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RESEARCH ARTICLE

Physiological and behavioral evidence of a capsaicin-sensitive TRPV-like channel in the medicinal leech

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ABSTRACT

Transient receptor potential vanilloid (TRPV) channels are found throughout the animal kingdom, where they play an important role in sensory transduction. In this study, we combined physiological studies with *in vivo* behavioral experiments to examine the presence of a putative TRPV-like receptor in the medicinal leech, building upon earlier studies in this lophotrochozoan invertebrate. The leech polymodal nociceptive neuron was activated by both peripheral and central application of the TRPV1-activator capsaicin in a concentration-dependent manner, with 100 µmol l^{-1} being the lowest effective concentration. Responses to capsaicin were inhibited by the selective TRPV1 antagonist SB366791. The polymodal nociceptive neuron also responded to noxious thermal stimuli (>40°C), and this response was also blocked by SB366791. Capsaicin sensitivity was selective to the polymodal nociceptor with no direct response being elicited in the mechanical nociceptive neuron or in the non-nociceptive touch- or pressure-sensitive neurons. Capsaicin also elicited nocifensive behavioral responses (withdrawals and locomotion) in a concentration-dependent manner, and these behavioral responses were significantly attenuated with SB366791. These results suggest the presence of a capsaicin-sensitive TRPV-like channel in the medicinal leech central nervous system and are relevant to the evolution of nociceptive signaling.

KEY WORDS: Leech, TRPV, Invertebrate, Nociception

INTRODUCTION

Transient receptor potential (TRP) channels are a diverse class of non-specific cation channels that are found throughout the animal kingdom (Damann et al., 2008). TRP channels are typically associated with sensory transduction and are often polymodal, such as the mammalian TRP-vanilloid 1 (TRPV1) channel, which responds to noxious thermal (>42°C), mechanical and chemical (low pH, capsaicin) stimuli (Szallasi and Blumberg, 1999). Capsaicin sensitivity varies greatly throughout metazoans, with mammals displaying the greatest response to capsaicin. Birds, once thought to be capsaicin insensitive, have been shown to respond to capsaicin at increased concentrations (30 µmol l^{-1}) compared with mammals (Kirifides et al., 2004). Even among mammals, capsaicin sensitivity varies greatly between species, and this variance is further complicated by ontogentic shifts in capsaicin sensitivity within specific strains of mice (Holzer, 1991). Invertebrate sensitivity to capsaicin is no less complicated because ecdysozoans, such as *Drosophila* (Vriens et al., 2004) and *Caenorhabditis elegans* (Tobin et al., 2002), are reported to be capsaicin insensitive, whereas lophotrochozoans, specifically the gastropod *Megalobulimus* (Kalil-Gaspar et al., 2007) and the medicinal leech (*Hirudo verbana*, Carena 1820) (Pastor et al., 1996; Yuan and Burrell, 2010) appear to be capsaicin sensitive, albeit at higher concentrations compared with mammals.

The medicinal leech is an interesting species to study TRP sensory function in because it possesses somatosensory neurons that are remarkably similar to those found in vertebrates (Smith and Lewin, 2009). Specifically, they have rapidly adapting cutaneous afferents that detect light touch (T cells), slower-adapting afferents that respond to sustained pressure (P cells) and both mechanical and polymodal nociceptive afferents (N cells) (Nicholls and Baylor, 1968; Blackshaw et al., 1982; Pastor et al., 1996). As in mammals, the polymodal N cells exhibit sensitivity to potentially damaging levels of mechanical, thermal and chemical stimuli (Blackshaw et al., 1982; Carlton and McVean, 1995; Pastor et al., 1996). In the case of chemical stimuli, these neurons respond to both low pH and capsaicin, albeit at relatively high concentrations of the latter compared with mammals (Pastor et al., 1996). This, along with responses to noxious heat, suggests the presence of a TRPV-like receptor in the polymodal N cells of the medicinal leech.

In the following study, we have confirmed these initial findings of Pastor et al. (Pastor et al., 1996) regarding capsaicin sensitivity in the leech and have expanded upon them by utilizing pharmacological approaches to block capsaicin-elicited activation of the polymodal N cell. Furthermore, we have characterized the nocifensive behavioral responses elicited by capsaicin *in vivo*.

RESULTS

TRPV-like receptor on the polymodal N cells

First, the peripheral and central effects of capsaicin were examined. Peripheral effects were examined using a body wall preparation, which consisted of a section of leech body wall (skin and muscle) pinned flat to the bottom of a recording chamber and still connected to the central nervous system (CNS) (one to three ganglia) via segmental nerve roots that project from the ganglia to the periphery (Fig. 1) (Nicholls and Baylor, 1968). Central effects were examined using isolated ganglia. Capsaicin (10 µmol l^{-1}–2 mmol l^{-1}) was applied to either the body wall preparations or isolated ganglia using a manual solution exchange system. Peripheral application of capsaicin elicited action potential firing in the polymodal lateral nociceptive (lN) cell in a concentration-dependent manner (Fig. 2A). Using two-way ANOVA analyses, we found a significant effect of capsaicin versus vehicle ($F_{1,53}$=176.792, P<0.001), a significant concentration effect ($F_{5,53}$=9.264, P<0.001) and a significant interaction effect, indicating an effect of increasing concentrations of capsaicin but not of increasing levels of dimethylsulfoxide (DMSO) ($F_{5,53}$=9.398, P<0.001). Central application of capsaicin produced a similar concentration-dependent increase in lN-cell activity (Fig. 2A; treatment effect $F_{1,51}$=340.311, P<0.001;

