Heat-Induced Action Potential Discharges in Nociceptive Primary Sensory Neurons of Rats

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1Division of Neurophysiology, Center of Biomedicine and Medical Technology Mannheim, Medical Faculty Mannheim, Ruprecht-Karls-University Heidelberg, Mannheim; 2Institute of Physiology and Pathophysiology, Johannes Gutenberg University, Mainz, Germany; 3Nottingham University Hospitals Trust, Nottingham, United Kingdom; and 4Texas Tech University–Health Sciences Center, Paul L. Foster School of Medicine, El Paso, Texas

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Greffrath W, Schwarz ST, Büsselberg D, Treede R-D. Heat-induced action potential discharges in nociceptive primary sensory neurons of rats. J Neurophysiol 102: 424–436, 2009. First published May 13, 2009; doi:10.1152/jn.90916.2008. Although several transducer molecules for noxious stimuli have been identified, little is known about the transformation of the resulting generator currents into action potentials (APs). Therefore we investigated the transformation process for stepped noxious heat stimuli (42–47°C, 3-s duration) into membrane potential changes and subsequent AP discharges using the somata of acutely dissociated small dorsal root ganglion (DRG) neurons (diameter ≤32.5 μm) of adult rats as a model for their own peripheral terminals. Three types of heat-induced membrane potential changes were differentiated: type 1, heat-induced AP discharges (~37% of the neurons); type 2, heat-induced membrane depolarization (40%); and type 3, responses not exceeding those of switching the superfusion (23%). Warming neurons from room temperature to 35°C increased their background conductance, nearly doubled the AP threshold current, and led to smaller and narrower APs. Adaptation of heat-induced AP discharges was seen in about half of the type 1 neurons. The remaining half displayed accelerating discharges to both heat stimuli and depolarizing current injection. Repeated heat stimulation induced marked suppression of AP discharges. Under rapid calcium buffering using BAPTA, repolarization of heat-induced APs stopped at a plateau potential slowly decreasing from +16.5 ± 2.9 to −2.2 ± 5.5 mV, resulting in no further AP discharges. This study demonstrates that heat-induced AP discharges can be elicited in the soma of a subgroup of DRG neurons. These discharges display suppression on repetitive stimulation, but either adaptation or sensitization during prolonged stimuli. AP threshold and AP shape during these discharges suggest temperature dependence of background conductance and repolarizing currents.

INTRODUCTION

The pseudo-unipolar ganglion cells in dorsal root and trigeminal sensory ganglia supply free and corpuscular nerve endings in the skin and other tissues. The vast majority of these neurons, particularly in the small- and medium-size range, are nociceptive and their peripheral terminals are specialized to detect actual or potential tissue damage. The somata of dorsal root ganglion (DRG) neurons have frequently been used for electrophysiological studies on sensory transduction mechanisms. They are accessible for patch-clamp recordings in dissociated or slice preparations (Baccaglini and Hogan 1983; Gold et al. 1996a; Scholz et al. 1998b; Vykylicky and Knotková-Urbancová 1996). DRG neurons respond with inward currents to a variety of noxious stimuli such as heat (Cesare and McNaughton 1996; Kirschstein et al. 1997, 1999; Liu and Simon 2000; Nagy and Rang 1999; Rau et al. 2007; Vykylický et al. 1999), cold (Reid and Flonta 2001; Viana et al. 2002), mechanical (Lewin and Moshourab 2004; McCarter et al. 1999), and various chemical agonists such as adenosine 5’-triphosphate (Cook and McCleskey 2002; Petruska et al. 2000; Piper and Docherty 2000), ethanol (Trevisani et al. 2002), nicotine (Liu and Simon 1996), and capsaicin (Hagenacker et al. 2005; Kirschstein et al. 1997, 1999; Liu and Simon 2000; Nagy and Rang 1999; Petersen and LaMotte 1991; Petruska et al. 2000; Piper and Docherty 2000).

