Axon Reflexes Evoked by Transient Receptor Potential Vanilloid 1 Activation Are Mediated by Tetrodotoxin-Resistant Voltage-Gated Na$^+$ Channels in Intestinal Afferent Nerves


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ABSTRACT
Capsaicin-sensitive nerves mediate axon vasodilator reflexes in the intestine, but the ion channels underlying action potential (AP) propagation are poorly understood. To examine the role of voltage-gated Na$^+$ channels underlying these reflexes, we measured vasomotor and electrophysiological responses elicited by capsaicin in guinea pig and mouse dorsal root ganglia (DRG) neurons, submucosal arterioles, and mesenteric arteries in vitro. Transient receptor potential vanilloid 1 (TRPV1) agonists dilated guinea pig ileal submucosal arterioles and were blocked by capsaepine and ruthenium red. In double-chamber baths, capsaicin-evoked activation of TRPV1 on proximal perivascular nerves in the left chamber evoked dilations of the distal segment of the submucosal arteriole in the right chamber. Dilations were tetrodotoxin (TTX) (1 $\mu$M)-resistant, but reducing extracellular Na$^+$ (10% solution) or applying the Na$_{\text{ inward}}$ antagonist A-803467 [5-(4-chlorophenyl)-N-(3,5-dimethoxyphenyl)fu- ran-2-carboxamide] (1 $\mu$M) in the proximal chamber blocked capsaicin-evoked dilations in the distal chamber (88%; $P = 0.01$ and 75% and $P < 0.02$, respectively). In mouse mesenteric arteries, electrical field stimulation and capsaicin (2 $\mu$M) evoked dilations that were also TTX-resistant. In perforated patch-clamp recordings, APs in mouse and guinea pig capsaicin-sensitive DRG neurons were TTX-resistant but blocked by 10% extracellular Na$^+$. When capsaicin-evoked AP conduction was studied in in vitro ileal multiunit afferent nerve preparations, capsaicin responses were elicited in the presence of TTX, whereas distention-evoked responses were almost completely blocked by TTX. Together, these data provide evidence for TTX-resistant AP conduction in extrinsic sensory neurons that innervate guinea pig and mouse intestine and suggest this neural propagation is sufficient to mediate axon reflexes in the intestine.

Capsaicin-sensitive extrinsic sensory nerves perform important afferent and efferent roles in multiple organs, including the intestine (Geppetti and Trevisani, 2004; Holzer, 2006, 2008; Khairatkar-Joshi and Szallasi, 2009). The effecter action results from the release of neuropeptides from axon collaterals (Holzer, 2007; Khairatkar-Joshi and Szallasi, 2009), which in turn stimulates a number of motor systems in the intestine, such as motility, secretion, and blood flow, and can also activate the immune system. The mechanisms underlying neuropeptide excitation--release coupling are unclear, but several models have been proposed. Depolarization may be restricted to nerve terminals, with neuropeptide release confined to that site (Maggi and Meli, 1988; Bevan and Geppetti, 1994; Khairatkar-Joshi and Szallasi, 2009). Alternatively, neuropeptides may be released after neural propagation from the initiation to release sites through “axon reflexes” (Holzer, 1991; Khairatkar-Joshi and Szallasi, 2009). Several studies suggest that these actions can be mediated, at least in part, by tetrodotoxin (TTX)-insensitive neuronal propagation (Szolcsányi, 1988; Vanner and Bolton, 1996). Determining which of these mechanisms underlies capsaicin-evoked responses, and the properties underlying neural propagation in axon reflexes, is fundamentally important to a better understanding of the effector role of these nerves in physiological and pathophysiological studies.

The excitatory actions of capsaicin are caused by the selective activation of transient receptor potential vanilloid 1 (TRPV1) cationic channels (Caterina et al., 1997; Holzer, 2008; Khairatkar-Joshi and Szallasi, 2009). TRPV1 receptors...
are found on peripheral axons of capsaicin-sensitive nerves (Khairatkar-Joshi and Szallasi, 2009), including those in the wall of the intestine (Vanner, 1993; Holzer, 2008). Autoradiographic studies suggest these receptors are located along the entire length of the axon (Szallasi, 1995). However, little is known about the effects of activation of these receptors and the neural mechanisms that underlie their actions.