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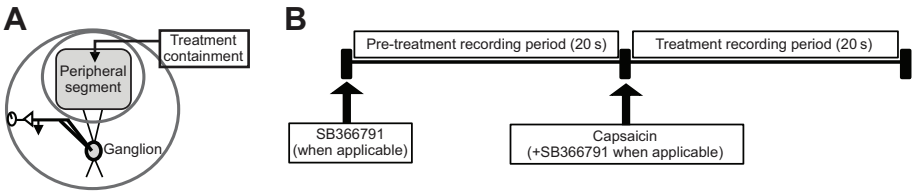

Fig. 1. Experimental methods for electrophysiology experiments. (A) Diagram of the body wall preparation. Intracellular recordings were made from the N, P or T cells within a ganglion that still innervated a piece of body wall. Treatments (capsaicin or thermal stimuli) and the TRPV1 antagonist (SB366791) were applied directly to the body wall, which had been isolated with a plastic containment ring and secured to the bottom of the dish with petroleum jelly. (B) Time frame of central and peripheral physiology experiments. Intracellular recordings of the N cells included 20 s of cell activity prior to capsaicin treatment, followed by 20 s of activity capsaicin treatment (capsaicin-elicited activity was calculated as the difference between these two periods). In some experiments, the preparation was pre-treated with the TRPV1 antagonist SB366791 prior to capsaicin treatment (SB366791 was also included in the capsaicin solution).

concentration effect $F_{5,51}$=15.697, P<0.001; and interaction effect $F_{5,51}$=16.023, P<0.001). The ability of both centrally and peripherally applied capsaicin to elicit lN activity and the effective concentration range observed are consistent with the previous findings of Pastor et al. (Pastor et al., 1996).

When 100 µmol l^{-1} capsaicin was co-administered with the selective TRPV1 antagonist SB366791 (100 µmol l^{-1}) (Varga et al., 2005), both peripheral and central responses to capsaicin were significantly attenuated in the polymodal lN cells (Fig. 2A,C; one-way ANOVA; peripheral, $F_{3,21}$=32.223, P<0.001; central, $F_{3,24}$=22.104, P<0.001). There was no effect on activity when SB366791 was applied alone. The concentration of SB366791 was chosen based on the elevated concentrations of capsaicin required to elicit a response in the lN cell. However, lower concentrations of SB366791 (10 µmol l^{-1}) have been shown previously to be effective at blocking the effects of capsaicin (10 µmol l^{-1}) on central synapses (Yuan and Burrell, 2012). These results are consistent with the hypothesis that peripheral and central responses to capsaicin are mediated by a TRPV-like receptor.

Mammalian TRPV channels undergo desensitization after exposure to capsaicin (Joseph et al., 2013; Lukacs et al., 2013). To determine whether desensitization of capsaicin-elicited responses occurs in the leech, experiments were conducted in which the initial response of lN cells to 100 µmol l^{-1} capsaicin was compared with that of a subsequent treatment with capsaicin (5 min inter-treatment interval). These experiments were performed using the same methods as the isolated ganglia experiments. The response of the lN cell to the second treatment with capsaicin was significantly reduced relative to that of the initial exposure, consistent with desensitization (Fig. 3A,B; two-way ANOVA; capsaicin versus DMSO treatment effect $F_{1,15}$=35.93, P<0.001; initial versus second exposure effect $F_{1,15}$=14.76, P<0.005; interaction $F_{1,15}$=12.5, P<0.005). Subsequent *post hoc* analysis confirmed a statistically significant difference in the responses to the initial versus the second exposure to capsaicin (P<0.001), whereas no such change was observed in cells that received two exposures to DMSO (P>0.05).

Previous experiments by Pastor et al. (Pastor et al., 1996) have indicated that noxious levels of thermal stimulation (>43°C) activated the capsaicin-sensitive lN cells but not the medial N (mN) cells (which are mechanical nociceptors and capsaicin insensitive). We repeated these experiments by applying saline that had been heated to 43°C to a patch of skin in a body wall preparation that had been isolated from the CNS using a Sylgard™ enclosure and sealed with Vaseline (see Fig. 1A). Heated saline perfused onto the periphery did cause the lN cells to fire, in agreement with Pastor et al. (Pastor et al., 1996). Furthermore, the lN cell response to noxious temperature was significantly attenuated with co-application of SB366791 (Fig. 4A,B; one-way ANOVA, $F_{1,18}$=19.858, P<0.001).

