Inhibition of Rapid Heat Responses in Nociceptive Primary Sensory Neurons of Rats by Vanilloid Receptor Antagonists

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Kirschstein, Timo, Wolfgang Greffrath, Dietrich Büsselberg, and Rolf-Detlef Treede. Inhibition of rapid heat responses in nociceptive primary sensory neurons of rats by vanilloid receptor antagonists. J. Neurophysiol. 82: 2853–2860, 1999. Recent studies demonstrated that heat-sensitive nociceptive primary sensory neurons respond to the vanilloid receptor (VR) agonist capsaicin, and the first cloned VR is a heat-sensitive ion channel. Therefore we studied to what extent heat-evoked currents in nociceptive dorsal root ganglion (DRG) neurons can be attributed to the activation of native vanilloid receptors. Heat-evoked currents were investigated in 89 neurons acutely dissociated from adult rat DRGs as models for their own terminals using the whole cell patch-clamp technique. Locally applied heated extracellular solution (effective temperature ∼53°C) rapidly activated reversible and reproducible inward currents in 80% (62/80) of small neurons (±32.5 μm), but in none of nine large neurons (P < 0.001, χ2 test). Heat and capsaicin sensitivity were significantly coexpressed in this subpopulation of small DRG neurons (P < 0.001. χ2 test). Heat-evoked currents were accompanied by an increase of membrane conductance (320 ± 115%; mean ± SE, n = 7), had a reversal potential of 5 ± 2 mV (n = 5), which did not differ from that of capsaicin-induced currents in the same neurons (4 ± 3 mV), and were carried at least by Na+ and Ca2+ (pCa2+ > pNa+). These observations are consistent with the opening of temperature-operated nonselective cation channels. The duration of action potentials was significantly higher in heat-sensitive (10–90% decay time: 4.45 ± 0.39 ms, n = 12) compared with heat-insensitive neurons (2.18 ± 0.19 ms, n = 6; P < 0.005, Student’s t-test), due to an inflection in the repolarizing phase. This property as well as capsaicin sensitivity and small cell size are characteristics of nociceptive DRG neurons. When coadministered with heat stimuli, the competitive VR antagonist capsazepine (1 μM to 1 mM) significantly reduced heat-evoked currents in a dose-dependent manner (IC50, 13 μM, Hill slope –0.58, maximum effect 75%). Preincubation for 12–15 s shifted the IC50 by –0.5 log10 units to an estimated IC50 of ∼4 μM. The noncompetitive VR antagonist ruthenium red (5 μM) significantly reduced heat-evoked currents by 33 ± 6%. The effects of both VR antagonists were rapidly reversible. Our results provide evidence for a specific activation of native VRs in nociceptive primary sensory neurons by noxious heat. The major proportion of the rapid heat-evoked currents can be attributed to the activation of these temperature-operated channels, and noxious heat may be the signal detected by VRs under physiological conditions.

INTRODUCTION

Polymodal C- and Aδ-fiber nociceptors in the skin of primates and humans respond within a few milliseconds to fast temperature increases generated by infrared lasers (Bromm and Treede 1983; Tillman et al. 1995). These short latencies observed in vivo suggest a rapid direct transduction mechanism for heat stimuli at the peripheral endings of small dorsal root ganglion (DRG) neurons (Treede et al. 1995, 1998). DRG neurons are primary sensory neurons comparable to olfactory or visual receptor cells, but in contrast to vision and olfaction, little is known about the transduction mechanisms of nociception and pain (Belmonte 1996).

Recent in vitro studies of the underlying membrane currents have supported the existence of a rapid heat transduction mechanism in nociceptive primary sensory neurons of the rat (Cesare and McNaughton 1996; Dittert et al. 1998; Kirschstein et al. 1997; Nagy and Rang 1999). The somata of DRG neurons are used as models for their own peripheral terminals (Vyklicky and Knotková-Urbancová 1996) because the terminals in the skin are inaccessible for adequate electrophysiological studies (patch-clamp) due to their small size and tough surrounding tissue. Brief heat stimuli of <1 s duration were found to elicit inward currents in DRG neurons of neonatal rats after 4–6 days in primary culture (Cesare and McNaughton 1996) and within 4–30 h after acute dissociation from adult rats (Kirschstein et al. 1997). The activation was rapid but not instantaneous with a half-time of maximal activation of 35 ms and with a threshold temperature of ∼43°C (Cesare and McNaughton 1996). These findings suggest a novel mechanism of rapid cellular signaling by temperature-operated ion channels.

