAMP, 0.1 leupeptin, and 3 Na_2-ATP, pH 7.3 (KOH). Membrane currents were recorded using an Axopatch 200B amplifier (Axon Instruments) and filtered at 2 kHz. Signals were digitized at 20 kHz using a Digidata 1200 interface (Axon Instruments) and the pClamp 9 software package. Excitatory postsynaptic currents (EPSCs) were recorded in all neurons at a holding potential of −60 to −70 mV. The addition of 30 μM 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX) abolished all synaptic events, indicating the involvement of non-NMDA glutamate receptors (data not shown). Synaptic responses were evoked using a concentric bipolar electrode, inner diameter 12.5 μm, outer diameter 125 μm, (FHC, Bowdoinham, ME) placed on the dorsal root. The stimulus intensity used to activate Aδ- and C-fibres was >500 μA and 500 μs (Morisset and Nagy, 1989). The stimulus was applied every 20–30 s, to avoid any activity-dependent modification of synaptic responses.

2.4. Analysis

All data were captured using pClamp 9 software (Axon Instruments Ltd) and analysed off-line using the programmes

Mini Analysis (Synaptosoft) and Clampfit (Axon Instruments). Data were compared using the non-parametric Kolmogorov–Smirnov test or the Analysis of Variance (ANOVA) test, with $P<0.05$ being taken as a measure of statistical significance. All data are expressed as means±S.E.M; *n* refers to the number of neurones studied.

2.5. Drugs

Drugs were applied by addition to the perfusing aCSF. Tetrodotoxin (TTX) and NBQX disodium salt were purchased from Tocris. SB-366791 (*N*-(3-methoxyphenyl)-4-chlorocinnamide) was synthesised in GSK medicinal chemistry laboratories, Harlow as previously described (Gunthorpe et al., 2004). Stock solutions were made up to 10 mM and then diluted into aCSF just before application. The final dimethyl sulphoxide (DMSO) concentration was never greater than 0.3%.

3. Results

3.1. Naïve animals

3.1.1. Effect of SB-366791 on spontaneous synaptic transmission

To define the role of TRPV1 in the transmission of nociceptive information from the periphery to the spinal cord, we began by assessing the effect of SB-366791 on spontaneous synaptic transmission in substantia gelatinosa neurones in spinal cord slices from naïve animals. Whole cell patch-clamp recordings were made from second order dorsal horn neurones or interneurones within the lamina II region of the spinal cord (see Materials and methods). All cells studied exhibited spontaneous excitatory post-synaptic currents (sEPSCs) at a holding potential of between −60 mV and −70 mV. In 10 cells, the sEPSCs occurred with mean frequency of 4.7±0.9 Hz (means ranging from 1.1 to 7.7 Hz) and had a mean amplitude of 26±4 pA (means ranging from 12 to 43 pA). SB-366791 (30 μM) was bath applied and a small but significant reduction in the frequency of spontaneous currents, to 82% of the control, was recorded in 1 cell out of 10, with no effect on sEPSCs in the other 9 cells. The mean data has been calculated for all 10 cells recorded and overall SB-366791 had no significant effect on sEPSC amplitude (94±2% of control), or frequency (102±5% of control) (Fig. 1A, mean data Fig. 1B). Moreover, for all the neurones recorded we did not find any change in the input resistance or holding current in response to compound application.

3.1.2. Effect of SB-366791 on evoked synaptic transmission

In the spinal cord slices taken from naïve animals, a single stimulation to the dorsal root evoked EPSCs with a mean peak amplitude of −340±160 pA ($n=5$). All of the neurones recorded had C-fibre inputs since the stimulation threshold to obtain a synaptic response was >500 μA in amplitude, 500 μs in duration. 30 μM SB-366791 had no significant effect on the evoked EPSC amplitude (102±5% of control, $n=5$) (see Fig. 1C and mean data of Fig. 1D, $P=0.53$).