Heat-evoked inward currents (Iheal) in a moderate noxious temperature range in nociceptive DRG neurons are probably carried by TRPV1, a member of the vanilloid receptor subfamily of the transient receptor potential channels. TRPV1 is a nonselective cation channel that is gated by capsaicin, noxious heat, and low pH (Caterina et al. 1997). Both TRPV1 and Iheal exhibit reversal potentials around 0 mV, a higher permeability for Ca2+ than for Na+, and outwardly rectifying voltage dependence (Caterina et al. 1997; Cesare and McNaughton 1996; Kirschstein et al. 1999; Schwarz et al. 2000; Tominaga et al. 1998). Iheal can be inhibited by the vanilloid receptor antagonists capsazepine and ruthenium red (Kirschstein et al. 1999; Liu and Simon 2000; Nagy and Rang 1999; Rau et al. 2007). Both Iheal and the heat-evoked currents through TRPV1 are inactivated during prolonged heat stimuli. Additionally, both currents show tachyphylaxis when repetitively stimulated. Inactivation and tachyphylaxis are independent of free intracellular calcium (Schwarz et al. 2000; Tominaga et al. 1998).

In peripheral nociceptive terminals, heat-evoked inward currents must be transformed into action potential (AP) discharges to be transmitted to the CNS. Without this essential step, the information concerning tissue damage would never reach the spinal cord or the brain. As a consequence there would be neither pain perception nor nociceptive reflexes. Furthermore, mutations of ion channels involved in AP-generation, such as the voltage-gated sodium channel Na1.7, lead to altered pain sensitivity in humans underlining the importance of this transformation process (see Waxman 2007 for review).

Very little is known about the transformation of heat-evoked currents into AP discharges. In native DRG neurons, peak values of heat-evoked depolarizing currents vary greatly between 50 and 10,000 pA (Cesare and McNaughton 1996;
The aim of this study was to investigate the transformation of heat-evoked inward currents into AP discharges using acutely dissociated DRG neurons as models for their own peripheral terminals. We recorded from neurons in current-clamp mode while superfusing them with extracellular solutions at room temperature or nonnoxious warm temperature (30–35°C), and during repeated noxious heat stimulation (42–47°C for 3 s, with an interstimulus interval [ISI] of about 40 s). We recorded the occurrence of heat-induced APs and their frequency adaptation and we documented the effects of increased temperatures on the shape of the electrically induced APs. Furthermore, we examined the influence of the intracellular free calcium concentration on the transformation process of the heat-evoked currents. We specifically addressed the following questions: Do moderate noxious heat pulses induce AP discharges in a subgroup of small DRG neurons? How do increasing temperatures affect the induction and shaping of APs? Do heat-induced AP discharges display adaptation and suppression in correspondence to the inactivation/tachyphylaxis of heat-evoked currents? If so, are these phenomena independent of rapid changes in intracellular calcium, as previously found for heat-evoked currents?

METHO DS

Acute dissociation of rat DRG neurons

The preparation of acutely dissociated DRG neurons was performed as previously described (Kirschstein et al. 1999; Schwarz et al. 2000). Adult Sprague–Dawley rats of either sex were deeply anesthetized with diethyl ether (Merck, Darmstadt, Germany) and rapidly decapitated. This method is in accordance with German National Law on the Principles for Animal Welfare and is approved by the representative for animal care and use of the University of Mainz. The spine was removed and transferred into chilled F12–Dulbecco’s modified Eagle’s medium (Sigma, Taufkirchen, Germany; adjusted to pH 7.4 by NaOH) containing 30 mM NaHCO3 (Merck), 100 IU streptomycin (Sigma) and equilibrated with 95% O2-5% CO2 throughout the entire preparation and dissociation procedure. Thoracic and lumbar DRGs were quickly isolated, and prepared with 95% O2-5% CO2 throughout the entire preparation and dishes, and stored at 37°C in a humidified 5% CO2 atmosphere for 1–10 h before being used for electrophysiological recordings. The addition of any growth factors was avoided, since they might change the response properties of nociceptors.