Responses to capsaicin in other sensory cells

Next, the responses of other leech mechanosensory cells were tested. The T cells, medial N (mN) cells (are only mechanosensitive), and medial P (mP) cells did not show a significant response to 100 µmol l^{-1} capsaicin when applied peripherally or centrally. However, the lateral P (lP) cells did respond when capsaicin was applied peripherally, but not when applied centrally (Fig. 5A). Although this is not in agreement with studies by Pastor et al. (Pastor et al., 1996), which did not find capsaicin-induced activation

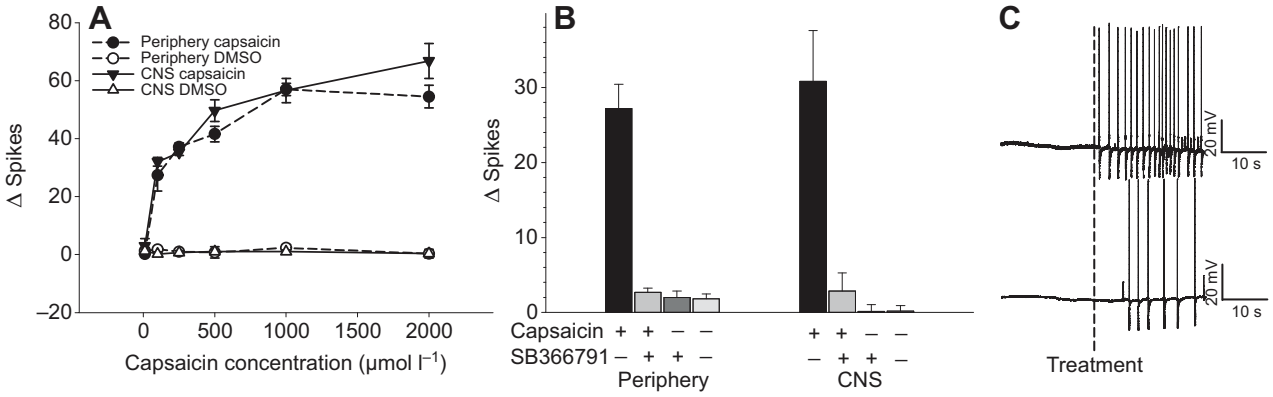

Fig. 2. Effect of the TRPV agonist capsaicin and the TRPV antagonist SB366791 in polymodal N cells. (A) The concentration-dependent response of lateral N (lN) cells (ΔSpikes) to peripherally and centrally applied capsaicin. (B) The selective TRPV1 antagonist SB366791 (100 µmol l^{-1}) blocked the response of lN cells to peripherally and centrally applied capsaicin (100 µmol l^{-1}). This concentration of SB366791 alone had no effect on lN-cell activity. (C) Sample traces of the lN-cell activity during capsaicin treatment (top) and during capsaicin treatment that included SB366791 (bottom).

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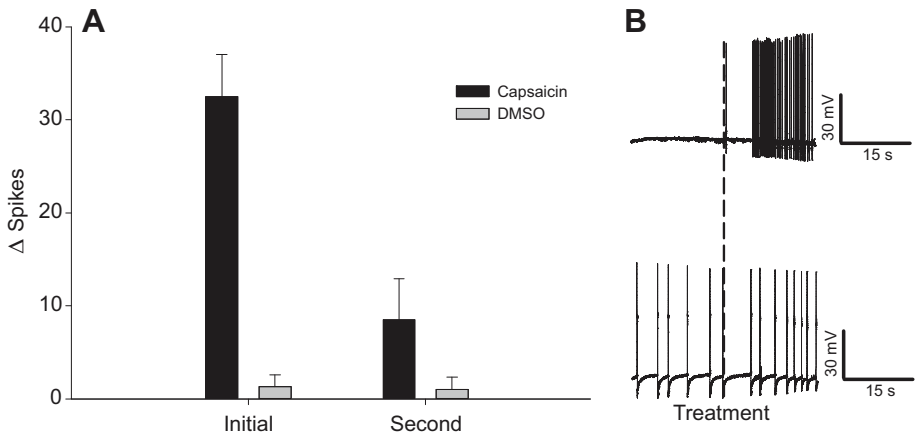

Fig. 3. Capsaicin-induced desensitization in TRPV-containing lN cells. (A) The robust response of the lN cells to centrally applied capsaicin (100 µmol l^{-1}) was reduced in a subsequent (Second) treatment, 5 min following the initial capsaicin exposure. (B) Traces of 100 µmol l^{-1} capsaicin-induced activity in the lN cells after the initial treatment (top) and a subsequent treatment 5 min post (bottom).

of the P cells, these previous studies did not differentiate between the lP and the mP cells. To further investigate the activation of the lP cells to peripherally applied capsaicin, the AMPA receptor antagonist CNQX (50 µmol l^{-1}) was applied to the CNS through bath application prior to peripheral application of 100 µmol l^{-1} capsaicin. CNQX has been used previously in the leech to block synaptic transmission in the CNS (Wessel et al., 1999; Li and Burrell, 2008; Yuan and Burrell, 2010). CNQX blocked the capsaicin-induced activation of the lP cell (Fig. 5B; one-way ANOVA, $F_{2,14}$=13.961, P<0.001). This result indicates that capsaicin activation of lP cells was not a direct effect, but instead due to peripheral activation of an unknown capsaicin-sensitive neuron that has glutamatergic synaptic input to the lP cells.