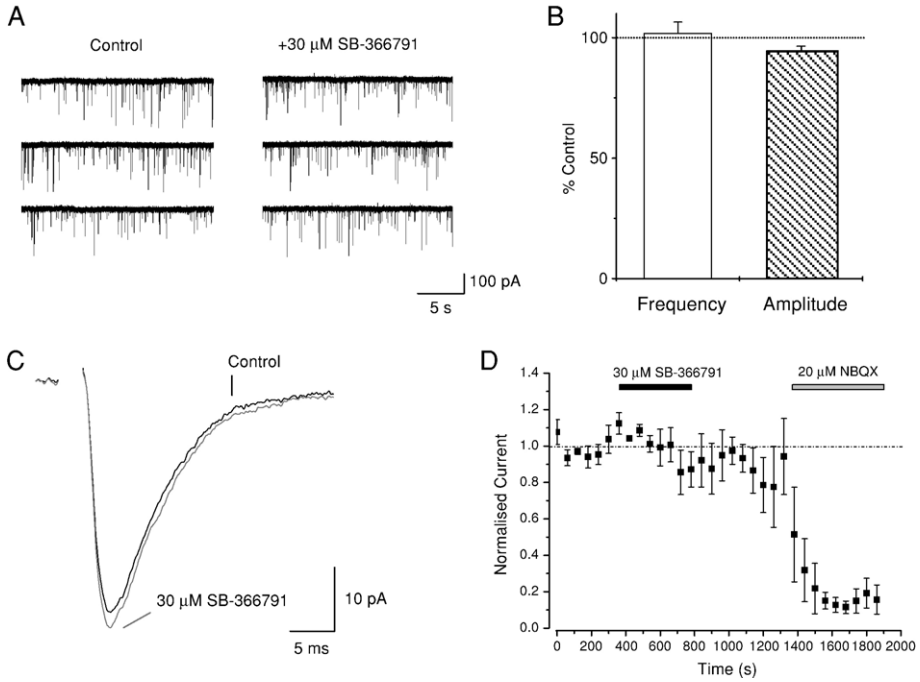

Fig. 1. Spontaneous EPSCs recorded from substantia gelatinosa neurones are not affected by the TRPV1 antagonist SB-366791. (A) An excerpt of recording showing glutamatergic spontaneous EPSCs recorded from a lamina II neurone over a 1 min period, in control conditions on the left and following bath application of 30 μM SB-366791 on the right. The addition of SB-366791 has no significant effect on the kinetics, frequency or amplitude of the EPSCs (see text). (B) Graph showing the mean data for the effects of SB-366791 on the frequency and amplitude of spontaneous EPSCs. (C) An excerpt of recording showing C-fibre evoked EPSCs recorded from a lamina II neurone, under control conditions, and in the presence of 30 μM SB-366791. The addition of SB-366791 had no significant effect on the amplitude of the eEPSC. (D) The graph below illustrates the time-course of the effects of SB-366791 and NBQX on the eEPSC peak amplitude from all of the neurones recorded. Each data point represents the average amplitude of evoked current, recorded over a 1 min period.

Table 1
Lack of effect of peripheral inflammation induced by FCA on the properties of spontaneous EPSCs recorded in lamina II of spinal cord

	Frequency (Hz)	Amplitude (pA)	Decay time constant (ms)
Naïve	4.7±0.9	26±4	3.2±0.3
FCA	3.2±0.5	23±3	4.1±0.6

Table detailing the mean frequency, amplitude and decay constant tau values for spontaneous EPSCs recorded in spinal cord slices from naïve animals ($n=10$) and from animals that had been treated with FCA ($n=10$). There was no significant difference, ($P>0.05$) between the events recorded for these two groups.

3.2. FCA-treated animals

3.2.1. Effect of FCA treatment on synaptic transmission

Spontaneous EPSCs were recorded from all neurones studied in the ipsilateral substantia gelatinosa of slices taken from the FCA-treated animals. The sEPSCs had a mean frequency of 3.2±0.5 Hz (means ranging from 0.6 to 5.4 Hz) and an amplitude of 23±3 pA (means ranging from 9 to 37 pA) ($n=10$). When compared to the sEPSCs recorded from slices taken from naïve animals, no significant difference in the frequency ($P=0.14$), amplitude ($P=0.54$) or decay kinetics (mean time constant=3.2±0.3 ms for slices from naïve animals and 4.1±0.6 ms for slices from FCA-treated animals, ($P=0.17$) was observed (Table 1). In addition to this, no significant difference was seen for the membrane resistance between naïve and FCA-treated animals (597±132 MΩ ($n=7$) and 681±61 MΩ ($n=5$), respectively; $P=0.58$).

3.2.2. Effect of SB-366791 on spontaneous and miniature synaptic transmission

Once a stable baseline of sEPSCs was established, SB-366791 (30 μM) was bath applied and a significant change in the sEPSCs was observed in a proportion of the neurones studied. In 5 out of the 10 neurones, SB-366791 addition resulted in a decrease in the frequency of sEPSCs (Fig. 2A and B) to 66±8% of the predrug level; the change in frequency was not accompanied by any significant change in the sEPSC amplitude (92±4% of control, Fig. 2B).

Given this effect, the origin of this modulation of synaptic transmission by a TRPV1 antagonist was further investigated by assessing the effect of SB-366791 on miniature EPSCs (mEPSCs) recorded in the presence of TTX (1 μM; Yoshimura and Jessell, 1990). Under these conditions, where all action potential driven neurotransmitter release had been eliminated, neither the frequency nor the amplitude of mEPSCs was altered significantly compared to sEPSCs. This suggests that the majority of the synaptic release of glutamate occurring in this in vitro preparation is not driven by action potential firing, but is most likely to be from spontaneous vesicular glutamate release.

The mEPSCs recorded had a mean frequency of 3.4±0.7 Hz (means range 1.0 to 5.9), and a mean amplitude of 19.0±1.7 pA (means range 11.9 to 25.1, $n=7$). As shown in Fig. 3A, SB-366791 reduced the frequency of mEPSCs, and this is illustrated in Fig. 3B by the rightward shift of the cumulative inter-event interval distribution plot ($P=0.0001$); the amplitude of the events was not significantly affected ($P=0.18$, Fig. 3A and C). On average SB-366791 (30 μM) reduced the frequency of mEPSCs in 6 out of 7 neurones to 63±4% of control values ($P=0.001$). No effect was seen on the amplitude (96±4% of control, $P=0.33$, Fig. 3B) suggesting a pre-synaptic site of action for SB-366791.

3.2.3. Effect of SB-366791 on evoked synaptic transmission

In spinal cord slices taken from FCA-treated animals, a single stimulation to the dorsal root evoked EPSCs with a mean peak amplitude of −86±17 pA ($n=5$). Of the 5 neurones recorded 4 had C-fibre inputs since the stimulation threshold to obtain a synaptic response was >500 μA in amplitude, 500 μs in duration. The other cell recorded had an Aδ-fibre input since the threshold for evoked EPSCs was below the C-fibre threshold. We repeated the stimulus every 30 s until the amplitude of the evoked EPSC stabilised, before studying the effects of SB-366791. In 1 out of 5 neurones SB-366791 had no effect, however in the other 4 neurones the amplitude of the current was significantly reduced to 76±6% of control in the presence of 30 μM SB-366791 (see Fig. 4A and B, $P<0.005$).