Electrophysiology

Whole cell patch-clamp experiments were performed in extracellular solution (ES) containing (in mM) 145 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 1.5 CaCl2, and 1.2 MgCl2 (pH adjusted to 7.4 at room temperature). Patch pipettes were fabricated from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) using a horizontal micropipette puller (P-87, Sutter Instruments, Novato, CA) and filled with a solution containing (in mM) 160 KCl, 10 HEPES, and either 8.13 EGTA or, in some experiments, 10 BAPTA adjusted to pH = 7.2 by KOH (Rion = 2.5–12 MΩ as measured and compensated in voltage-clamp mode in the bath). In experiments with BAPTA a period of 10 min was kept before applying heat stimuli to allow proper equilibration with the intracellular solution; no such fixed time interval was kept in neurons recorded with EGTA-containing solutions but the recording period before heat stimulation also amounted to several minutes here. Diameter, membrane capacitance, and resting membrane potential (RMP) were measured for each neuron investigated. Only round or oval-shaped neurons with a maximum diameter of 32.5 μm and without any processes were used for this study. The neuronal size was determined by averaging the major and the minor diameters of the neuron. Nociceptive neurons are generally small (Gold et al. 1996a; Petersen and LaMotte 1991) and heat-sensitive neurons were found to have diameters ≤32.5 μm (Kirschstein et al. 1997, 1999).

Recordings were performed using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and pCLAMP 8 Software (Axon Instruments; sampling rate: 20 kHz in current-clamp mode, 4 kHz in voltage-clamp mode).

Heat stimulation

The setup for the application of stepped heat stimuli has been described in detail before (Schwarz et al. 2000). Briefly, extracellular solutions were applied through four parallel glass tubes (outer diameter: 1.6 mm, inner diameter: 1.05 mm; flow rate 0.6–0.7 ml min−1). Two of the tubes (tubes 1 and 4) could be heated with a resistive device (HT 1.2; VETEC, Dümmerdor, Germany). Tubes 2 and 3 were warmed by the adjacent tubes 1 and 4 to about 30°C. Each tube was grounded and contained a miniature thermocouple (Physitemp IT-1E, Clifton, NJ) to measure the temperature of the solution near the outlet (adjusted to 42–47°C). In off-line experiments, when a further miniature thermocouple was positioned in place of the neuron at a distance of about 150 μm from the outlets, there was a difference of 0.2 ± 0.3°C (mean ± SD; see Schwarz et al. 2000) between the temperatures measured by the thermocouple inside and in front of the outlet. ES in the two reservoirs connected to the heated tubes was adjusted to a pH of 7.4 at 50°C. A stepper-motor was used to switch between the four tubes controlled by pCLAMP software. The time course of the heat stimulus of this setup, as measured by temperature-dependent change of the junction potential at the tip of open patch pipettes (see Fig. 1, A and C), had a rise time of 114 ± 6 ms to reach the plateau temperature and a fall time of 146 ± 13 ms to return to the baseline temperature.

Protocol

Neurons were clamped at −80 mV in voltage-clamp mode. In current-clamp mode, neurons were adjusted to a membrane potential of about −60 mV by injection of a DC current. Series resistance and membrane capacitance were manually compensated in voltage-clamp mode but not in current-clamp mode (see Technical considerations in Discussion for distortions of AP recordings when using patch-clamp amplifiers). Excitability was tested by depolarizing current steps (ΔI = 200 pA) at room temperature. Cells without the capability of
generating APs elicited by depolarizing current pulses of 40-ms duration were excluded from further investigation. A subset of neurons was additionally tested with longer-lasting rectangular depolarizing pulses of 750-ms duration. Subsequently, neurons were superfused with ES at 30–35°C (33.5 ± 0.2°C) and excitability was retested. Repeated noxious heat stimuli of 3-s duration were applied with constant stimulus temperatures of 42–47°C and an ISI of about 40 s. In experiments when neurons were tested repetitively, a maximum variation of 1°C in the application tube was tolerated between the repeated heat stimulations. In every dish only one neuron was tested for heat responses.