We have previously demonstrated that heat sensitivity in acutely dissociated DRG neurons is coexpressed with sensitivity to capsaicin (Kirschstein et al. 1997), the active ingredient in hot chili peppers that is a selective activator of nociceptive afferents (Szallasi and Blumberg 1996). This observation would be compatible with two possibilities: 1) capsaicin and heat act on different transduction pathways that are co-localized in the same nociceptive neurons, or 2) capsaicin and heat act on a common transduction pathway. The second hypothesis is more likely because the first cloned vanilloid receptor (VR1) is a nonselective cation channel that is activated by the vanilloid receptor agonist capsaicin and by raising the temperature to 40–45°C (Caterina et al. 1997; Tominaga et al. 1998). On the other hand, additional heat-transduction mechanisms have been described, which differ from the rapid transduction pathway with respect to 1) threshold temperature and response latency (Treede et al. 1995, 1998), 2) correlation with VR expression (Nagy and Rang 1999), and 3) dependence on intracellular calcium (Reichling and Levine 1997).

The aim of this study was to test whether and to what extent heat-evoked inward currents in nociceptive DRG neurons can
be attributed to the activation of native vanilloid receptors. For this purpose, we characterized the electrophysiological properties of heat-evoked inward currents and of the neurons that express these currents, tested responses of the neurons to the VR-agonist capsaicin, and determined the effects of the two known vanilloid receptor antagonists, ruthenium red (RR) (Amann and Maggi 1991) and capsazepine (CPZ) (Bevan et al. 1992), on rapid heat-evoked currents in acutely dissociated DRG neurons of adult rats. Preliminary accounts of this study have appeared in abstract form (Kirschstein et al. 1998).

This paper contains essential parts of the dissertation of T. Kirschstein.

METHODS

The preparation, neuron dissociation, electrophysiological recordings, and heat stimulation were done as previously described (Kirschstein et al. 1997). Briefly, adult Sprague-Dawley rats (120–270 g) of both sexes were deeply anesthetized with diethylether and rapidly decapitated (see Greffrath et al. 1998 for detail). This method is in accordance with German national law and the rules of local ethical committees. The spine was chilled at 4°C in F12-Dulbecco’s modified Eagle’s medium (Sigma) saturated with carbogen gas (95% O2-5% CO2) and additionally containing 30 mM NaHCO3 (Merck, Darmstadt, Germany), 100,000 units I-1 penicillin and 100 mg I-1 streptomycin (Sigma). Thoracic and lumbar DRGs (8–15) were quickly dissected and freed from connective tissue. Neurons were dissociated in an incubation chamber enriched with carbogen gas at 37°C using collagenase CLS II (5–10 mg ml-1, 40–50 min; Biochrom, Berlin, Germany) and trypsin (0.2–1 mg ml-1, 10–12 min; Sigma) dissolved in the F12 medium. After trituration (4–6 times with a Pasteur pipette) neurons were plated on 35-mm-diam culture dishes, which also served as recording chambers, and stored at 37°C in a humidified 5% CO2 atmosphere before being used for electrophysiological recordings 3–12 h (up to 30 h in some cases) after dissociation.

Electrophysiology

Only round or oval-shaped neurons without any processes were included in this study. The average of the major and the minor diameter was used to measure the size of oval shaped neurons. Whole cell patch-clamp experiments were performed in carbogen gas saturated F12 medium (pH 7.4) at room temperature (RT) using an Axopatch 200A amplifier (Axon Instruments) in voltage-clamp mode at a holding potential of ~80 mV controlled by pCLAMP software. Data were also registered on a chart recorder (L6512, Linseis, Selb, Germany). Patch pipettes were fabricated from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) using a horizontal micropipette puller (RP-87, Sutter) and filled with a solution containing (in mM) 140 CsCl, 10 HEPES, 10 EGTA, and 4 MgCl2, saturated in F12 medium. After trituration (4–6 times with a Pasteur pipette) neurons were plated on 35-mm-diam culture dishes, which also served as recording chambers, and stored at 37°C in a humidified 5% CO2 atmosphere before being used for electrophysiological recordings 3–12 h (up to 30 h in some cases) after dissociation.