Studies of the ionic conductance underlying action potentials (APs) in capsaicin-sensitive peptidergic dorsal root ganglia (DRG) neurons innervating the viscera have been confined largely to the cell body (Rush et al., 2007; Dib-Hajj et al., 2009) and have demonstrated that the somatic action potential is TTX-resistant. Molecular studies using laser-capture microdissected intestinal DRG neurons have confirmed the presence of Na,1.8 transcript in the soma (King et al., 2009); however, it remains controversial as to whether these channels can sustain neural transmission in peripheral axons (Pinto et al., 2008). Moreover, little is known about the role of these channels in nerve terminals within the target organ, such as the intestine, where the effenter function occurs. Therefore, in the present study we sought to confirm whether neural transmission in the distal axons of DRG neurons underlying vasodilator axon reflexes in the intestine involved TTX-resistant mechanisms and, if so, to identify the major ion conductance underlying this neural propagation.

We found conclusive evidence for such a pathway and used in vitro vasodilatory measurement techniques and electrophysiological techniques to examine whether TTX-resistant Na⁺ channels are involved.

Materials and Methods

The methods were approved by the Animal Care Committee at Queen’s University and conform to the guidelines of the Canadian Council of Animal Care. In vitro studies were conducted on guinea pigs (150–225 g) and CD1 mice (20–25 g) obtained from Charles River Canada, Montreal, QC, Canada. Guinea pigs and mice were anesthetized by isoflurane inhalation and euthanized by cervical transection.

Video Microscopy of Guinea Pig Submucosal Arteriole Dilatation. After euthanasia, a midline laparotomy was performed, and the distal ileum was removed. Submucosal preparations were dissected as described previously (Vanner, 1993). In brief, the ileum was opened along the antimesenteric border and pinned flat in a Sylgard-lined Petri dish (Dow-Corning, Midland, MI) containing Krebs physiological saline solution. The mucosa was removed, and the submucosa was dissected free of the underlying muscle layers. The preparations were pinned in a small organ bath (3–4 ml). In double-chamber preparations, a small Plexiglas divider was placed between the proximal and distal portions of the parent submucosal arteriole and sealed with a silicone gel as described previously (Van-nder and Bolton, 1996). Organ bath chambers were continuously perfused with Krebs physiological saline solution (126 mM NaCl, 2.5 mM NaH₂PO₄, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 5 mM KCl, 25 mM NaHCO₃, and 11 mM glucose) gassed with 95% O₂–5% CO₂ at 35 to 36°C. In some experiments, a modified extracellular solution was used containing 10% normal Na⁺ was used as described above. After a 30-min equilibration, arteries were normalized to an internal circumference equivalent to 90% of that of the blood vessel under a transmural pressure of 100 mm Hg (Mulvany and Halpern, 1977). Vessels were left to equilibrate for approximately 1 h before experimentation began.

Neurotransmitter release from perivascular nerves was evoked by electrical field stimulation (EFS) using platinum electrodes connected to a Grass (Brossard, QC, Canada) SD9 stimulator (10 Hz for 10 s, 0.5 ms pulse width). Sympathetic vasoconstrictions were measured in vessels at rest (i.e., not constricted) and expressed as a percentage of the amplitude of vasoconstriction in response to 100 nM prostaglandin F₂α. Vasodilations caused by release of neuropeptides from the nerve terminals of extrinsic afferent neurons were measured after preconstriction with prostaglandin F₂α, and the amplitude of dilation was expressed as a percentage of the constriction to prostaglandin F₂α.

Multiunit Extracellular Recordings from Ileal Afferents. Experiments were performed on guinea pig ileum dissected with attached mesenteric tissue from euthanized animals. The preparation was placed in a Sylgard-lined organ chamber that was continuously perfused with oxygenated Krebs solution (118.4 mM NaCl, 24.9 mM NaHCO₃, 1.9 mM CaCl₂, 7 mM MgSO₄, 1.2 mM H₂O₂, 1.2 mM KH₂PO₄, and 11.7 mM d-glucose) at a flow rate of 6–7 ml/min and maintained at 33 to 34°C. Proximal and distal ends of the bowel were securely attached to an input and outlet port. The input port was connected to a perfusion syringe pump, which allowed continuous intraluminal perfusion of Krebs solution through the segments (0.2 ml/min) or periodic distension when closed. Intraluminal pressure was recorded via a pressure amplifier (NL 108; Digitimer, Welwyn Garden City, UK) connected in parallel with the input port. The mesenteric bundle was pinned to the base of the chamber, and a mesenteric nerve was dissected from the bundle and drawn into a suction electrode. Electrical activity was recorded by a Neurolog system (Digitimer) bandpass-filtered 0.2 to 3 kHz and acquired at a 20-kHz sampling rate to a personal computer through a Micro 1401 MKII interface running Spike 2 software (CED, Cambridge, UK).