Altogether, 48 neurons were tested with heat stimuli of 3-s duration in current-clamp mode using the standard intracellular solution. Repetitive heat responses were investigated in 23 of these neurons. In 25 of the 48 neurons, properties of APs induced by depolarizing electrical pulses of 40 ms duration were studied at both room temperature and 30–35°C. Repetitive AP discharges to depolarizing electrical square pulses of 750-ms duration at room temperature were studied in 17 heat-sensitive neurons. Ten additional neurons not stimulated with heat were investigated by injection of reconstructed heat-evoked generator currents at room temperature. These generator currents were obtained by averaging heat responses from 18 heat-sensitive neurons investigated in the voltage-clamp mode (Schwarz et al. 2000). Another 15 small DRG neurons were tested with noxious heat stimuli using BAPTA (10 mM) to rapidly bind free intracellular calcium. A period of 10 min was kept before stimulation to allow equilibration of the fast calcium chelator with the intracellular milieu.

**Data analysis and AP variables**

Statistical analysis was performed using pCLAMP 8 (Axon Instruments), Excel 2003 (Microsoft), Statistica 4.5 (StatSoft), and Origin 5.0 with pCLAMP module (Microcal Software). AP threshold was determined as the minimum current needed for AP induction using depolarizing current steps. The leakage current was determined as the minimum current needed for AP induction using −80 mV in voltage-clamp mode. The holding current needed to stabilize the membrane potential at about −60 mV in current-clamp mode was determined and analyzed as a measure for background conductance. For interpretation of the discharge rate of heat-induced AP bursts, the absolute number of overshooting APs was determined in periods of 200-ms duration as a measure of instantaneous discharge rates during the stimulus.

To characterize the shape of APs induced by electrical or heat stimuli the following parameters were determined: amplitude of AP (maximum AP depolarization compared with baseline), maximum rate of AP rise (highest speed of depolarization), maximum rate of AP fall (highest speed of repolarization), and decay time (time to repolarize from 90 to 10% of maximum AP amplitude to minimum depolarization).

Data are presented as means ± SE. Treatment effects were statistically analyzed by Student’s t-test, for paired or unpaired data, or by ANOVA with a post hoc evaluation using the least-significant difference test. Error probabilities $P < 0.05$ were considered statistically significant.

**RESULTS**

The inward currents of 18 heat-sensitive neurons in response to a 3-s heat stimulus of 44.8 ± 0.19°C were averaged (Fig. 1A). This heat-evoked current exhibited pronounced inactivation during the constant-temperature pulse. When the averaged heat-evoked current was applied as depolarizing current to small DRG neurons at room temperature (Fig. 1B, top trace),
during current injection to 6.5 nV, polarization of 31.3 mV; median: depolarization of 2.7 mV, nonnoxious temperature (range: depolarization of 9.3 to hyper-
those induced by switching between solutions of the same
F response types did not differ with respect to diameter [F(2,45) = 0.74, RMP [F(2,45) = 0.66, P = 0.52], leakage current at −80 mV [F(2,45) = 0.04, P = 0.96], pipette resistance [F(2,45) = 0.87, P = 0.42], or serial resistance [F(2,45) = 0.41, P = 0.67].
Furthermore, neither stimulus temperature [F(2,45) = 1.07, P = 0.35] nor the temperature change during heat stimulation [F(2,45) = 1.31, P = 0.28] differed significantly between groups.

Influence of temperature on AP shape and excitability

Action potential generation in response to the heat pulse occurred at 42–47°C, i.e., a much higher temperature than electrically induced APs (room temperature). Therefore we
investigated the effects of warming the neurons by 10°C from room temperature to 33.8 ± 0.2°C on AP shape and excitability in 25 neurons (9 type 1, 10 type 2, and 6 type 3). APs were elicited by short (40-ms) depolarizing current injections of increasing intensity until the first AP occurred. Baseline AP properties did not differ across the three types of neurons, except for a trend for the decay time to be longer in type 3 than that in type 1 or type 2 neurons (Table 2). Warming by 10°C significantly increased not only the holding current but also the current necessary for AP induction, indicating an increase in background conductance. AP amplitudes and decay times were significantly reduced, leading to smaller and narrower APs (Fig. 3A).