Heat stimulation, drug application, and solutions

Application of ~50 µl of heated extracellular solution through a puffing system fixed on a micromanipulator was used to elicit heat-evoked currents. Control measurements with a fast temperature sensor (BAT-12, Physitemp; τ = 5 ms) in place of the neurons revealed an effective peak temperature of ~53°C, a rise time of ~250 ms, and a decay with a time constant of ~20 s. Effects were compared with those of application of the same amount of medium at RT. Heat stimuli with or without vanilloid receptor antagonists and control applications at RT were repeated 2–10 times, and the elicited currents were averaged. A neuron was considered heat sensitive when the heat-evoked inward current was significantly greater than any fluctuations caused by superfusion of solution at RT (unpaired Student’s t-test for each neuron, P < 0.05). Heating the buffered extracellular solution may change its pH, and acid solutions of pH 6.2 are known to activate nociceptive DRG neurons (Bevan and Yeats 1991). The pH of a HEPES-buffered solution decreases while heating (e.g., pH 7.1 at 50°C according to the Henderson-Hasselbalch equation). In contrast higher temperatures increase the pH of a NaHCO3/CO2 buffer, because the solubility of CO2 is reduced and thus reverses the HEPES effect. Off-line measurements showed that the pH of the F12 medium maximally changed in a range of 7.28–7.52 while heating to 50°C and cooling down to RT. Furthermore, the absence of color changes of the pH indicator phenol red, which was present in the extracellular solution, verified on-line that pH throughout all experiments remained well above 6.8. The membrane conductance was measured in voltage-clamp mode by hyperpolarizing pulses (5 mV, 10 ms, 50 s-1), and conductance changes were determined at the maximum amplitude of heat evoked currents.

Reversal potentials of heat- and capsaicin-induced currents were measured as described by Liu et al. (1997) using fast depolarizing ramps (~80 to ~300 mV in 200 ms every 550 ms). In these experiments patch pipettes were filled with a potassium-free solution containing (in mM) 140 CsCl, 10 HEPES, 10 EGTA, and 4 MgCl2, adjusted to pH 7.2). Tetrodotoxin (TX, 100 µM; Sigma) and nifedipine (1 µM; Sigma) were added to the extracellular solution to block voltage-gated Na+ and Ca2+ channels. These neurons were not included in the general statistics of heat-sensitive cells. To examine charge carriers of heat-induced currents, Na+ in the extracellular solution was replaced by N-methyl-D-glucamine (NMDG; Sigma). In some of these experiments extracellular Ca2+ was raised to 10 mM. Reversibility of ion replacement experiments was tested by final application of heated solution at physiological ion composition. Capsaicin (Sigma) was initially dissolved in ethanol, diluted to its final concentration in F12 medium, and applied through the puffing system as described above. CPZ (dissolved in DMSO; purchased from RBI, Cologne, Germany) and RR (in extracellular solution; RBI) were prepared as concentrated stock solutions, diluted to final concentration in F12 medium, and applied either at RT or ~53°C in the same way. Because CPZ solutions contained final concentrations of 0.01–10% DMSO, the same amount was added to all solutions applied in CPZ experiments, too. In the concentration range of 0.01–1%, DMSO alone did not elicit any visible effects, but with 10% DMSO the proportion of neurons that could not be kept for the full protocol was increased. Reversibility of antagonist action was tested by reapplication of heated extracellular solution without any agents (n = 21). Only neurons tested for reversibility were included for further statistical analysis.

Data analysis

Off-line measurements and statistical analysis were done using pCLAMP6 (Axon Instruments) and EXCEL 5.0 (Microsoft). Data are presented as means ± SE. Treatment effects were statistically analyzed by Student’s t-test for paired data and χ2 test for analysis of incidences. Dose dependence of the CPZ-induced inhibition was
analyzed by a repeated measures ANOVA with Student-Newmann-Keuls post hoc test for ordered means (STATISTICA 4.5, StatSoft). Error probabilities $P \leq 0.05$ were considered statistically significant. Fitting of the dose-response function was done using GraphPad Prism 2.01 (GraphPad Software).