Overall these results are consistent with SB-366791 acting pre-synaptically via the TRPV1 receptors located on primary afferent neurones terminating in the dorsal horn of the spinal

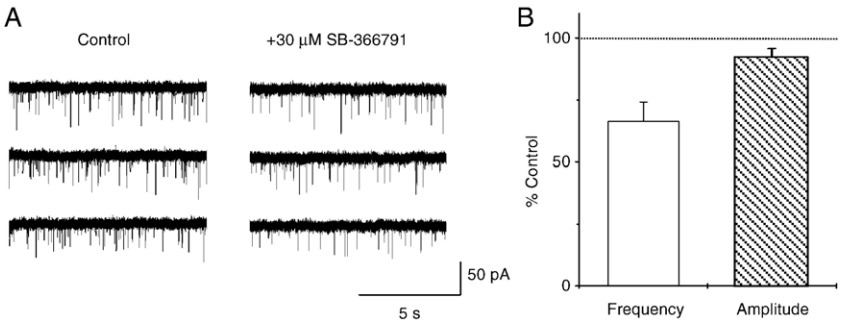

Fig. 2. SB-366791 reduces the frequency of spontaneous EPSCs in the spinal cord slices from FCA-treated rats. (A) An excerpt of recording showing spontaneous EPSCs recorded from a lamina II neurone over a 30 s period, under control conditions on the left and following bath application of 30 μM SB-366791 on the right. (B) Graph illustrating the mean data for the effects of SB-366791 on the frequency and amplitude of spontaneous EPSCs compared to control in 5 out of 10 neurones.

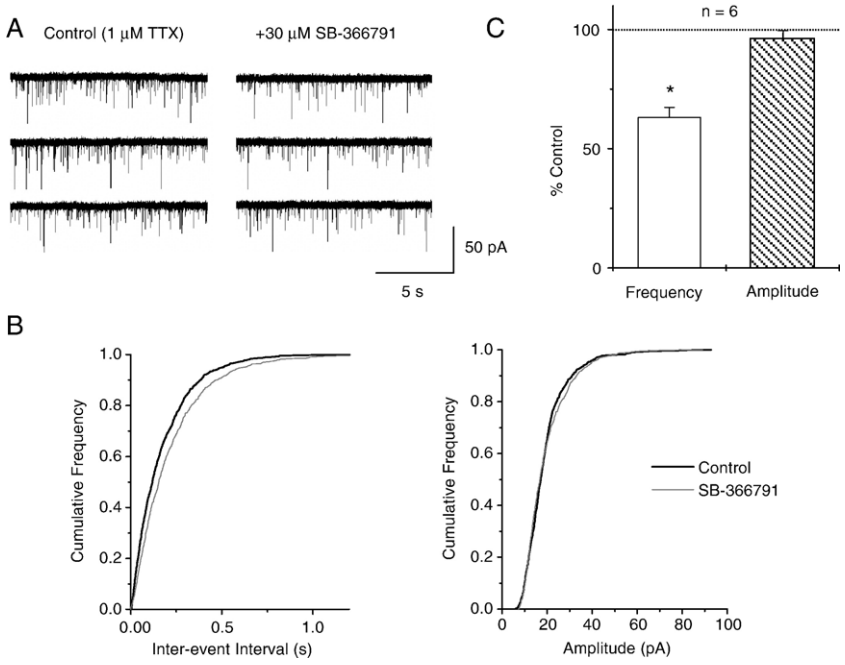

Fig. 3. SB-366791 inhibits the frequency of miniature EPSCs. (A) An excerpt of recording showing miniature EPSCs recorded from a lamina II neurone over a 30 s period, in the presence of 1 μM TTX. The events recorded under control conditions on the left and following bath application of 30 μM SB-366791 on the right. Following the addition of SB-366791, the frequency of the miniature EPSCs was reduced. (B) Cumulative distribution plots of the amplitude and inter-event interval of mEPSCs. SB-366791 produced a rightward shift in the inter-event interval, indicating a decrease in the mEPSC frequency ($P=0.0001$), and had no significant effect on the amplitude distribution ($P=0.18$). (C) Graph illustrating the mean data for the effects of SB-366791 on the frequency ($P=0.001$) and amplitude ($P=0.33$) of miniature EPSCs in 6 out of 7 neurones.

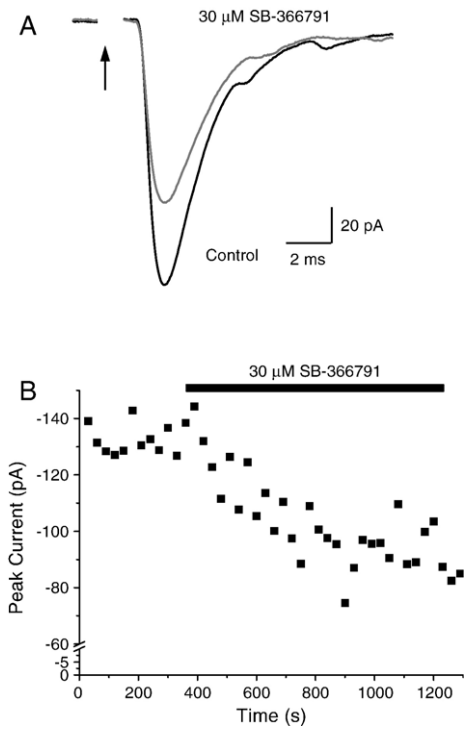

Fig. 4. SB-366791 inhibits the amplitude of C-fibre evoked EPSCs. (A) An excerpt of recording showing C-fibre evoked EPSCs recorded from a lamina II neurone, under control conditions, and in the presence of 30 μM SB-366791. The addition of SB-366791 significantly reduced the amplitude of the eEPSC. (B) Time-course for the neurones recorded in (A) showing the baseline recording followed by the addition of 30 μM SB-366791.

cord that are tonically active following a peripheral inflammatory insult.