The preparation was stabilized for 60 min and distended to an intraluminal pressure of 60 mm Hg by closing the outlet port. This procedure was repeated at 10-min intervals to test the viability of the preparation and the reproducibility of theafferent response to distension.
Perforated Patch-Clamp Recordings. The vertebral column was removed, and DRG from thoracic vertebra T9 to T13 were isolated bilaterally and placed into ice-cold Hanks’ balanced salt solution. DRG were then dissociated as described previously (Moore et al., 2002), using a sequential treatment with papain (69 U/1.5 ml) and collagenase/dispase (12 and 14 mg/3 ml) for 10 min/37°C each. Ganglia were triturated gently through a flame-polished Pasteur pipette, and neurons were placed onto round coverslips (precoated with sterile 20 μg/ml laminin plus 2 mg/ml poly-D-lysine) in F12 medium, containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 ng/ml nerve growth factor and maintained at 37°C in a humidified atmosphere of 5% CO₂ until they were used for electrophysiological experiments.

Electrophysiological experiments were conducted on small neurons (<40 pF). Neurons were recorded by using an Amphotericin B perforated patch clamp. Patch pipettes were made with a final resistance of 2 to 5 MΩ. Currents were amplified with a Multiclamp 700B amplifier and digitized by a Digidata 1440A AD converter (both by Molecular Devices, Sunnyvale, CA). Pipette solution was 110 mM K-gluconate, 30 mM KCl, 10 mM HEPES, 1 mM MgCl₂, and 2 mM CaCl₂, pH to 7.25 with KOH. External solution was 100 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 1 mM MgCl₂, and 2 mM CaCl₂, pH to 7.4 with NaOH. Experiments conducted in 10% sodium were made by lowering the NaCl concentration to the desired percentage and substituting it with N-methyl-D-glucamine (pH corrected with HCl).

Experiments were performed at room temperature (~23°C). Only one cell was recorded from each coverslip to prevent TRPV1 desensitization by prior capsaicin exposure. 

Drugs. All chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated. Hanks’ balanced salt solution and F12 medium were purchased from Invitrogen (Montreal, QC, Canada). Papain was purchased from Worthington Biochemicals (Freehold, NJ). A-803467 [5-(4-chlorophenyl-N-(3,5-dimethoxyphenyl)furan-2-carboxamide] was obtained from Tocris Bioscience (Ellisville, MO).

Statistical Analysis. Population data are expressed as mean ± S.E.M. Statistical analysis was carried out in Prism (GraphPad Software Inc., San Diego, CA) using paired and unpaired t tests, two-way ANOVA, and the Mann-Whitney U test for nonparametric data. Significance was set at p < 0.05. Dose–response curves were fit by using the function \( y = y_{\text{min}} + (y_{\text{max}} - y_{\text{min}})/(1 + 10^{((\log EC_{50} - x)/Hill\ slope)}) \).

Results

Activation of TRPV1 Receptors on Perivascular Capsaicin-Sensitive Nerves in Guinea Pig Ileum

General Properties. The resting outside diameter of guinea pig ileal submucosal arterioles at the recording site ranged from 43 to 84 μm (n = 68). In experiments where double-chamber experiments were performed (see Fig. 3) the integrity of the chemical isolation between the chambers was established by superfusing trypan blue into the capsaicin-stimulating chamber and ensuring that leakage did not occur between the chambers. Only those experiments where leakage between the chambers did not occur were included for analysis. Previous studies (Vanner and Bolton, 1996) used selective surgical denervation techniques to demonstrate...
that dilations observed in response to capsaicin stimulation were mediated by capsaicin-sensitive extrinsic afferent nerves.