RESULTS

Electrophysiological properties of heat-sensitive DRG neurons

Whole cell patch-clamp experiments were performed on 89 acutely dissociated DRG neurons clamped at $-80$ mV. Brief superfusion of heated extracellular solution (effective temperature $\sim 53{^\circ}C$) activated a significantly greater inward current than the same amount of solution applied at RT (see METHODS) in 62 of the 80 small neurons (80%; see Fig. 1A for an example). In contrast, none of nine large neurons (diameter $>32.5 \, \mu m$) significantly responded to heat ($P < 0.001, \chi^2$ test). In the majority of heat-sensitive neurons examined (47 of 62) application of solution at RT did not elicit any change in holding currents. Currents elicited by control applications at RT were even significantly smaller in heat-sensitive (10 ± 3 pA, $n = 62$) than in heat-insensitive neurons (53 ± 17 pA, $n = 18$, $P < 0.001$). Within heat-insensitive neurons, however, the inward currents elicited by solutions at either $\sim 53{^\circ}C$ (62 ± 16 pA) or RT (53 ± 17 pA) did not differ significantly. Heat-evoked currents were reproducible at 1 to 15 s interstimulus intervals (1st: 279 ± 43 pA; 2nd: 310 ± 44 pA, $n = 62$, n.s.) and were accompanied by an increase in membrane conductance (Fig. 1B), consistent with the opening of temperature-operated channels. The averaged maximum increase of the membrane conductance during the peak of heat-evoked currents was $320 \pm 115\%$ ($n = 7$).

Within this subpopulation of small DRG neurons (diameter $\leq 32.5 \, \mu m$), heat-sensitive and heat-insensitive neurons did not differ in diameter ($27.5 \pm 0.3 \, \mu m$ for heat-sensitive, $27.4 \pm 0.8 \, \mu m$ for heat-insensitive neurons) nor in whole cell capacitance ($23.2 \pm 0.9 \, pF$ vs. $21.2 \pm 1.7 \, pF$). RMP was more depolarized in heat-sensitive ($-46 \pm 1 \, mV$) than in heat-insensitive small neurons ($-52 \pm 2 \, mV$, $P = 0.033$), but there was no correlation between RMP, measured in current-clamp mode, and heat-evoked current in neurons clamped at $-80 \, mV$ in voltage-clamp mode ($r = -0.17$, n.s., $n = 62$). Moreover, the three cells with the most negative RMP of about $-70 \, mV$ exhibited three of the largest heat responses (>600 pA). Therefore heat sensitivity is not a function of a depolarized membrane potential.

A prominent inflection in the repolarizing phase of APs...
evoked by constant current injection was observed in 11 of 12 heat-sensitive neurons (Fig. 2), whereas a smaller inflection was detectable only in 1 of 6 heat-insensitive small neurons ($P$, 0.005, $\chi^2$ test). As a consequence of this “shoulder,” action potentials in heat-sensitive neurons displayed significantly longer repolarizing phases (10–90% decay time: 4.45 ± 0.39 ms, $n$ = 12) than heat-insensitive neurons (2.18 ± 0.19 ms, $n$ = 6; $P$ < 0.005).

To test whether heat and capsaicin sensitivity are coexpressed in DRG neurons, the effect of capsaicin (1–10 μM) was examined in 29 neurons. None of 5 large neurons (diameter >32.5 μm) but 16 of 24 small neurons (<32.5 μm) were excited by capsaicin ($P$, 0.01, $\chi^2$ test). Only 1 of 15 heat-sensitive neurons was not excited by capsaicin, whereas 12 of 14 heat-insensitive neurons were also capsaicin insensitive ($P$, 0.001, $\chi^2$ test). Two heat-insensitive neurons were ex-
cited by capsaicin, but one of those neurons developed heat sensitivity after the capsaicin-induced excitation, the remaining neuron was not tested with heat after capsaicin. Time-to-peak of capsaicin-induced currents varied between 300 ms and 65 s with a mean of 14 ± 5 s (n = 14). Capsaicin often induced multiple current peaks, and for the value above the time to the maximum current was measured (2.950 ± 700 pA). Whereas some capsaicin-induced currents were almost as fast as heat-evoked currents, others were as slow as the long-latency capsaicin currents reported by others (Liu and Simon 1996; Petersen et al. 1996).

The reversal potentials of heat-evoked currents and capsaicin-induced currents were determined in five small neurons (diameter 28 ± 0.8 μm) with current-voltage (I-V) curves elicited by fast depolarizing ramps using Cs⁺ in the intracellular and TTX (100 μM) and nifedipine (1 μM) in the extracellular solution. These agents did not abolish all inward currents elicited by the initial depolarizing ramp, which may be due to the presence of, e.g., TTX-resistant sodium channels. However, these small currents disappeared during repeated ramps. Therefore the repeated-ramps protocol was run until those currents had inactivated (within ~100 cycles), before heat or capsaicin were applied to determine reversal potentials. The reversal potential of heat-evoked currents was 5 ± 2 mV, that of the initial phase of capsaicin-induced currents (1 μM) in the same neurons 4 ± 3 mV (n.s.). Within the variability between neurons, the reversal potentials of heat-evoked and capsaicin-induced currents were highly correlated (r = 0.93, P < 0.05, n = 5). The reversal potential near 0 mV can be explained by a nonselective cation channel, that is outwardly permeable to Cs⁺. In conclusion, heat-evoked currents and capsaicin-induced currents are both carried through similar nonspecific cation channels.