4. Discussion

In this study we used the selective TRPV1 antagonist SB-366791 to investigate the role of TRPV1 in synaptic transmission in substantia gelatinosa of the rat spinal cord. We found that following peripheral inflammation, but not under naïve conditions, SB-366791 inhibited glutamatergic transmission in a subset of neurones via a pre-synaptic mechanism. SB-366791 has been reported to be a potent and selective inhibitor of both recombinant ($IC_{50}=7.5$ nM at rat TRPV1 channels expressed in a HEK293 cells), and native TRPV1 channels (1 μM abolishes capsaicin evoked TRPV1 current in rat dorsal root ganglia neurones), and lacks many of the selectivity issues and modality specific effects associated with capsazepine (Docherty et al., 1997; Liu and Simon, 1997; Gill et al., 2004; Lopez-Rodriguez et al., 2003; Gunthorpe et al., 2004, Behrendt et al., 2004; Weil et al., 2005). SB-366791 has also been used in vivo to assess the potential analgesic action of the inhibition of TRPV1, and significantly inhibited capsaicin-induced hypothermia, eye wiping movements and vasodilatation in the knee joint (Varga et al., 2005). There is also other evidence for the efficacy of TRPV1 antagonists in vivo but as yet, the post-inflammatory role of TRPV1 has not been extensively investigated at the first level of integration of peripheral transmission, the substantia gelatinosa.

In these studies we have shown that SB-366791 inhibits glutamatergic transmission via an apparently pre-synaptic mechanism(s). These data are supported by in situ hybridisation and immunocytochemical data showing that TRPV1 mRNA and protein are localised on small- and medium-sized dorsal root ganglia neurones (Caterina et al., 1999; Helliwell et al., 1998; Michael and Priestley, 1999), as well as on central terminals of primary afferent neurones, which terminate in lamina I and II of the spinal dorsal horn (Tominaga et al., 1998). Although a study by Valtschanoff et al. (2001) suggested that TRPV1 is located on intrinsic spinal neurones, our data do not confirm these findings due to the lack of effect on the amplitude of miniature EPSCs, holding current and membrane resistance.

The reduction in transmitter release from the pre-synaptic terminal in the presence of SB-366791 may result from a number of mechanisms: the first mechanism to be considered is an inhibition of voltage gated Ca^{2+} channels. Although this has been shown in other areas of the central nervous system including locus coeruleus (Marinelli et al., 2002), and the substantia nigra (Marinelli et al., 2003), it is unlikely to be the case in the spinal cord since mEPSCs in this region are reported to be independent of the activity of pre-synaptic Ca^{2+} channels (Sillar and Simmers, 1994). Moreover, the values for the reduction in the frequency of spontaneous and miniature EPSCs are not significantly different (66±8% of control values and 63 ±4% of control, respectively, $P=0.73$) and previous work concluded that SB-366791 had no effect on voltage-gated Ca^{2+} channels found in acutely dissociated dorsal root ganglia neurones (Gunthorpe et al., 2004). This suggests that additional modulating mechanisms are occurring to affect the exocytotic pathway in the pre-synaptic terminal. One candidate mechanism, as previously suggested by Marinelli et al. (2003), is that the Ca^{2+} entry directly through TRPV1 may produce a facilitatory effect on the probability of neurotransmitter release. Subsequently, inhibition of this Ca^{2+} flux through tonically active TRPV1 by SB-366791 would thus decrease glutamate release.

As reported here, the effect of SB-366791 on EPSC frequency is present in a proportion of neurones in spinal cord slices taken from rats that had undergone a peripheral inflammation and no effect was recorded in naïve rats. We hypothesise that the peripheral inflammation sensitises TRPV1 producing a tonic activation of the receptors on sensory terminals within SG, and it is this additional activity that is inhibited in the presence SB-366791. If this hypothesis is correct then in the "non-responding" subset of neurones, where SB-366791 has no effect, it is likely that the pre-synaptic primary afferent neurone was not sensitised by the FCA injection in the periphery and/or was not a TRPV1 expressing neurone.

The involvement of TRPV1 in inflammatory processes has already been widely described in the literature. In whole animal studies, TRPV1 null mice have been shown to lack hypersensitivity to thermal stimuli following an inflammatory insult (Caterina et al., 2000; Davis et al., 2000) providing evidence for the essential role of TRPV1 in the development of thermal hyperalgesia. There are also changes in the expression levels of TRPV1 following a peripheral inflammatory insult: TRPV1 protein expression is increased at the DRG level (Amaya et al., 2003; Luo et al., 2004), and there is a subsequent axonal transport of TRPV1 mRNA in primary afferents to the central terminals (Tohda et al., 2001), where an increase in TRPV1 protein has been reported in the superficial layers of the spinal dorsal horn (Luo et al., 2004). Although the increase in the TRPV1 receptor expression may well result in a greater nociceptive response to peripheral inputs, the ability of pharmacological agents to activate TRPV1 present in substantia gelatinosa in naïve animals (Yang et al., 1998, 1999, 2000), indicates that the receptor is expressed at the cell membrane in the dorsal horn and is able to be activated under naïve conditions. Therefore an increase in the number of channels expressed is unlikely to account for the change in control of glutamatergic transmission alone. Instead the effects of inflammation on receptor function and sensitisation must also be considered.