Activation of TRPV1 Receptors on Peripheral Nerve Terminals. In single-chamber bath experiments, superfusion of capsaicin (20 nM–2 μM) evoked dose-dependent dilations of guinea pig submucosal arterioles (Fig. 1) as described previously (Vanner 1993). In separate preparations, superfusion of resiniferatoxin (60 nM–2 μM) also evoked dose-dependent dilations (Fig. 1). Dilations completely desensitized during a 2-min superfusion period as described previously with capsaicin (Vanner, 1993). After a 20-min washout, reapplication of resiniferatoxin had no effect (n = 4). Therefore, only single applications of resiniferatoxin were applied in each preparation. Resiniferatoxin-evoked dilations of similar amplitude compared with capsaicin-evoked dilations, but its potency was ~100-fold increased compared with capsaicin (EC50 = 280 nM versus 32 nM, respectively; p < 0.0001 by two-way ANOVA; n = 4 or more, one animal for each preparation).

Cross-desensitization by the TRPV1 agonists was examined in a single-chamber bath (data not shown). Superfusion of capsaicin (600 nM) evoked typical dilations. After a 10-min washout period, resiniferatoxin (2 nM; n = 4) was superfused in the same preparation, and no dilation was observed. In a similar series of experiments, superfusion of resiniferatoxin (2 nM; n = 4) evoked typical dilations, but subsequent application of capsaicin (600 nM) had no effect.

Effect of TRPV1 Antagonists Ruthenium Red and Capsazepine. When capsaicin (600 nM) was applied in the presence of capsaicin (10 μM; n = 5) dilations were not observed (Fig. 2). After a 10-min washout of capsaicin, capsaicin-evoked dilations were observed (mean = 65.0 ± 10.2%). Superfusion of resiniferatoxin (2 nM) also did not evoke responses in the recording chamber in the presence of capsaicin (3 μM; n = 4). In contrast to capsaicin responses, resiniferatoxin failed to elicit dilations after washout of capsaicin in three of the four preparations (Fig. 2). A small dilation was observed in one preparation (21% dilation).

The effect of ruthenium red (10 μM) on the capsaicin- and resiniferatoxin-evoked dilations was also studied. Dilations evoked by capsaicin (2 μM; n = 6) were completely blocked in four of the six preparations (mean dilation = 15%). After a 10-min washout of ruthenium red, reapplication of capsaicin evoked a typical dilation (mean = 63 ± 9.0%). Dilations evoked by resiniferatoxin (2 nM) were also markedly reduced in the presence of ruthenium red (10 μM) (mean dilation = 3.5 ± 3.5%; n = 6). After a 10-min washout period, resiniferatoxin (2 nM) alone was reapplied but had no effect. To provide a positive control in these studies, the effects of resiniferatoxin (2 nM) alone were studied in separate prep-
arations, and typical dilations were observed (mean dilation = 61.5 ± 9.2%; n = 6).

**Ionic Mechanism Underlying TRPV1-Evoked Neural Propagation**

**TTX Sensitivity.** Previous studies in guinea pig ileum (Vanner and Bolton, 1996) suggested that neural propagation underlying axon reflexes in capsaicin-sensitive nerves may involve TTX-resistant action potentials, but this was not examined in a systematic fashion. Therefore, in the current study we examined whether neural propagation from the stimulating to recording chamber involved TTX-sensitive and/or TTX-resistant components by studying paired preparations from the same animal (Fig. 3A). One preparation was superfused with capsaicin (600 nM) in the stimulating chamber, and the other preparation was superfused with capsaicin (600 nM) in the presence of TTX (1 μM) in the stimulating chamber. TTX had no significant effect on the capsaicin-evoked dilations (n = 7 paired preparations) compared with those evoked by capsaicin alone, suggesting that neural propagation occurs by mechanisms independent of TTX-sensitive Na⁺ channels.

In mouse mesenteric vessels, electrical field stimulation evoked large transient constrictions of mesenteric arteries. Incubation of artery segments with 1 μM TTX abolished constrictions to EFS (Fig. 3C; n = 10 arteries from five mice; P > 0.001, paired t test). However, in the same arteries, EFS-evoked vasodilations of preconstricted arteries were resistant to TTX (P = 0.25, paired t test).

**Afferent Nerve Recording**

To further examine TTX-resistant nerve conduction in distal axons of capsaicin-sensitive sensory afferents innervating the guinea pig intestine, multiunit afferent recordings were obtained from perivascular nerves. These multiunit recordings exhibited baseline activity that was markedly increased by luminal distention (Fig. 4, C and D) and application of capsaicin (2 μM) to the bath (Fig. 4, A and B). When TTX...
(1 μM) was superfused, basal activity was markedly reduced \(p = 0.002\) by Mann-Whitney U test; \(n = 6\) preparations from six animals). Luminal distention also elicited responses in the presence of TTX but they were markedly reduced compared with controls, suggesting that the majority of responses were mediated by TTX-sensitive nerves. However,
stimulation with capsaicin (2 μM) in the presence of TTX still elicited robust action potential discharge (Fig. 4), although this discharge occurred later during the superfusion of capsaicin than those elicited without TTX (see Fig. 4A). This delay may reflect differences in the voltage dependence of activation of slow TTX-resistant Na⁺ currents compared with fast TTX-sensitive Na⁺ currents (Beyak et al., 2004).