We therefore tested whether the rapid heat-induced inward currents are carried by Na⁺ and/or Ca²⁺. When Na⁺ was replaced by NMDG (n = 15), heat-evoked currents were reversibly reduced to 34 ± 4% of the mean currents before and after ion replacement (P < 0.001). When Ca²⁺ in the Na⁺-free extracellular solution was raised to 10 mM (n = 7), heat-evoked currents were enhanced to 478 ± 120% (P < 0.05). Although in these experiments the proportion of putative charge carriers was smaller than in physiological extracellular solution (10 vs. 145 mM) heat-evoked currents did not differ significantly from the normalized controls (129 ± 38% vs. 100%; n.s.), indicating a higher permeability for Ca²⁺ than for Na⁺ ions.

**Effects of vanilloid receptor antagonists on heat-evoked currents**

Heat-sensitive neurons were tested for the effects of vanilloid receptor agonists on heat-evoked inward currents to examine pharmacologically, whether and to what extent native vanilloid receptors contribute to rapid heat-transduction mechanisms. When the competitive antagonist CPZ (10 μM) was added to the heated extracellular solution (Fig. 3, A–C) heat-evoked inward currents were significantly reduced by 31 ± 5% (P < 0.01, n = 12). This inhibition was rapidly and fully reversible, as demonstrated by the response to the application of heated control solution ≤60 s after the CPZ application, which did not differ from the initial heat response (Fig. 3D).

Furthermore, CPZ significantly increased the time-to-peak of heat-evoked currents from 124 ± 5 ms to 154 ± 10 ms (P < 0.01; n = 12). In contrast, in normal extracellular solution, smaller currents were associated with shorter time-to-peak (r = 0.64, P < 0.05, n = 12).

The CPZ-induced inhibition of heat-evoked currents was dose dependent (1 μM to 1 mM; F4,38 = 6.42, P < 0.001, ANOVA; see Fig. 3E) with an IC₅₀ of 13 μM (log₁₀ IC₅₀ = −4.88 ± 0.18), a Hill slope of −0.58 ± 0.10, and an extrapolated efficacy of 75 ± 5%, suggesting that most of the rapid heat-evoked current was inhibited by CPZ, although CPZ binding probably was not at equilibrium with simultaneous application. When neurons were preincubated with 10 μM CPZ (i.e., at the steepest part of the dose-response function) for 12–15 s, heat-evoked currents were reversibly reduced by 47.2 ± 8% of the normalized controls (P < 0.001; n = 7). The difference between the inhibiting effect of CPZ with and without preincubation was marginally significant (47 vs. 31%; P = 0.11), indicating a very fast action of the competitive antagonist on heat-evoked currents. This difference corresponded to a shift of the dose-response curve by −0.5 log₁₀ units resulting in an estimated IC₅₀ of ~4 μM. Application of CPZ at room temperature did not elicit any currents (0 ± 0 pA, n = 2, data not shown).

Application of RR (5 μM) significantly reduced heat responses by 33 ± 6% (Fig. 4; P < 0.005, n = 7), likewise in a fully reversible manner and without affecting neurons when applied at RT (0 ± 0 pA, n = 3, data not shown). RR specifically inhibits the vanilloid receptors at lower concentrations only in a narrow range (estimated to 0.1–10 μM) and may also reduce intracellular calcium release (Amann and Maggi 1991; Maggi 1991). Thus we did not use higher concentrations.

**DISCUSSION**

A subpopulation of small neurons acutely dissociated from rat dorsal root ganglia responded to brief noxious heat stimuli with inward currents. In contrast to heat-insensitive small and large neurons, this population was excited by the vanilloid receptor agonist capsaicin and exhibited a prolonged action potential duration with a prominent inflection in the repolarization phase. Heat-sensitive DRG neurons therefore fulfilled properties of heat-sensitive DRG neurons.