There is evidence to show that TRPV1 can be directly activated by endogenous ligands, such as anandamide (Smart et al., 2000; Zygmunt et al., 1999) and arachidonic acid metabolites such as *N*-arachidonoyldopamine (NADA) and 12-hydroperoxyeicosatetraenoic acid (12-HPETE) (Toth et al., 2003; Hwang et al., 2000). In the spinal cord, the frequency of glutamatergic sEPSCs and mEPSCs is reported to increase in the presence of relatively high concentrations of anandamide (Morisset et al., 2001) or the anandamide analogue, AM404 (Yue et al., 2004). This effect, thought to be TRPV1 mediated, mimics that seen with capsaicin (Yang et al., 1998; Nakatsuka et al., 2002) and is sensitive to inhibition by capsazepine. As reported previously, the potency of "endovanilloids" such as anandamide may be relatively low, (for review see Van Der Stelt and Di Marzo, 2004) but under inflammatory conditions numerous mediators are released and can act synergistically. Secondly, TRPV1 can be modulated and sensitised by endogenous inflammatory mediators as well as directly activated. Phosphorylation of TRPV1 via protein kinase C (PKC)- and protein kinase A (PKA)-dependent mechanisms sensitises the receptor (increasing the probability of opening) to capsaicin, protons and anandamide (Premkumar and Ahern, 2000; De Petrocellis et al., 2001; Vellani et al., 2001), ATP (Tominaga et al., 1998), bradykinin and nerve growth factor (NGF) (Chuang et al., 2001; see Di Marzo et al., 2002 for review), and the level of phosphorylation (of ε-isozyme) of PKC has been shown to be upregulated in dorsal root ganglion neurones following FCA-induced inflammation (Zhou et al., 2003). Many of these modulators will be released in an inflammatory state, along with prostaglandins such as PGE_2 or PGI_2, and by reducing the temperature threshold for TRPV1 activation (Tominaga et al., 1998; Sugiura et al., 2002; Moriyama et al., 2005), will lead to sensitisation and even tonic activity of TRPV1 receptors at body temperature. Although we have proposed the involvement of many inflammatory mediators in the direct activation and sensitisation of TRPV1, more work must be done to address which ones specifically affect TRPV1 expression and modulate its function in this preparation.

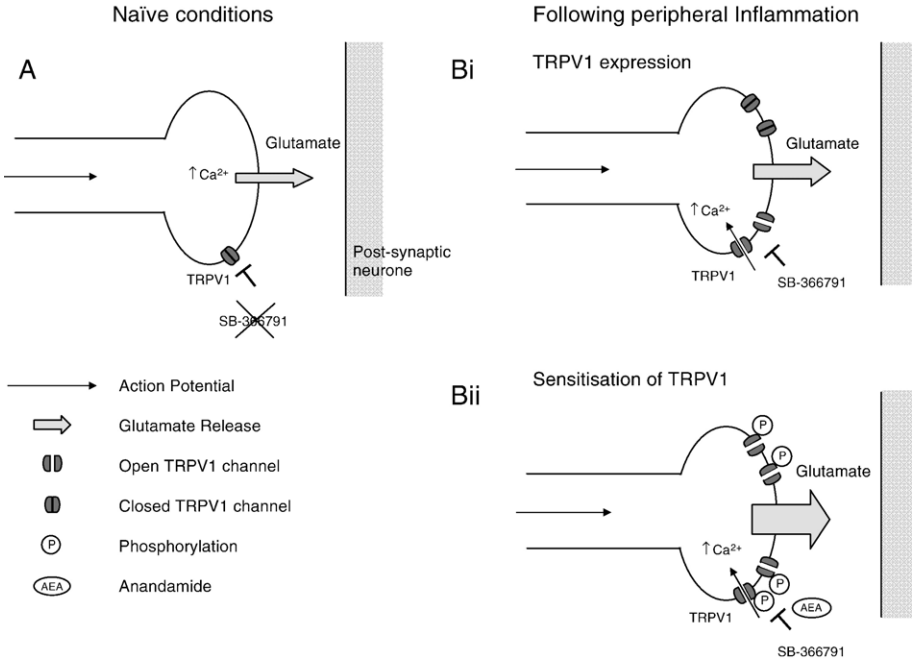

Fig. 5. Model of sensitisation and upregulation of TRPV1 following a peripheral inflammation. The central terminal of a primary afferent neurone terminating onto a spinal intrinsic neurone in substantia gelatinosa of the dorsal horn. (A) Naïve conditions: TRPV1 is present in the central terminals and is shown in the closed state under normal conditions. Action potentials result in glutamate release from the primary afferent neurone via other receptors (not shown) as indicated by the arrow. SB-366791 has no effect on glutamate release as the role of TRPV1 is limited under these conditions. (B) Following peripheral inflammation: (Bi) the expression of TRPV1 protein increases at the central terminals (Luo et al., 2004), resulting in greater nociceptive response to peripheral inputs. The magnitude of the glutamate release is denoted by the size of the arrow. (Bii) In combination with the changes occurring in Bi, the TRPV1 protein becomes phosphorylated by PKC and PKA, leading to an increase in channel open probability, sensitisation of the receptor to ligands such as anandamide (Di Marzo et al., 2002 for review) and a shift in the thermal activation threshold to more physiological temperatures (Tominaga et al., 1998; Sugiura et al., 2002; Moriyama et al., 2005). This change in both Bi and Bii increases the activity of TRPV1 and provides a mechanistic basis for the inhibitory effect seen in the presence of SB-366791.