Behavioral effects of capsaicin

Next, the functional relevance of putative TRPV channel activation was examined by monitoring behavioral responses to increasing capsaicin concentrations (10–2000 µmol l^{-1}) in intact animals. Application of capsaicin (1 ml) to the posterior sucker of the leech produced a concentration-dependent display of nocifensive behaviors that most typically began with posterior sucker withdraw and was then followed by locomotion. The posterior sucker withdraw was present starting at 100 µmol l^{-1} capsaicin (Fig. 6A,B), comparable to the threshold at which the lN cell was activated by capsaicin. The extent of withdraw was characterized as a partial withdraw, where the sucker does not completely retract beneath the body, or a full withdrawal, where the sucker completely retracts beneath the animal's body (Fig. 6A). A full withdrawal of the posterior sucker was found in all animals treated with ≥500 µmol l^{-1} capsaicin, whereas partial withdrawals were only observed in animals that were treated with ≤250 µmol l^{-1} capsaicin. Capsaicin at <100 µmol l^{-1} did not elicit any withdrawal response. Co-treatment with SB366791 attenuated responses to capsaicin. At 100 and 250 µmol l^{-1} capsaicin, SB366791 reduced the proportion of animals responding with full withdrawals and increased the proportion of no responses (Fig. 6B). Using 500 and 1000 µmol l^{-1} capsaicin, SB366791 reduced the proportion of full withdrawals to capsaicin so that both partial withdrawals and no responses were observed at these capsaicin concentrations. At 2000 µmol l^{-1} capsaicin, SB366791 reduced the proportion of full withdrawals so that partial withdrawals were now observed at this concentration.

The latency to initiate a withdrawal response decreased with increasing capsaicin concentration. This is consistent with the idea that capsaicin becomes more noxious as the concentration increases. Co-application of 100 µmol l^{-1} SB366791 with capsaicin increased the latency to respond and decreased the number of animals that responded to capsaicin (Fig. 6B,C). Two-way ANOVA analyses detected a statistically significant effect of treatment ($F_{2,107}$=108.36, P<0.001), a significant effect of concentration ($F_{5,107}$=26.225, P<0.001) and a significant interaction effect between the treatment and concentration ($F_{10,107}$=11.116, P<0.001). A subsequent Student–Newman–Keuls *post hoc* test of the treatment effect confirmed that the capsaicin group was significantly different from the capsaicin-SB366791 and DMSO groups (P<0.001) and that the capsaicin-SB366791 group was significantly different from the DMSO group (P<0.001).

Following posterior sucker withdrawal, leeches would propel themselves (crawl) away from the site of treatment, beginning at 250 µmol l^{-1} capsaicin (Fig. 6D). Again, the duration of this locomotion period increased with increasing capsaicin

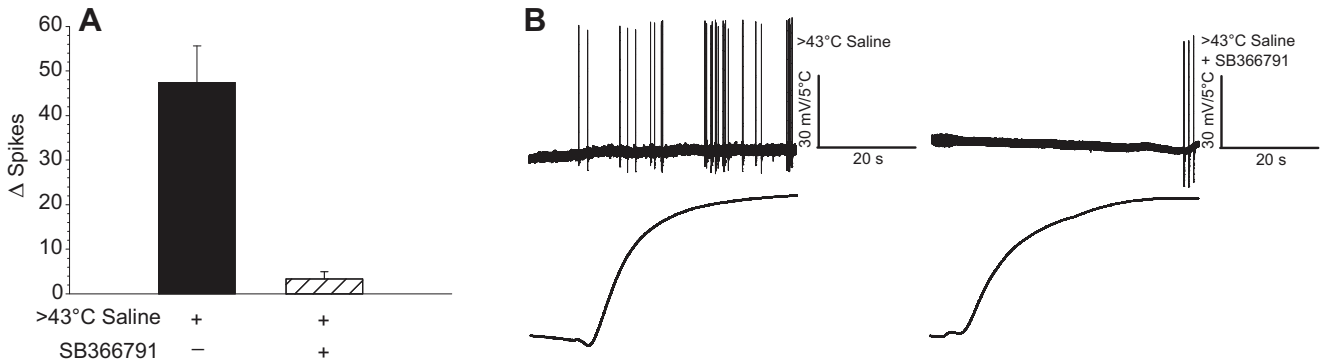

Fig. 4. Effect of noxious thermal stimuli on polymodal N cells. (A) Application of saline heated to >43°C elicited lN activity, and this activity was blocked by SB366791. (B) Traces of noxious heat activation of the lN cells (left). Peripherally applied SB366791 blocked noxious heat activation in lN cells (right).

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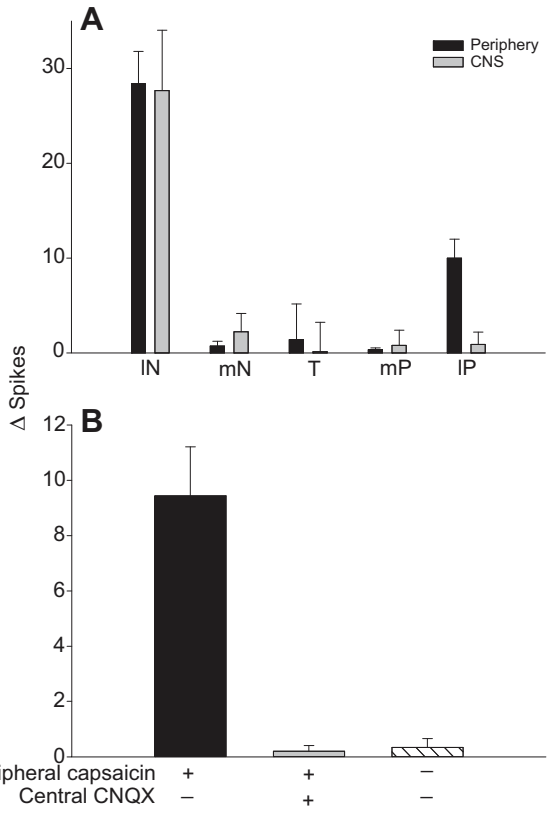