As in our previous study (Kirschstein et al. 1997; see also Dittert et al. 1998), we found rapid heat-evoked currents only in small neurons (≤32.5 μm). Nociceptive neurons are generally small, consistent with their small axons (Gold et al. 1996; Harper and Lawson 1985; Petersen and LaMotte 1991). In their
pioneering study on heat-evoked currents, Cesare and McNaughton (1996) therefore restricted their sample to small DRG neurons. Two other studies reported heat-evoked currents in some larger DRG neurons (Nagy and Rang 1999; Reichling and Levine 1997), but these responses are likely due to other heat transduction mechanisms (see *Multiple heat transduction pathways*).

Rapid heat-evoked inward currents in DRG neurons were associated with comparably fast responses to the vanilloid receptor agonist capsaicin, confirming the results of our previous study (Kirschstein et al. 1997). A similar coexpression has been reported in rat and monkey nociceptive afferents in vivo (Baumann et al. 1991; Szolcsányi et al. 1988). The finding that vanilloid receptor antagonists substantially reduced all rapid heat responses in the present study provides another line of evidence that heat-sensitive neurons express vanilloid receptors.

The repolarization phase of the AP was significantly longer in heat-sensitive than heat-insensitive small DRG neurons, due to a shoulder that was exhibited by 11 of 12 heat-sensitive but only 1 of 6 heat-insensitive neurons. This AP shape has been associated with small soma diameter and slow axonal conduction velocity in the C-fiber range (Harper and Lawson 1985), the presence of TTX-resistant sodium channels (Waddell and Lawson 1990), as well as responsiveness to the vanilloid receptor agonist capsaicin (Del Mar et al. 1996). Our data support the view that somal AP shape is associated with functional characteristics of the cell as a nociceptive primary sensory neuron rather than simply with its size.

**Properties of rapid heat-evoked currents**

The suggestion that nociceptive primary sensory neurons possess a rapid transduction mechanism for noxious heat stimuli that is independent from tissue damage (Treede et al. 1995) was supported by the demonstration that noxious heat elicited inward currents in small DRG neurons (Cesare and McNaughton 1996). Two possible mechanisms may be responsible for the inward currents: 1) the heat-induced opening of membrane channels or 2) the heat-induced inactivation of persistent outward currents. Because in our data the heat-evoked inward currents were accompanied by significant increases in whole cell conductance, we conclude that they were due to channel opening. Likewise, a preliminary report on cell-attached patch recordings has demonstrated channel opening by heat stimuli of 40–48°C (Nagy and Rang 1998).

Noxious heat may either induce a direct opening of temperature-operated channels or trigger an intracellular signal cascade resulting in channel opening. A direct transduction mechanism seems more probable, because the time-to-peak of heat-evoked currents was at least as fast as the temperature peak of the stimulus used. This finding is consistent with observations on primary nociceptive afferents in vivo, that the initial burst of action potentials is already generated during the rise time of the cutaneous heat stimulus (Treede et al. 1995). To determine the activation kinetics exactly, a heat stimulus has to be established, which is much faster than the rapid heat-evoked currents (e.g., a laser-stimulator described by Baumann and Martenson 1994).

The reversal potential of heat-evoked currents in our study was similar to that in cultured DRG neurons of neonatal rats (Cesare and McNaughton 1996), which resembled that of the rapid component of capsaicin-induced currents (Liu and Simon 1996; Oh et al. 1996). Ion replacement experiments showed that the temperature-operated channels are permeable to Na++, Ca++, and Cs⁺ (cf. Cesare and McNaughton 1996). Finally, rapid heat-evoked currents had a higher permeability for Ca++ than for Na++ ions, which is also typical for capsaicin-induced currents (Bevan and Szolcsányi 1990; Jung et al. 1999). From changes of reversal potentials of heat-evoked currents after ion replacement, the permeability ratio pNa+/pCa2+ was calculated to be 1:1.28 (Cesare and McNaughton 1996). In addition to these similarities of heat-evoked and capsaicin-induced currents described in different studies, we now have demonstrated that the reversal potential of both currents is not distinguishable in the same neuron. Thus both currents may be conducted at least in part by identical channels.