**Role of Na⁺ Channels in Neural Propagation.** To examine the role of Na⁺ in action potential electrogenesis of capsaicin-sensitive nerves, the effect of TTX and reduced Na⁺ concentration in the external solutions was studied by using perforated patch-clamp recordings (Fig. 5). Results were confined to recordings from small neurons (< 40 pF) that were depolarized by 2 μM capsaicin, properties typical of peptidergic C fiber nociceptors (n = 5). Ninety-seven percent (34 of 35) of small guinea pig DRG neurons exhibited TTX-resistant action potentials (Fig. 5) and the characteristic “hump” on its falling phase. In a 10% Na⁺ solution only a small prepotential was evident at the rheobase. This potential increased in amplitude at two and three times the rheobase but did not elicit an action potential (Fig. 5A), presumably representing the activation of voltage-gated calcium channels.

The effect of the reduced Na⁺ concentration was studied in parallel on the capsaicin-evoked vasodilator responses in the guinea pig ileum double-bath preparations to determine whether the capsaicin-evoked neural propagation between the two chambers was mediated by TTX-insensitive Na⁺ channels (Na,1.8). Capsaicin (2 μM; n = 5 paired preparations from five animals) superfused into the stimulating chamber evoked typical vasodilations in the recording chamber, but when applied in the presence of the 10% Na⁺ solution only a small depolarization was observed at the rheobase. Reapplication of capsaicin in 10% Na⁺ still evoked large dilations (mouse DRG neuron mean depolarization = 9.3 ± 4.0 mV; guinea pig DRG neurons mean depolarization = 10.3 ± 5.8 mV; n values and animal numbers as above). The lack of obvious desensitization to repeat capsaicin probably is caused by the use of a fast flow system (see Materials and Methods) compared with the standard superfusion system in the vasodilation experiments described above. These data suggest that capsaicin can still cause depolarization in the lowered sodium conditions used. To determine whether this depolarization was sufficient to evoke neurotransmitter release from nerve terminals when capsaicin was directly applied, we studied the effects of 10% Na⁺ on capsaicin-evoked dilations on the mouse mesenteric arteries. Capsaicin-evoked dilations in 10% Na⁺ were not significantly different from those obtained in control solutions (n = 8 vessels from four mice for each treatment; p = 0.235 Mann-Whitney U test).

**Role of Na⁺ Ions in Capsaicin-Evoked Depolarization.** The effect of low Na⁺ could be caused by two possible effects: blocking depolarization response to capsaicin or blocking voltage-dependent Na⁺ channels. We used patch-clamp recordings to examine whether capsaicin still depolarized neurons in low Na⁺. Capsaicin (2 μM) evoked large depolarizations and action potential discharge with control solutions (guinea pig neurons mean depolarization = 13.8 ± 6.6 mV, n = 4 from four animals; mouse DRG neuron mean depolarization = 21.6 ± 5.0 mV, n = 6 from two mice; see Fig. 6). Reapplication of capsaicin in 10% Na⁺ still evoked large depolarizations (mouse DRG neuron mean depolarization = 21.6 ± 5.0 mV, guinea pig DRG neurons mean depolarization = 10.3 ± 5.8 mV; n values and animal numbers as above).

To further examine the role for TTX-resistant voltage-gated Na⁺ channels we studied the effect of the Na,1.8 channel blocker A-803467 (Jarvis et al., 2007) (Fig. 7). Perforated patch-clamp recordings were obtained from Fast Blue-labeled mouse and guinea pig intestinal DRG neurons. Action potentials were elicited by intracellular pulses (100-ms duration, 10-pA pulses increasing to a maximum of twice the