In type 1 neurons, AP amplitude was further reduced at 42–47°C \( F_{(2,16)} = 15.0, P < 0.001 \), Fig. 3, A and B). Maximum rate of depolarization decreased slightly \( F_{(2,16)} = 2.21, P = 0.14 \) and maximum rate of repolarization increased significantly \( F_{(2,16)} = 12.9, P < 0.001 \), Fig. 3C). The decay time decreased significantly \( F_{(2,16)} = 48.35, P < 0.001 \), leading to additional shortening of AP duration (Fig. 3, A and D).

Discharge pattern of heat-induced APs

The mean time from heat stimulus onset to the first heat-induced AP was 960 ± 226 ms across all 14 type 1 neurons. The mean discharge rate across all of these neurons rapidly increased to 35 ± 10 Hz and continuously increased until the end of the heat stimulus to 46 ± 12 Hz (n.s., Student’s t-test; data not shown). However, when focusing on the individual AP-discharge patterns of the neurons, there was an obvious heterogeneity: 6 neurons displayed an early peak and decelerating discharge rates (decelerating response type 1A, Fig. 4A), whereas 8 neurons exhibited an accelerating response (type 1B, Fig. 4B). The peak discharge rate in type 1A neurons was reached within 500 ms (latency to first AP, 321 ± 37 ms), whereas the peak discharge rate in type 1B neurons was reached at the end of the heat pulse and the latency to the first AP was 1,345 ± 318 ms. There was no difference in the stimulus intensity between these two subgroups: 44.2 ± 0.7°C for type 1A versus 45.0 ± 0.3°C for type 1B \( P = 0.28 \). However, although not different with respect to diameter, type 1B neurons displayed a significantly larger membrane capacitance (28.9 ± 1.4 vs. 21.9 ± 2.2 pF, \( P < 0.05 \), unpaired t-test). Thus only about half of the heat-induced AP discharges (type 1A neurons) reflected the inactivation of heat-evoked inward currents (Fig. 1A). The accelerating discharge in the type 1B neurons suggests that additional mechanisms in the transformation stage contribute to forming heat-induced AP discharges. Therefore we measured repetitive AP discharges elicited by long rectangular depolarizing current pulses (750-ms duration) at room temperature in 2 type 1A, 3 type 1B, and 12 type 2 neurons (Fig. 5). Both of the type 1A neurons and 8 of the type 2 neurons exhibited a steady AP discharge rate (Fig. 5, A, C, and D). In contrast, all 3 type 1B neurons and 4 of the type 2 neurons exhibited a delayed accelerating discharge to steady current injection (Fig. 5, B, E, and F).

Responses to repeated noxious heat stimulation

Of the 48 neurons recorded, 23 were investigated with three repeated heat stimuli of the same temperature (mean: 44.8 ± 0.2°C; 43–45°C with an IS of 39 ± 1 s (10 type 1, 6 type 2, 7 type 3). Of the 10 type 1 neurons, 5 still exhibited APs during the second heat pulse and 3 during the third heat pulse. Thus the number of neurons that generate AP discharges declined with repetitive stimulation (Fig. 6A). The number of APs discharged during a heat pulse also decreased by 68 ± 22% from the first stimulus to the third stimulus \( P < 0.05, \) paired t-test; Fig. 6B). Thus type 1 neurons displayed distinct suppression on repetitive heat stimulation (Fig. 6C). The suppression could be explained by a significant reduction of the maximum depolarization across stimuli \( F_{(2,18)} = 6.615, P < 0.01; \) 26.7 ± 4.4 mV (first stimulus), 23.7 ± 4.7 mV (second stimulus), 17.7 ± 3.0 mV (third stimulus), suggesting that depolarization may have dropped below AP threshold.