**Vanilloid receptors and heat transduction**

A candidate for such a channel is the vanilloid receptor VR1, a nonselective cation channel that is activated by heating the cell to 40–45°C and that is selectively expressed in DRGs and trigeminal ganglia (Caterina et al. 1997; Tominaga et al. 1998). The heterologous expression of VR1 in both human embryonic kidney cells (HEK 293) and frog oocytes conveyed heat sensitivity to these cells (Caterina et al. 1997), consistent with a direct action of noxious heat on capsaicin-sensitive membrane channels. Single-channel openings were observed in excised patches of these VR1-transfected cells to both heat and capsaicin (Tominaga et al. 1998). In the present study heat-evoked currents in DRG neurons were inhibited by 75% by the competitive VR antagonist CPZ and by 33% by the noncompetitive VR antagonist RR. Thus we have now provided pharmacological evidence that native vanilloid receptors are a key element in the responses of nociceptive primary sensory neurons to noxious heat stimuli.

The reduction in heat-evoked currents by 10 µM CPZ (by 31% with co-application and 47% with preincubation) was smaller than that reported for currents elicited by 0.5 µM capsaicin (by 96%) in neonatal rat DRG neurons (Bevan et al. 1992). Whereas the reduction of heat-evoked currents by CPZ was fully reversible on wash out, the capsaicin-evoked currents recovered only by ~50%; this was attributed to desensitization induced by the first application of capsaicin (Bevan et al. 1992). Interestingly, in *Xenopus* oocytes expressing the cloned VR1, 10 µM CPZ had 70% efficacy against 44°C (Tominaga et al. 1998), but >95% efficacy against 0.6 µM capsaicin (Caterina et al. 1997). In HEK 293 cells, CPZ had 90% efficacy against moderate noxious heat and 97% against capsaicin, but the preincubation with CPZ was four times longer for the heat tests than for capsaicin, recovery was incomplete, and repetition of the 46°C heat stimulus by itself reduced the response by 44% (Tominaga et al. 1998). Thus a reduced efficacy of CPZ to inhibit VR1 activation by heat compared with capsaicin is supported by these findings, too. Because protein structures are highly sensitive to temperature, the affinity of vanilloid receptors for CPZ is likely to be reduced at the temperature used in the present study. For enzymes such as lactate dehydrogenase, the *K_m* for substrate binding is about three to five times higher at 50°C than at room temperature (Somero 1995). The temperature dependence of ligand-receptor interactions may explain...
most of the difference between the IC_{50} described here at \(-53^\circ\text{C}\) (4–13 \(\mu\text{M}\)) and the binding constant of CPZ to vanilloid receptors at room temperature \(K_d\) of 0.1–0.7 \(\mu\text{M}\), that was estimated from Schild plots of the competitive antagonism of CPZ versus capsaicin and resiniferatoxin in rat DRG neurons (Bevan et al. 1992). Unfortunately, a Schild analysis to determine the \(K_d\) for CPZ-induced inhibition of heat-evoked currents is impracticable, because the upper end of useful heat intensities (i.e., the “agonists” concentration) is limited by activation of another transduction mechanism that is VR independent (Caterina et al. 1999; Nagy and Rang 1999) (see Multiple heat transduction pathways) and ultimately by cell damage. Therefore we did not determine, whether CPZ inhibits heat-evoked currents competitively like it antagonizes capsaicin-induced currents.

Alternatively, the apparent difference in affinity raises the question, whether the inhibition of heat-evoked currents may have been unrelated to a specific inhibition of vanilloid receptors. CPZ had no effects on GABA-receptors, ATP-receptors, and depolarization-induced ion fluxes at RT (Bevan et al. 1992). Recently, however, nonspecific effects of CPZ have been described on voltage-activated calcium channels with an \(E_{D_{50}} = 8 \mu\text{M}\) (Docherty et al. 1997) and on nicotinic acetylcholine receptors at 10 \(\mu\text{M}\) (Liu and Simon 1997). The time to reach half-maximum effect on voltage-activated calcium channels was \(-5\text{ min}\) at 1–10 \(\mu\text{M}\) and 1 min at 100 \(\mu\text{M}\), and this effect was irreversible. Likewise, the inhibition of nicotinic acetylcholine receptors by 10 \(\mu\text{M}\) CPZ was found with a 2-min preincubation period of the antagonist, and it took \(-10\text{ min}\) wash out to reverse this effect. Because in our data the antagonists were preincubated for a few seconds only and the inhibition of the “agonist” heat was fully reversible as soon as tested \(-1\text{ min}\) or less), the inhibition of heat-evoked inward currents by CPZ is unlikely to be due to nonspecific effects. A further argument for the inhibition of heat-evoked currents by a specific interaction with vanilloid receptors derives from the significant effect of 5 \(\mu\text{M}\) RR. RR interacts with a different site of the vanilloid receptors (Bevan et al. 1992; Dray et al. 1990), and our dose was within the range that is considered specific for vanilloid receptors (0.1–10 \(\mu\text{M}\)) (Maggi 1991).