To relate this back to the in vitro spinal cord preparation we have studied here, we suggest a number of processes that may account for the differential effect seen with SB-366791 in slices from naïve and FCA-treated animals. Under naïve conditions (Fig. 5A), the TRPV1 receptor will be inactive and present mainly in the closed state and so not susceptible to antagonism by SB-366791. Therefore the presence of this antagonist will have no effect on the pre-synaptic release of glutamate (Fig. 5A). Following a peripheral inflammation, the increase in TRPV1 expression at the central terminals of primary afferent neurones will enhance the nociceptive response to peripheral inputs (Fig. 5Bi). In combination with this, the sensitisation of TRPV1 would result in an increase in open channel probability and functionally active receptors at lower temperatures, including body temperature (Fig. 5Bii). This activity is a likely candidate effect underlying the behavioural readout of hyperalgesia seen in e.g. the FCA model of inflammatory pain. Here the effects of SB-366791 on this activity would be considered anti-hyperalgesic. Together these changes alter the role of TRPV1 in substantia gelatinosa, but the balance between them and the degree to which they occur in different pain states may vary and is yet to be established.

In summary, our results suggest that TRPV1 becomes functionally active in the substantia gelatinosa following peripheral inflammation, and promotes the synaptic release of glutamate. Our data provide an additional cellular basis for the anti-hyperalgesic effects of TRPV1 receptor antagonists and give further confidence for their therapeutic use in the treatment of pain indications and in particular inflammatory pain.

References

Almasi, R., Petho, G., Bolcskei, K., Szolcsanyi, J., 2003. Effect of resiniferatoxin on the noxious heat threshold temperature in the rat: a novel heat allodynia model sensitive to analgesics. Br. J. Pharmacol. 139, 49–58.

Amaya, F., Oh-hashi, K., Naruse, Y., Iijima, N., Ueda, M., Shimosato, G., Tominaga, M., Tanaka, Y., Tanaka, M., 2003. Local inflammation increases vanilloid receptor 1 expression within distinct subgroups of DRG neurons. Brain Res. 963, 190–196.

Behrendt, H.J., Germann, T., Gillen, C., Hatt, H., Jostock, R., 2004. Characterization of the mouse cold-menthol receptor TRPM8 and vanilloid receptor type-1 VR1 using a fluorometric imaging plate reader (FLIPR) assay. Br. J. Pharmacol. 141, 737–745.

Calixto, J.B., Kassuya, C.A., Andre, E., Ferreira, J., 2005. Contribution of natural products to the discovery of the transient receptor potential (TRP) channels family and their functions. Pharmacol. Ther. 106, 179–208.

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.

Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A.J., Julius, D., 1999. A capsaicin-receptor homologue with a high threshold for noxious heat. Nature 398, 436–441.

Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Petersen-Zeitz, K.R., Koltzenburg, M., Basbaum, A.I., Julius, D., 2000. Impaired

nociception and pain sensation in mice lacking the capsaicin receptor. Science 288, 306–313.

Chuang, H.H., Prescott, E.D., Kong, H., Shields, S., Jordt, S.E., Basbaum, A.I., Chao, M.V., Julius, D., 2001. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. Nature 411, 957–962.

Cruz, F., 2004. Mechanisms involved in new therapies for overactive bladder. Urology 63, 65–73.

Davis, J.B., Gray, J., Gunthorpe, M.J., Hatcher, J.P., Davey, P.T., Overend, P., Harries, M.H., Latcham, J., Clapham, C., Atkinson, K., Hughes, S.A., Rance, K., Grau, E., Harper, A.J., Pugh, P.L., Rogers, D.C., Bingham, S., Randall, A., Sheardown, S.A., 2000. Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. Nature 405, 183–187.

De Petrocellis, L., Harrison, S., Bisogno, T., Tognetto, M., Brandi, I., Smith, G.D., Creminon, C., Davis, J.B., Geppetti, P., Di Marzo, V., 2001. The vanilloid receptor (VR1)-mediated effects of anandamide are potently enhanced by the cAMP-dependent protein kinase. J. Neurochem. 77, 1660–1663.

Di Marzo, V., Blumberg, P.M., Szallasi, A., 2002. Endovanilloid signaling in pain. Curr. Opin. Neurobiol. 12, 372–379.

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.

El Kouhen, R., Surowy, C.S., Bianchi, B., Neelands, T., McDonald, H., Niforatos, W., Gomtsyan, A., Lee, C.H., Honore, P., Sullivan, J., Jarvis, M., Faltynek, C., 2005. A-425619, a novel and selective TRPV1 receptor antagonist, blocks channel activation by vanilloids, heat and acid. J. Pharmacol. Exp. Ther. 314, 400–409.

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.

Gill, C.H., Randall, A.D., Bates, S.A., Hill, K., Owen, D., Larkman, P.M., Cairns, W., Yusaf, S.P., Murdock, P.R., Strijbos, P.J., Powell, A.J., Benham, C.D., Davies, C.H., 2004. Characterization of the human HCN1 channel and its inhibition by capsazepine. Br. J. Pharmacol. 143, 411–421.

Gunthorpe, M.J., Rami, H.K., Jerman, J.C., Smart, D., Gill, C.H., Soffin, E.M., Luis, H.S., Lappin, S.C., Egerton, J., Smith, G.D., Worby, A., Howett, L., Owen, D., Nasir, S., Davies, C.H., Thompson, M., Wyman, P.A., Randall, A.D., Davis, J.B., 2004. Identification and characterisation of SB-366791, a potent and selective vanilloid receptor (VR1/TRPV1) antagonist. Neuropharmacology 46, 133–149.

Hayes, P., Meadows, H.J., Gunthorpe, M.J., Harries, M.H., Duckworth, D.M., Cairns, W., Harrison, D.C., Clarke, C.E., Ellington, K., Prinjha, R.K., Barton, A.J., Medhurst, A.D., Smith, G.D., Topp, S., Murdock, P., Sanger, G.J., Terrett, J., Jenkins, O., Benham, C.D., Randall, A.D., Gloger, I.S., Davis, J.B., 2000. Cloning and functional expression of a human orthologue of rat vanilloid receptor-1. Pain 88, 205–215.