Similarly, repeated heat stimulation in type 2 neurons induced a depolarization by 18.7 ± 2.8 mV during the first stimulus, by 14.3 ± 2.3 mV during the second stimulus, and by 14.4 ± 1.7 mV during the third stimulus \( n = 6 \). This decrease, however, just missed statistical significance \( P = 0.07, \) paired t-test, first stimulus to third stimulus). In type 3 neurons, the heat-stimulus–induced nonspecific depolarization did not differ across stimuli (first: 6.4 ± 12 mV; second: 8.2 ± 1.6 mV; third: 7.8 ± 1.8 mV; \( n = 7 \), n.s., paired t-test).

Six neurons that had generated AP discharges in response to injection of the mean generator current of heat-sensitive neurons (Fig. 1B) were subjected to repeated generator current injections of the same amplitude with a mean IS of 34 ± 1 s (Fig. 7). This experiment mimics opening of \( f_{\text{heat}} \) channels at room temperature without temperature effects on other channels involved in the transformation process. All six neurons generated APs in response to the second and third stimuli (Fig. 7A). The mean number of APs did not decrease; instead it

<table>
<thead>
<tr>
<th>Property</th>
<th>Type 1 (n = 18): Heat-Induced AP Discharges</th>
<th>Type 2 (n = 19): Heat-Specific Depolarization</th>
<th>Type 3 (n = 11): Nonspecific</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential, mV</td>
<td>−56.90 ± 2.00</td>
<td>−58.80 ± 1.90</td>
<td>−55.30 ± 2.60</td>
</tr>
<tr>
<td>Diameter, μm</td>
<td>28.90 ± 0.50</td>
<td>28.20 ± 0.40</td>
<td>29.30 ± 0.60</td>
</tr>
<tr>
<td>Membrane capacitance, pF</td>
<td>26.20 ± 1.20</td>
<td>26.70 ± 1.00</td>
<td>27.70 ± 1.80</td>
</tr>
<tr>
<td>Leakage current, nA</td>
<td>−0.12 ± 0.04</td>
<td>−0.14 ± 0.05</td>
<td>−0.12 ± 0.03</td>
</tr>
<tr>
<td>Pipette resistance, MΩ</td>
<td>3.39 ± 0.29</td>
<td>3.48 ± 0.24</td>
<td>3.93 ± 0.28</td>
</tr>
<tr>
<td>Serial resistance, MΩ</td>
<td>6.60 ± 0.57</td>
<td>6.71 ± 0.60</td>
<td>7.41 ± 0.65</td>
</tr>
<tr>
<td>Peak stimulus temperature, °C</td>
<td>44.70 ± 0.30</td>
<td>45.20 ± 0.20</td>
<td>44.90 ± 0.10</td>
</tr>
<tr>
<td>Temperature change, °C</td>
<td>10.90 ± 0.40</td>
<td>11.60 ± 0.40</td>
<td>10.70 ± 0.20</td>
</tr>
</tbody>
</table>

Values are means ± SE; n values are in parentheses. Leakage current is the current applied to keep the membrane potential at −80 mV in voltage-clamp mode; pipette resistance is the resistance of the patch pipettes used; serial resistance is the resistance consisting of membrane resistance and pipette resistance.
tended to increase from the first stimulus (range 3–54 APs) to the third stimulus [range 2–130; ANOVA, F(2,10) = 3.62, P = 0.07; Fig. 7, B and C]. Thus response decrements to repetitive heat stimulation were likely due to tachyphylaxis of generator currents and not due to tachyphylaxis in the transformation process.