Fig. 5. Effect of capsaicin in non-nociceptive neurons. (A) Peripherally and centrally applied capsaicin (100 μmol l^{-1}) directly activated the lN cells, but did not activate the medial N (mN), touch (T) or medial pressure (mP) neurons. The lateral P (lP) cell was activated by capsaicin; however, this activation is the result of indirect actions of an unknown TRPV-containing neuron, as shown by co-treatment with CNQX blocking the capsaicin-induced activity (B).

concentrations. Co-application of SB366791 prevented locomotion during treatments with 250 μmol l^{-1} and 500 μmol l^{-1} capsaicin and significantly reduced locomotion at concentrations of 1000 μmol l^{-1} and 2000 μmol l^{-1} (two-way ANOVA; treatment effect $F_{2,108}$=38.85, P<0.001; concentration effect $F_{5,108}$=15.876, P<0.001; interaction effect $F_{10,108}$=6.189, P<0.001). A *post hoc* analysis of the treatment effect confirmed that the capsaicin group was significantly different from the capsaicin-SB366791 and DMSO groups (P<0.001) and that the capsaicin-SB366791 group was significantly different from the DMSO group (P<0.001).

DISCUSSION

This study found pharmacological evidence of capsaicin sensitivity in the leech. Specifically, capsaicin was observed to selectively activate polymodal N cells and to elicit nocifensive behaviors. These findings are consistent with the hypothesis that the leech CNS possesses a TRPV-like receptor and are in agreement with previous work in the leech by Pastor et al. (Pastor et al., 1996). Similar to mammalian TRPV1-channel-containing neurons, the leech polymodal lN cell responds to capsaicin and noxious temperatures exceeding 43°C, and both capsaicin and thermal responses can be attenuated by treatment with SB366791 (Julius, 2013). Unlike mammalian TRPV1 channels, which can respond to capsaicin at concentrations as low as 0.1 μmol l^{-1} (Caterina et al., 1997), the capsaicin-sensitive neurons in the leech were not reliably activated by capsaicin concentrations below 25 μmol l^{-1}. Despite a lower sensitivity to capsaicin, these results are, probably, not due to non-TRPV-mediated effects. First, the direct activation by capsaicin was only observed in the polymodal N cell and not the other cutaneous afferent neurons, including the mechanical-only nociceptive neuron. Second, the selective TRPV1 antagonist SB366791 inhibited both the physiological and behavioral effects of capsaicin. Third, SB366791 also blocked the polymodal cell response to noxious thermal stimulation. Fourth, the response of the lN cell to capsaicin exhibited desensitization, a property observed in other capsaicin-sensitive TRPV channels (Joseph et al., 2013; Lukacs et al., 2013).

Traditionally, it has been thought that invertebrates were not sensitive to capsaicin and thus that they do not have a TRPV channel that is functionally similar to mammalian TRPV1 channels. Although it is true that capsaicin has been tested in other invertebrates, such as *C. elegans*, without effect (Tobin et al., 2002), it is possible that these previous studies did not use high enough concentrations. This is reminiscent of the past controversies regarding invertebrates that were thought to lack *N*-methyl-D-aspartate (NMDA) receptors as a result of their lower sensitivity to NMDA compared to that of vertebrates. Subsequent molecular and physiological studies have confirmed that invertebrates do have an NMDA receptor that not only responds well to other specific NMDA receptor agonists and antagonists but also fulfills the traditional NMDA receptor functional role regarding synaptic plasticity (Glantz and Pfeiffer-Linn, 1992; Brockie et al., 2001; Glanzman, 2010). Although it is not possible to definitively determine whether there is a TRPV homolog in the leech using solely pharmacological evidence it is unlikely that the capsaicin-induced activity found exclusively in lN cells and the ability of SB366791 to inhibit this activity would be due to off-target effects. The next step in verifying a TRPV channel in the leech will require using molecular tools to clone the channel and perform physiological experiments to determine its similarity to mammalian TRPV1 channels.

TRPV homologs are present in other invertebrates, such as OSM-9 in *C. elegans*, which is responsible for olfaction and mechanosensation (Colbert et al., 1997). Although OSM-9 does not respond to capsaicin at concentrations that elicit responses in mammals, it does respond to noxious temperatures, and this response can be enhanced by capsaicin or blocked by the TRPV1 antagonist capsazepine (Wittenburg and Baumeister, 1999). The *Drosophila* TRPV channels, Inactive and Nanchung, do not respond to noxious temperature or capsaicin and are primarily involved in hearing (Kim et al., 2003). This variance in function among invertebrate homologous TRPV channels could be due to an evolutionary divergence between ecdysozoans and lophotrochozoans because both the leech and the mollusk (both lophotrochozoans) respond to capsaicin (Pastor et al., 1996; Kalil-Gaspar et al., 2007; Yuan and Burrell, 2010). One possible reason for these differences in TRP channel function, at least in insects, is that the high surface area to volume ratio of insects makes them more sensitive to thermal changes, potentially leading to the need for different mechanisms to detect temperature change (Liu et al., 2001).