Helliwell, R.J., McLatchie, L.M., Clarke, M., Winter, J., Bevan, S., McIntyre, P., 1998. Capsaicin sensitivity is associated with the expression of the vanilloid (capsaicin) receptor (VR1) mRNA in adult rat sensory ganglia. Neurosci. Lett. 250, 177–180.

Honore, P., Wismer, C.T., Mikusa, J.P., Zhu, C.Z., Zhong, C., Gauvin, D.M., Gomtsyan, A., El Kouhen, R., Lee, C.H., Marsh, K., Sullivan, J.P., Faltynek, C.R., Jarvis, M.F., 2005. A-425619, a novel TRPV1 receptor antagonist, relieves pathophysiological pain associated with inflammation and tissue injury in rats. J. Pharmacol. Exp. Ther. 314, 410–421.

Hwang, S.W., Cho, H., Kwak, J., Lee, S.Y., Kang, C.J., Jung, J., Cho, S., Min, K.H., Suh, Y.G., Kim, D., Oh, U., 2000. Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. Proc. Natl Acad. Sci. U. S. A. 97, 6155–6160.

Kanai, Y., Nakazato, E., Fujiuchi, A., Hara, T., Imai, A., 2005. Involvement of an increased spinal TRPV1 sensitization through its up-regulation in mechanical allodynia of CCI rats. Neuropharmacology 49, 977–984.

Liu, L., Simon, S.A., 1997. Capsazepine, a vanilloid receptor antagonist, inhibits nicotinic acetylcholine receptors in rat trigeminal ganglia. Neurosci. Lett. 228, 29–32.

Lopez-Rodriguez, M.L., Viso, A., Ortega-Gutierrez, S., 2003. VR1 receptor modulators as potential drugs for neuropathic pain. Mini Rev. Med. Chem. 3, 729–748.

Luo, H., Cheng, J., Han, J.S., Wan, Y., 2004. Change of vanilloid receptor 1 expression in dorsal root ganglion and spinal dorsal horn during inflammatory nociception induced by complete Freund's adjuvant in rats. Neuroreport 15, 655–658.

Marinelli, S., Vaughan, C.W., Christie, M.J., Connor, M., 2002. Capsaicin activation of glutamatergic synaptic transmission in the rat locus coeruleus in vitro. J. Physiol. 543, 531–540.

Marinelli, S., Di Marzo, V., Berretta, N., Matias, I., Maccarrone, M., Bernardi, G., Mercuri, N.B., 2003. Presynaptic facilitation of glutamatergic synapses to dopaminergic neurons of the rat substantia nigra by endogenous stimulation of vanilloid receptors. J. Neurosci. 23, 3136–3144.

Mason, L., Moore, R.A., Derry, S., Edwards, J.E., McQuay, H.J., 2004. Systematic review of topical capsaicin for the treatment of chronic pain. Br. Med. J. 328, 991–996.

Michael, G.J., Priestley, J.V., 1999. Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. J .Neurosci. 19, 1844–1854.

Morisset, V., Nagy, F., 1989. Nociceptive integration in the rat spinal cord: role of non-linear membrane properties of deep dorsal horn neurons. Eur. J. Neurosci. 10, 3642–3652.

Morisset, V., Ahluwalia, J., Nagy, I., Urban, L., 2001. Possible mechanisms of cannabinoid-induced antinociception in the spinal cord. Eur. J. Pharmacol. 429, 93–100.

Moriyama, T., Higashi, T., Togashi, K., Iida, T., Segi, E., Sugimoto, Y., Tominaga, T., Narumiya, S., Tominaga, M., 2005. Sensitization of TRPV1 by EP1 and IP reveals peripheral nociceptive mechanism of prostaglandins. Mol. Pain 1, 1–13.

Nakatsuka, T., Furue, H., Yoshimura, M., Gu, J.G., 2002. Activation of central terminal vanilloid receptor-1 receptors and alpha beta-methylene-ATP-sensitive P2X receptors reveals a converged synaptic activity onto the deep dorsal horn neurons of the spinal cord. J. Neurosci. 22, 1228–1237.

Nolano, M., Simone, D.A., Wendelschafer-Crabb, G., Johnson, T., Hazen, E., Kennedy, W.R., 1999. Topical capsaicin in humans: parallel loss of epidermal nerve fibers and pain sensation. Pain 81, 135–145.

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.

Premkumar, L.S., Ahern, G.P., 2000. Induction of vanilloid receptor channel activity by protein kinase C. Nature 408, 985–990.

Rami, H.K., Gunthorpe, M.J., 2004. The therapeutic potential of TRPV1 (VR1) antagonists: clinical answers await. Drug Discov. Today 1, 97–104.

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.

Seabrook, G.R., Sutton, K.G., Jarolimek, W., Hollingworth, G.J., Teague, S., Webb, J., Clark, N., Boyce, S., Kerby, J., Ali, Z., Chou, M., Middleton, R., Kaczorowski, G., Jones, A.B., 2002. Functional properties of the high-affinity TRPV1 (VR1) vanilloid receptor antagonist (4-hydroxy-5-iodo-3-methoxyphenylacetate ester) iodo-resiniferatoxin. J. Pharmacol. Exp. Ther. 303, 1052–1060.

Shimizu, I., Iida, T., Horiuchi, N., Caterina, M.J., 2005. 5-Iodoresiniferatoxin evokes hypothermia in mice and is a partial TRPV1 agonist in vitro. J. Pharmacol. Exp. Ther. 314, 1378–1385.