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**Fig. 5.** Effect of a low Na⁺ solution on action potentials and vasodilations evoked by capsaicin applied at a site remote from the recording. A, representative perforated patch-clamp recordings of an action potential from a small mouse dorsal root ganglia neuron (30 pF) that was elicited by intracellular current pulses. The amplitude of the action potential was not affected by TTX (1 μM), but when the extracellular solution was switched to a 10% Na⁺ solution the action potential was blocked, even at two and three times the rheobase (150 pA). After a washout of the 10% Na⁺ solution, the action potential was again elicited by intracellular current pulse. B, representative traces of capsaicin-evoked dilations using the double-chamber bath shown in Fig. 4. When capsaicin was superfused into the left chamber (left) a typical dilation was evoked in the right chamber, as described in Fig. 4. In a separate preparation (center), when capsaicin was superfused into the left chamber in the presence of a 10% Na⁺ solution in that chamber no dilation was observed in the right chamber. Right, summary of the mean dilator responses to capsaicin alone (control) and capsaicin plus a 10% Na⁺ solution (n = 5 paired preparations from five animals) showing that capsaicin-evoked dilations were blocked by the 10% Na⁺ solution (p = 0.0097, Mann-Whitney U test).
rheobase) in the presence of TTX (1 μM). After a 3-min perfusion of A-803467 (1 μM), action potentials were blocked in 44% of guinea pig neurons (4/9) and 64% of mouse neurons (7/11). The effect of A-803467 was also tested on capsaicin-evoked depolarizations (Fig. 7B). Action potentials elicited by a brief capsaicin (2 μM) application were blocked by A-803467 in five/six mouse neurons (from four mice) and three/four guinea pig neurons (from three guinea pigs), and the amplitude was reduced in the remainder. Capsaicin-evoked depolarizations were not affected by A-803467. To directly test the effects of A-803467 on capsaicin-evoked dilations, studies were conducted by using the guinea pig submucosal preparation in the double-chamber bath (see Fig. 3A). Mean submucosal arteriolar dilations evoked by capsaicin (2 μM) applied in the left chamber peak and recorded in the right chamber were reduced by 75% in the presence of A-803467 (1 μM) and TTX (1 μM) compared with capsaicin alone (p < 0.02; n = 5 preparations in each group).

**Discussion**

The release of neuropeptides from terminals of capsaicin-sensitive afferent nerves has been shown to have an important effect on a number of gut functions, including blood flow, vascular permeability, secretion, motility, and the immune system (Szallasi, 1995; Holzer, 2006, 2008; Bartho et al., 2008; Khairatkar-Joshi and Szallasi, 2009). There is growing evidence that these actions can involve local “axon reflexes” within the distal axons of extrinsic afferent nerves, which implies that neural propagation underlies this effector signaling. However, there is little known about the mechanisms involved. In the current study we have shown that activation of TRPV1 channels on distal axons of extrinsic sensory nerves innervating the intestine of the guinea pig can release vasodilator neurotransmitters at remote sites from the stimulus. This axon reflex was resistant to TTX but blocked by low Na⁺ solutions, suggesting TTX-resistant voltage-gated Na⁺ channels underlie this action.

The selective actions of vanilloid compounds including capsaicin and the more potent agonist resiniferatoxin are mediated by TRPV1 (Holzer, 2004; Khairatkar-Joshi and Szallasi, 2009). These nonselective cation channels have a high permeability for Ca²⁺, and sustained signaling leads to a reduction in the effector function of the neuron. We found that application of capsaicin and resiniferatoxin to guinea pig and mouse perivascular nerves evoked vasodilations that rapidly desensitized. In the guinea pig, we found that stimulation of the receptors by capsaicin and resiniferatoxin was blocked by TRPV1 antagonists capsazepine and ruthenium red, further supporting their selective actions. We also found, as described previously (Vanner and Bolton, 1996), that stimulation with capsaicin, at a site remote and isolated by a dividing chamber from the recording site, evoked vasodilator responses. These actions could not be reproduced with exogenous application of the putative neurotransmitter substance P (Vanner and Bolton, 1996) into the stimulating chamber, thereby excluding electrotonic coupling as the mechanism underlying the signaling between the two chambers. Taken together, these studies show that functional TRPV1 receptors exist on distal axons and their activation evokes action potentials resulting in vasodilatory neurotransmitter release.