Role of intracellular calcium for heat-induced AP discharges

We first analyzed heat responses based on heat-evoked generator currents $I_{\text{heat}}$ obtained in voltage-clamp mode experiments (reanalysis of data from Schwarz et al. 2000). We found that the number of heat-sensitive neurons decreased only slightly from stimulus to stimulus (Fig. 8A, left), but $I_{\text{heat}}$ displayed pronounced tachyphylaxis (Fig. 8A, right). When rapidly chelating intracellular calcium with BAPTA, tachyphylaxis of $I_{\text{heat}}$ was unchanged, both for the number of heat-sensitive neurons and for the area under the curve of $I_{\text{heat}}$ (Fig. 8B). Therefore we also investigated the effects of intracellular BAPTA (10 mM) on heat-induced AP discharges in 15 small DRG neurons in current-clamp mode. The percentage of neurons that exhibited AP discharges in response to the heat stimuli (peak temperatures between 44 and 46°C) was similar (5/15; 33%) to that of experiments without BAPTA (18/48 = 37%). These data indicate that heat sensitivity of the neuron itself was not affected by rapidly chelating intracellular free calcium.

Four of the heat-sensitive neurons investigated with intracellular BAPTA were also tested with three repeated heat pulses. This protocol revealed a degree of suppression (Fig. 8C, left) similar to that with EGTA-containing intracellular solution (Fig. 6A). However, the absolute number of APs discharged was surprisingly low during heat stimulation and did not change significantly when neurons were repeatedly stimulated (Fig. 8C, right). This phenomenon was due to the fact that AP discharges in all heat-sensitive neurons tested with BAPTA terminated rapidly after one to three APs (Fig. 8D). The membrane potential subsequently remained at a plateau that peaked at $+16.5 \pm 2.9$ mV (peak plateau depolarization) and slowly decreased to $-2.2 \pm 5.5$ mV during heat stimulation (end of plateau potential; $n = 5$, $P < 0.01$, paired t-test). On cessation of the heat pulse, the membrane potential rapidly returned to the baseline. Depolarizations at the beginning of the plateau and at its end were significantly higher (both $P < 0.001$, unpaired t-test) than the membrane depolarization measured between initial APs in the type 1 neurons investigated without BAPTA ($-37.5 \pm 3.7$ mV; $n = 14$). A comparable blockade of repolarization was never observed in the heat-induced AP discharges of the type 1 neurons investigated in the presence of EGTA (5 of 5 with BAPTA vs. 0 of 18 with EGTA; $P < 0.001$, Fisher’s exact test). Only one of 11 neurons tested by electrical stimulation of 750-ms duration in the presence of BAPTA at room temperature displayed such a blockade ($P < 0.005$, Fisher’s exact test). Therefore the phenomenon of blockade of repolarization was attributed to a combination of rapidly chelating intracellular calcium with BAPTA and the heat-induced membrane depolarization. In summary, intracellular calcium can be regarded as essential for a repetitive heat-induced AP discharge since it is necessary for a regular repolarization of the heat-induced APs.

<table>
<thead>
<tr>
<th>Property</th>
<th>Holding current, pA</th>
<th>AP threshold current, nA</th>
<th>Maximum rate of AP rise, mV/ms</th>
<th>Amplitude of action potential, mV</th>
<th>Maximum rate of AP fall, mV ms</th>
<th>Decay time, ms</th>
</tr>
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<tbody>
<tr>
<td>Group</td>
<td>RT</td>
<td>34°C</td>
<td>RT</td>
<td>RT</td>
<td>RT</td>
<td>RT</td>
</tr>
<tr>
<td>Type 1</td>
<td>12.0, 0.35</td>
<td>0.3</td>
<td>112.2, 0.5</td>
<td>11.0, 0.0</td>
<td>110.0, 0.3</td>
<td>25.0, 0.3</td>
</tr>
<tr>
<td>Type 2</td>
<td>22.0, 0.35</td>
<td>0.4</td>
<td>112.2, 0.4</td>
<td>11.0, 0.8</td>
<td>110.0, 0.2</td>
<td>25.0, 0.5</td>
</tr>
<tr>
<td>Type 3</td>
<td>11.0, 0.3</td>
<td>0.3</td>
<td>112.2, 0.3</td>
<td>11.0, 0.7</td>
<td>110.0, 0.1</td>
<td>25.0, 0.3</td>
</tr>
</tbody>
</table>