The peripheral TRPV1 channel in mammals acts as a nociceptor to alert the animal that they have encountered noxious stimuli and need to react defensively (Julius, 2013). In this study the functional relevance of the leech TRPV-like channel was investigated for the first time by observing the behavioral responses of leeches to increasing capsaicin concentrations applied to their posterior sucker. Leeches responded to capsaicin by withdrawing their sucker and propelling away from the site of treatment, indicating a nocifensive response to capsaicin. The withdrawal response is indicative of the animal attempting to pull away from the noxious quality of capsaicin, but it is possible that the enhanced locomotion following

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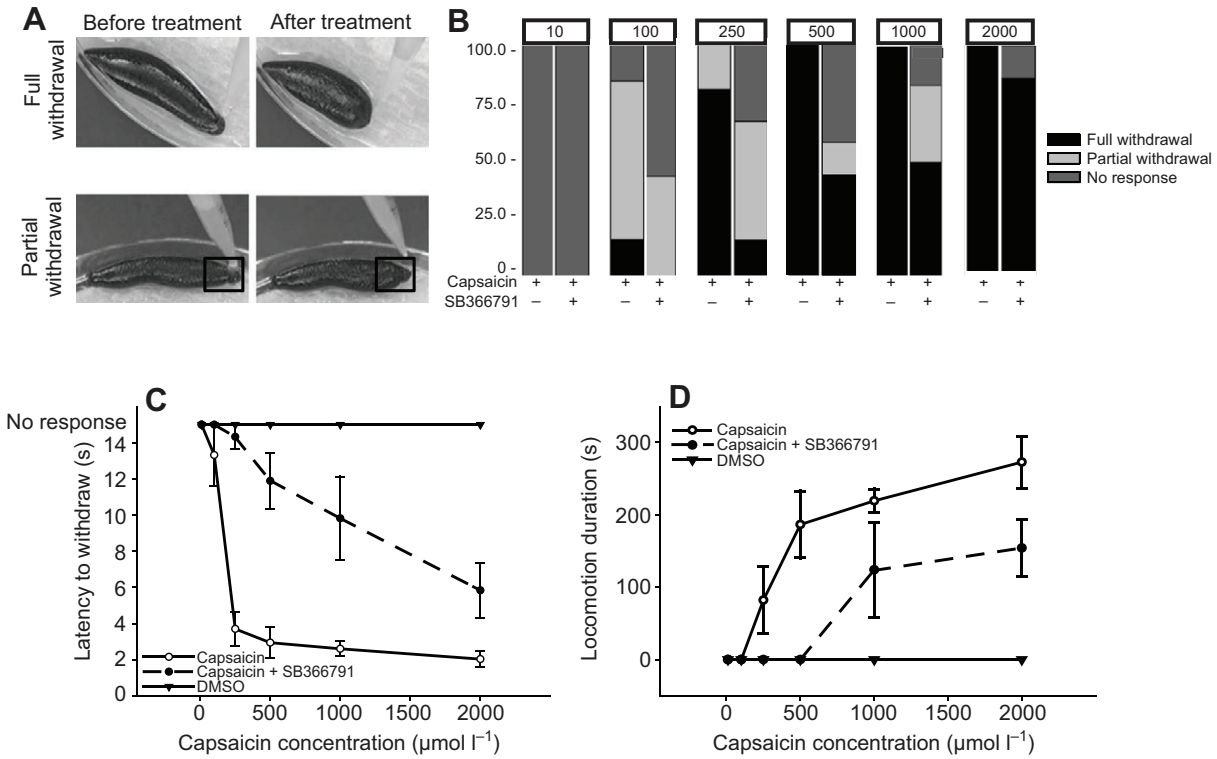

Fig. 6. Capsaicin elicited nocifensive responses in leeches that increased in magnitude with increasing capsaicin concentration and were blocked or reduced when pre-treated with SB366791. (A) Categorization of withdrawal behavior. (Top) Display of a full withdrawal elicited by treatment with 1 mmol l^{-1} capsaicin delivered to the posterior sucker (1 ml). A full withdrawal was achieved if the posterior sucker fully retracted beneath the animal. (Bottom) Display of a partial withdrawal elicited by treatment with 100 μmol l^{-1} of capsaicin delivered to the posterior sucker (1 ml). A partial withdrawal was achieved if the sucker moved after treatment but did not completely retract beneath the body. The boxes shown are placed in the same position to help demonstrate the extent of the partial withdrawal. (B) The proportion of partial and then full withdrawals increased with capsaicin concentration (indicated above the bars, μmol l^{-1}). Co-application of SB366791 attenuated the intensity of the response to capsaicin at each concentration. (C) The latency to initiate withdrawal of the posterior sucker decreased with increasing capsaicin concentration. Co-application of SB366791 increased the latency to withdraw to a given capsaicin concentration. Animals that did not respond with a full withdrawal within 15 s of capsaicin application were recorded as showing no response. (D) The duration of capsaicin-induced locomotion (crawling) increased with increasing capsaicin concentration. Co-application of SB366791 eliminated locomotion at lower capsaicin concentrations and reduced the locomotion duration at higher concentrations.

capsaicin treatment is a coping mechanism that is used to facilitate pain relief, similar to how capsaicin application to the paw in rodents causes a concentration-dependent licking response (Caterina et al., 2000). Furthermore, these behavioral results are in agreement with the physiological results, where the animals responded to capsaicin at concentrations exceeding 100 μmol l^{-1} and where treatment with SB366791 attenuated these responses.