Our studies suggest that the neural propagation initiated by activation of TRPV1 in these visceral extrinsic afferent nerves is not sensitive to TTX but depends on Na⁺ currents. We found in both the guinea pig and mouse that vasodilator responses evoked by capsaicin, and EFS in the mouse, were not blocked by 1 μM TTX. Furthermore, capsaicin-evoked vasodilator responses in guinea pig ileum were blocked by low Na⁺ solutions and markedly inhibited by the Na⁺,1.8 antagonist A-893467 (Jarvis et al., 2007), directly implicating TTX-resistant Na⁺,1.8 channels. It is thought unlikely that low Na⁺ solution would inhibit the depolarization caused by opening of the TRPV1 channel (Holzer, 1991). We also tested this possibility directly by using perforated patch-clamp recordings from the cell bodies of capsaicin-sensitive DRG nerves. We found that low Na⁺ blocked TTX-resistant action potentials and the Na⁺,1.8 antagonist A-893467 blocked a significant proportion, but application of capsaicin still evoked large depolarizations in the presence of combined TTX and low Na⁺ solution or A-893467. Furthermore, in our studies of mouse mesenteric arterioles (see Fig. 6B) we found that low Na⁺ solution did not block capsaicin-evoked dilations. Recent studies of somatic extrinsic afferent nerves have provided important insights into the relative role of TTX-sensitive and -insensitive Na⁺ channels in axonal neurotransmission (Zimmermann et al., 2007; Pinto et al., 2008).
In those studies axonal neurotransmission in Aδ and C fibers evoked by electrical stimulation were dominated by TTX-sensitive Na⁺ channels at physiological temperatures. TTX-resistant channels did not support propagation of full-amplitude APs that could trigger synaptic release in the spinal cord (Pinto et al., 2008). Those studies have also suggested that these channels, at least in the somatic nervous system, are physiologically relevant only at low temperatures. In contrast, our study examines the efferent actions of these nerves in a visceral organ. In this setting, our findings suggest that visceral nerves have sufficient density of TTX-resistant Na⁺ channels to support the local axonal propagation of APs sufficient to evoke release of neurotransmitter within the intestine.

It is unclear at present whether the subpopulation of capsaicin-sensitive nerves that subserve the efferent function of these nerves, e.g., vasodilator nerves, represent the same population of capsaicin-sensitive extrinsic afferent nerves underlying nociceptive signaling in the intestine. We examined whether afferent nerve recordings from the ileum were able to generate TTX-resistant APs and found that capsaicin-evoked responses, although reduced, still exhibited robust action potential discharge. In contrast, distention-induced responses were almost completely blocked by TTX, suggesting these mechanical responses were mediated by extrinsic afferent nerves dominated by TTX-sensitive Na⁺ channels. These latter findings are similar to those reported by others in the mouse and rat somatic nerves (Yoshida and Matsuda, 1979; Villiére and McLachlan, 1996). It is possible that capsaicin activates a subpopulation of nerves with sufficient density of TTX-resistant Na⁺ channels to support neural propagation, at least in distal nerves over short distances, and/or neural propagation in visceral nerves differs from that observed in somatic nerves.

In summary, the results of this study demonstrate that activation of TRPV1 on intestinal extrinsic afferent neurons initiates TTX-resistant neural propagation with ensuing neurotransmitter release and resulting vasodilation. Previous electrophysiological and immunohistochemical studies suggested that Na⁺ channels are present in the soma and axons of these neurons and may underline the TTX-resistant neural propagation. The effect of the A-803467 antagonist observed in the present study supports these conclusions (Rush et al., 2005, 2007; King et al., 2009). Our findings differ from other studies of neural propagation DRG axons in the somatic nervous system where these channels seem to be predominantly active during hypothermia. These differences may reflect the relative evolutionary survival advantages of hypothermia signaling in the skin versus the viscera and/or differences in efferent as opposed to afferent actions of the nerve terminals in the end organ. These voltage-gated Na⁺ channels are highly regulated during inflammation (Stewart et al., 2003; Beyak et al., 2004), including colitis and ileitis, either by phosphorylation of existing channels and/or increased expression of channels in the membrane (King et al., 2009). These events have been documented through studies of the cell bodies of the extrinsic sensory afferents. It will be important to determine whether similar changes occur in...
their axon terminals, resulting in increased local axon reflexes in the intestine. The findings of this study also highlight that using Na\textsubscript{1.8} antagonists, such as A-803467, for the treatment of clinical pain syndromes may also disrupt peptidergic axon reflexes within the intestine and possibly other organs. Depending on the clinical setting, current data suggest this might be detrimental if Na\textsubscript{1.8} antagonists are involved in protective actions, such as mucosal protection, or advantageous, if they are stimulating proinflammatory responses (Holzer, 2006).

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