Sillar, K.T., Simmers, A.J., 1994. Presynaptic inhibition of primary afferent transmitter release by 5-hydroxytryptamine at a mechanosensory synapse in the vertebrate spinal cord. J. Neurosci. 14, 2636–2647.

Simone, D.A., Nolano, M., Johnson, T., Wendelschafer-Crabb, G., Kennedy, W.R., 1998. Intradermal injection of capsaicin in humans produces

degeneration and subsequent reinnervation of epidermal nerve fibers: correlation with sensory function. J. Neurosci. 18, 8947–8959.

Smart, D., Gunthorpe, M.J., Jerman, J.C., Nasir, S., Gray, J., Muir, A.I., Chambers, J.K., Randall, A.D., Davis, J.B., 2000. The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). Br. J. Pharmacol. 129, 227–230.

Sugiura, Y., Lee, C.L., Perl, E.R., 1986. Central projections of identified, unmyelinated (C) afferent fibers innervating mammalian skin. Science 234, 358–361.

Sugiura, T., Tominaga, M., Katsuya, H., Mizumura, K., 2002. Bradykinin lowers the threshold temperature for heat activation of vanilloid receptor 1. J. Neurophysiol. 88, 544–548.

Tohda, C., Sasaki, M., Konemura, T., Sasamura, T., Itoh, M., Kuraishi, Y., 2001. Axonal transport of VR1 capsaicin receptor mRNA in primary afferents and its participation in inflammation-induced increase in capsaicin sensitivity. J. Neurochem. 76, 1628–1635.

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.

Tominaga, M., Wada, M., Masu, M., 2001. Potentiation of capsaicin receptor activity by metabotropic ATP receptors as a possible mechanism for ATP-evoked pain and hyperalgesia. Proc. Natl. Acad. Sci. U. S. A. 98, 6951–6956.

Toth, A., Kedei, N., Wang, Y., Blumberg, P.M., 2003. Arachidonyl dopamine as a ligand for the vanilloid receptor VR1 of the rat. Life Sci. 73, 487–498.

Valenzano, K.J., Grant, E.R., Wu, G., Hachicha, M., Schmid, L., Tafesse, L., Sun, Q., Rotshteyn, Y., Francis, J., Limberis, J., Malik, S., Whittemore, E.R., Hodges, D., 2003. *N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carbox-amide (BCTC), a novel, orally effective vanilloid receptor 1 antagonist with analgesic properties: I. In vitro characterization and pharmacokinetic properties. J. Pharmacol. Exp. Ther. 306, 377–386.

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.

Van Der Stelt, M., Di Marzo, V., 2004. Endovanilloids. Putative endogenous ligands of transient receptor potential vanilloid 1 channels. Eur. J. Biochem. 271, 1827–1834.

Varga, A., Nemeth, J., Szabo, A., McDougall, J.J., Zhang, C., Elekes, K., Pinter, E., Szolcsanyi, J., Helyes, Z., 2005. Effects of the novel TRPV1 receptor antagonist SB366791 in vitro and in vivo in the rat. Neurosci. Lett. 385, 137–142.

Vellani, V., Mapplebeck, S., Moriondo, A., Davis, J.B., McNaughton, P.A., 2001. Protein kinase C activation potentiates gating of the vanilloid receptor VR1 by capsaicin, protons, heat and anandamide. J. Physiol. 534, 813–825.

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

Walker, K., Bowes, M., Panesar, M., Davis, A., Gentry, C., Kesingland, A., Gasparini, F., Spooren, W., Stoehr, N., Pagano, A., Flor, P.J., Vranesic, I., Lingenhoehl, K., Johnson, E.C., Varney, M., Urban, L., Kuhn, R., 2001. Metabotropic glutamate receptor subtype 5 (mGlu5) and nociceptive function: I. Selective blockade of mGlu5 receptors in models of acute, persistent and chronic pain. Neuropharmacology 40, 1–9.

Weil, A., Moore, S.E., Waite, N.J., Randall, A., Gunthorpe, M.J., 2005. Conservation of functional and pharmacological properties in the distantly related temperature sensors TRPV1 and TRPM8. Mol. Pharmacol. 68, 518–527.

Willis Jr., W.D., Coggeshall, R.E., 1991. Sensory Mechanisms of the Spinal Cord. Plenum Press, New York, NY.

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.

Yang, K., Kumamoto, E., Furue, H., Li, Y.Q., Yoshimura, M., 1999. Action of capsaicin on dorsal root-evoked synaptic transmission to substantia gelatinosa neurons in adult rat spinal cord slices. Brain Res. 830, 268–273.

Yang, K., Kumamoto, E., Furue, H., Li, Y.Q., Yoshimura, M., 2000. Capsaicin induces a slow inward current which is not mediated by substance P in substantia gelatinosa neurons of the rat spinal cord. Neuropharmacology 39, 2185–2194.

Yoshimura, M., Jessell, T., 1990. Amino acid-mediated EPSPs at primary afferent synapses with substantia gelatinosa neurones in the rat spinal cord. J. Physiol. 430, 315–335.

Yue, H.Y., Fujita, T., Kawasaki, Y., Kumamoto, E., 2004. AM404 enhances the spontaneous release of L-glutamate in a manner sensitive to capsazepine in adult rat substantia gelatinosa neurones. Brain Res. 1018, 283–287.

Zhou, Y., Li, G.D., Zhao, Z.Q., 2003. State-dependent phosphorylation of epsilon-isozyme of protein kinase C in adult rat dorsal root ganglia after inflammation and nerve injury. J. Neurochem. 85, 571–580.

Zygmunt, P.M., Petersson, J., Andersson, D.A., Chuang, H., Sorgard, M., Di Marzo, V., Julius, D., Hogestatt, E.D., 1999. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature 400, 452–457.