One novel finding from this study was the observation that the lP cell, and not the mP cell, was activated by capsaicin, but only when applied peripherally. This capsaicin response was inhibited when CNQX was applied centrally, indicating that P-cell activity was being driven by glutamatergic synaptic signaling and not direct activation of the lP cell itself. This synaptically driven activation of the lP cell did not appear to be driven by the lN cell because central application of capsaicin to the isolated ganglia activated the lN cells but not the lP cells. The ability of central CNQX treatment to block peripheral capsaicin-induced activity indicates the presence of an unknown capsaicin-sensitive cell in the periphery that projects to the lP cells. The leech has many peripheral sensory cells that project to the CNS, such as the light-sensitive sensilla or stretch-sensitive Hoovers cells that could be potential candidates (Blackshaw and Thompson, 1988).

The peripheral TRPV-like receptor in the leech is presumably similar to the peripheral mammalian TRPV1 and serves as a nociceptor, as indicated by the behavioral findings in this study. However, it must be asked what the function of the central TRPV-like channels is in the leech. In mammals, TRPV1 is expressed throughout the CNS and exerts a wide range of neuromodulatory effects (Cristino et al., 2006; Kauer and Gibson, 2009; Di Marzo and De Petrocellis, 2012). Capsaicin activation of presynaptic TRPV1 produces short-term synaptic facilitation (seconds to minutes in duration) that is likely to be a result of Ca^{2+} influx through these channels (Sikand and Premkumar, 2007; Medvedeva et al., 2008). Mammalian TRPV1 is also activated by lipid neurotransmitters, referred to as endocannabinoids or endovanilloids, such as 2-arachidonoylglycerol (2-AG) and anandamide (AEA) (De Petrocellis et al., 2001a; De Petrocellis et al., 2001b; Di Marzo et al., 2001; Qin et al., 2008; Zygmunt et al., 2013). This endovanilloid signaling has been linked to long-lasting synaptic plasticity in a number of regions in the brain and spinal cord (Gibson et al., 2008; Jensen and Edwards, 2012; Kim et al., 2012; Brown et al., 2013), and to modulating a variety of behavioral processes, such as pain, learning and memory, and stress (Starowicz and Przewlocka, 2012; Kulisch and Albrecht, 2013; Laricchiuta et al., 2013). The endocannabinoids and endovanilloids AEA and 2-AG are present in the leech CNS (Matias et al., 2001), yet the leech and other protostomal invertebrates lack orthologs to the CB1 and CB2 receptors (Elphick, 2012). There is, however, evidence that central TRPV-like channels mediate synaptic plasticity in the leech via mechanisms that are similar to those observed in vertebrates (Li and Burrell, 2010; Yuan and Burrell, 2010; Li and Burrell, 2011; Yuan and Burrell, 2012; Higgins et al., 2013; Yuan and Burrell, 2013).

Relevant to the present study, 2-AG has been shown to mediate TRPV-dependent depression of nociceptive synapses and nociceptor-elicited behavior. This capacity for lipid signaling molecules to activate TRP channels is observed in other invertebrates, such as *C. elegans* and *Drosophila* (Kahn-Kirby et al., 2004; Leung et al., 2008), suggesting that the neuromodulatory role of central TRP channels is evolutionarily conserved in the animal kingdom.

It is already appreciated that TRPV channels are multifunctional in terms of the stimuli they transduce. Furthermore, studies in invertebrates such as *Drosophila* and *C. elegans* have shown that the function of TRPV as a peripheral sensory element has changed over evolutionary time. However, the present findings, although pharmacological in nature, suggest that some elements of TRPV function thought to be restricted to vertebrates, such as responsiveness to thermal and chemical stimuli, may also exist in lophotrochozoans. In addition, TRPV channels are now known to have functions in the CNS that are related to synaptic plasticity, and these functions appear to be well conserved between mammals and the leech. In addition to this conserved neuromodulatory role, there is now evidence of a highly conserved metabolic role for TRPV channels between vertebrates and invertebrates (Riera et al., 2014). It is possible that the CNS functions of the TRPV channels in animals such as the leech represent the beginning of the endovanilloid system that would eventually be supplemented by the cannabinoid receptor system in the lower pre-chordates (Elphick, 2012). Consequently, the leech provides a useful model system in which to study the physiological processes and evolutionary changes of both peripheral sensory and central neuromodulatory function.