Thus whereas nonspecific effects of CPZ have been reported, the temporal characteristics of those effects are sufficiently different from the rapid and fully reversible inhibition of heat-evoked inward currents by CPZ to warrant the conclusion that vanilloid receptors are essentially involved in the rapid heat transduction pathway. The receptor subtype need not necessarily be VR1, because multiple vanilloid receptors exist (Szallasi 1994), which may be coexpressed in the same neuron (Liu and Simon 1996; Liu et al. 1997; Petersen et al. 1996), and subtype specific antagonists are not available.

Multiple heat transduction pathways

The major proportion of the rapid heat-evoked current of the present study can be attributed to vanilloid receptor activation. However, the extrapolated efficacy of CPZ (75 \(\pm\) 5\%) raises the question of whether this reflects a true incomplete antagonism (e.g., because heat acts at a different site of VR1 than CPZ) or whether the remaining fraction was due to another transduction mechanism that was independent of VRs.

A subpopulation of cultured DRG neurons has recently been described, that were heat-sensitive but not excited by capsaicin (Nagy and Rang 1999), and a membrane channel with similar properties has been cloned and called vanilloid receptor--like channel (VRL-1) (Caterina et al. 1999). These neurons had a higher heat threshold, smaller maximum currents, and larger diameters. A corresponding heat transduction mechanism is present in a subclass of Aδ-fiber nociceptors that respond to heat only after prolonged stimulus duration or subsequent to tissue damage and are called HTM for high-threshold mechanoreceptor or type 1 AMH (the 1st type of A-fiber mechanoreceptor discovered) (Fitzgerald and Lynn 1977; Perl 1968; Treede et al. 1995). In an in vivo study on the rat saphenous nerve, all polymodal C-fibers and the majority of polymodal Aδ-fibers responded to close arterial injection of capsaicin, whereas the Aδ-fiber HTMs were not affected (Szolcsányi et al. 1988). Thus in type 1 AMH the slow heat transduction mechanism appears to be independent of vanilloid receptors and may be mediated by VRL-1.

The remaining current after maximum blockade of VRs in the present study was significantly delayed, but the time-to-peak was still an order of magnitude faster than that of currents presented by Nagy and Rang (1999) and Caterina et al. (1999). Moreover, the size distributions of neurons expressing VR1 and VRL-1 do not suggest a widespread coexpression but an expression of either VR1 or VRL-1 (Caterina et al. 1999). In contrast to Nagy and Rang (1999) we found only one capsaicin-insensitive neuron that responded to heat. This difference between the studies may be due to several reasons. 1) The temperature used by us may not sufficiently activate the high-threshold current. 2) The stimulus used may have been too short; activation of the corresponding neurons in vivo (i.e., type 1 AMH) depends on a stimulus duration of several seconds (Treede et al. 1998). 3) Culture conditions were different (acutely dissociated vs. several days in culture with nerve growth factor, NGF); in the absence of NGF, type 1 AMHs in vivo change their phenotype into D-hair receptors (Ritter et al. 1993).

Still another mechanism seems to underly the heat-induced inward current \(I_{\text{heat}}\) reported by Reichling and Levine (1997). Peak currents (50 vs. 280 pA) and conductance increases (25 vs. 320\%) were about one order of magnitude smaller than in the present and other studies (Cesare and McNaughton 1996; Kirschstein et al. 1997). Their \(I_{\text{heat}}\) was blocked by extracellular cesium ions, whereas the nonspecific cation channel activated by brief noxious heat stimuli was even more permeable to Cs\(^+\) than to Na\(^+\) ions (Cesare and McNaughton 1996). Moreover, their \(I_{\text{heat}}\) exhibited a linear increase from room temperature to 45°C. This temperature dependence is not easily compatible with nociceptive neurons.

In summary, rapid heat responses in nociceptive primary sensory neurons appear to depend predominantly on a vanilloid receptor as the heat-sensing element. Conversely, our results support the hypothesis that the physiological function of the native vanilloid receptor VR1 is to detect noxious heat. This mechanism is different from two other heat transduction pathways that have been partly described, and the mechanisms of which await further studies.

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