MATERIALS AND METHODS

Animal preparation

Leeches (~3 g each) were obtained from a commercial supplier (Niagara Medicinal Leeches, Cheyenne, WY, USA) and maintained in a vented plastic container (30 cm long, 21 cm wide, 9 cm deep) filled halfway with artificial pond water (0.52 g l^{-1} H_2O Instant Ocean, replaced every 2 days) on a 12 h light:dark cycle at 18°C. Animals were used within approximately a month of being received from the supplier and were not fed because feeding can elicit significant changes in behavioral responsiveness (Kristan et al., 2005). Prior to dissection, animals were placed in an ice-lined dissecting tray filled with ice-cold leech saline. Dissections were started when the animal ceased spontaneous movement. Dissections and recordings were performed in ice-cold leech saline solution (in mmol l^{-1}: 114 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 NaOH and 10 HEPES, pH 7.4). For electrophysiological experiments using isolated ganglia, individual ganglia were dissected and placed within a recording chamber (2 ml). All pharmacological treatments were applied through rapid replacement of normal saline with treatment saline using a two-syringe manual fluid exchange system. For body wall preparation experiments, ganglia found posterior to the reproductive segments (segments 5–6) were dissected with the lateral segmental nerves still connected to a portion of the body wall. All pharmacological treatments and thermal stimuli [leech saline heated to 43°C using a heating and cooling perfusion pre-stage (ALA Scientific Instruments Inc., Westbury, NY, USA)] were restricted to the peripheral body-wall portion of the preparation using a Sylgard™ enclosure that was placed around the body wall and sealed to the bottom of the recording chamber with petroleum jelly. For all pharmacological experiments, drugs were dissolved in leech saline from frozen stock solutions. Final concentrations were made from stock solutions just prior to the individual experiments. Control experiments were conducted using ascending concentrations of 0.0001–0.02% DMSO. The following drugs were obtained from Sigma-Aldrich (St Louis, MO, USA): capsaicin, CNQX and DMSO. SB366791 was purchased from Tocris (Ellisville, MO, USA).

Electrophysiology

Current clamp (bridge balanced) intracellular recordings were made using sharp glass microelectrodes (35–40 MΩ) fabricated from borosilicate capillary tubing (1.0 mm outer diameter, 0.75 mm inner diameter; FHC, Bowdoinham, ME, USA) using a horizontal puller (Sutter Instruments P-97; Novato, CA, USA). Each microelectrode was filled with 3 mol l^{-1} K^+ acetate. Impalement of individual neurons was performed using a manual micropositioner (Model 1480; Siskiyou Inc., Grants Pass, OR, USA). Signals were recorded using a bridge amplifier (BA-1S; NPI, Tamm, Germany) and then digitally converted (Digidata 1322A A/D converter) for observation and analysis (Axoscope; Molecular Devices, Sunnyvale, CA, USA).

T, lN, mN, lP and mP cells were identified based on their size, position within the ganglion and action potential shape (Muller et al., 1981). In these experiments, the ganglion was pinned ventral side up in the recording chamber. Activity in these cells was recorded for 20 s in normal leech saline followed by 20 s in capsaicin. The capsaicin-elicited activity was determined by subtracting the amount of activity (number of action potentials) during the initial normal saline period from the activity during the capsaicin treatment period. Experiments using SB366791 involved pretreating the preparation immediately prior to recording (1 ml; 100 µmol l^{-1} SB366791), followed by co-application of SB366791 and capsaicin during the recorded treatment period. In desensitization experiments, recordings from the isolated ganglia were made during an initial treatment with 100 µmol l^{-1} capsaicin and then repeated 5 min later.

Behavior experiments

Intact animals (each weighing ~3 g) were placed in a plastic Petri dish (14.5 cm diameter, 165 cm^2 area) lined with filter paper that had been saturated with pond water (0.5 g l^{-1} Instant Ocean). This chamber was of sufficient size to permit the leeches, which are ~4 cm in length, ample room to move. All animals were acclimated to the arena for 20 min prior to the start of the experiments. Capsaicin (1 ml) was applied to the posterior sucker. In experiments requiring an antagonist, leeches were pre-treated 5 s prior to the start of the experiment; the antagonist was then co-applied with capsaicin. Each animal was only exposed to a single concentration of capsaicin to avoid the effects of desensitization. Behavioral observations were recorded using a digitalized video camera (SONY Handycam HDR-CX580) and analyzed using NOLDUS Ethovision software. The behaviors that were analyzed included the degree of withdrawal of the posterior sucker, the latency to withdraw the posterior sucker and the locomotion duration. The degree of withdrawal was characterized as either partial (posterior sucker did not fully retract beneath the animal) or full (posterior sucker fully retracted beneath the animal) (Fig. 6A,B). The withdraw latency was measured as the period between the start of the capsaicin application (which could be observed in the video recordings) and the time at which the animal initiated a withdrawal from the noxious stimuli. Animals that failed to initiate a withdrawal within 15 s of the capsaicin application were scored as non-responsive. Locomotion duration was determined by the duration of time the animal spent moving, starting once the animal begun to move from the treatment application area and concluded once the animal stopped moving its posterior sucker for 15 s.

Statistics

Data are presented as means ± s.e.m. Statistical analyses using one-way analysis of variance (ANOVA) were performed to determine the main effects with Newman–Keuls *post hoc* tests to confirm the ANOVA results. All significance was determined at an α level of at least $P<0.05$.

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Competing interests

The authors declare no competing financial interests.

Author contributions

B.D.B. and T.S. conceived and designed the experiments. T.S. and S.H. conducted the experiments and subsequent data analysis. B.D.B. and T.S. interpreted the findings and drafted/revised the manuscript.

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