Values for types 1, 2, and 3 are means ± SE; $n$ values as in parentheses. Analysis was done by two-way ANOVA (group x temperature) with repeated measures for the factor temperature (room temperature).
DISCUSSION

Heat-induced AP discharges were elicited in a subpopulation of primary nociceptive neurons, although heating interfered with AP induction by increasing background conductance. A second group of heat-sensitive neurons was found that displayed a heat-induced depolarization that did not reach AP threshold voltage. Heating amplified the repolarization phase of APs and reduced the duration and amplitude of APs, suggesting activation of repolarizing currents and/or an increase in sodium channel inactivation by heat. Calcium-dependent repolarization processes were essential for the continuation of heat-induced repetitive AP discharges. Adaptation during constant stimulation (i.e., decelerating response pattern) was found in a subpopulation of heat-sensitive neurons only; others displayed accelerating discharges. Repeated heat stimulation reliably induced suppression of AP discharges, which was not dependent on intracellular calcium.

Heat-induced AP discharges

About one third (37%) of all small neurons investigated responded with overshooting AP discharges and another 40% responded to heat with depolarization while no APs were generated. Thus altogether about 77% of neurons were heat sensitive. Previous studies looking at heat-evoked currents in voltage-clamp mode revealed a proportion of heat-sensitive neurons in the range of 40–80% (e.g., Cesare and McNaughton 1996; Kirschstein et al. 1999; Nagy and Rang 1999; Schwarz et al. 2000). These proportions are compatible with our findings, assuming that the subthreshold depolarization is also due to $I_{\text{heat}}$. This assumption is supported by the finding that the depolarization in type 2 neurons displayed suppression similar to that in type 1 neurons reflecting tachyphylaxis of $I_{\text{heat}}$ (Schwarz et al. 2000), whereas the nonspecific depolarizations seen in type 3 neurons did not.

There were no significant differences between type 1, type 2, and type 3 neurons for the parameters determined at resting membrane potential (RMP, diameter), in clamped neurons (membrane capacitance, leakage current, serial resistance) or in the bath (pipette resistance). This confirms that the different types of heat responses were independent of the electrophysiological, mechanical, and morphological characteristics of the neurons or recordings. Consequently, specific transduction mechanisms are accountable for the heat-induced depolarization and, if sufficient, for the AP discharges.

Different expression levels of the heat-sensitive membrane channel TRPV1 may explain why generator currents in response to the heat stimuli were suprathreshold in type 1 neurons and below AP threshold in type 2 neurons. Differences in sodium channel accommodation during the slow slope of the
Depolarizing generator current may also exist across neurons, since application of a depolarizing $I_{\text{heat}}$-shaped generator current at room temperature induced APs in only 60% of neurons tested. Moreover, the current necessary for the induction of APs is higher at higher temperatures (see Effects of heat on the waveform of APs). Thus the finding that only about half of the heat-sensitive neurons generated APs in response to heat is easily explained. Since all our data are from the soma of nociceptive neurons (as a surrogate model of their own peripheral terminals), it remains uncertain to what extent heat-induced depolarizations reach AP threshold at the terminals due to their different geometry.

**Heat-induced AP discharges do not regularly display adaptation**

About half the type 1 neurons displayed decelerating AP discharges during a constant moderate noxious heat stimulus (type 1A), whereas the other half displayed accelerating discharges (type 1B). Therefore only one half of the neurons showed heat-induced AP discharges, which resembled the kinetics of inactivating heat-evoked currents. The discharges observed in type 1B neurons displayed longer latencies as well as accelerating discharge patterns and thus clearly differed from the kinetics of the presumably heat-evoked generator currents that had a peak latency of about 625 ms and inactivated by 45% with time constants of 4–5 s (Schwarz et al. 2000). Although the response pattern of type 1A neurons faithfully reflected inactivation of the generator currents, the response pattern of type 1B neurons suggests that other mechanisms in addition to the inactivating generator current contribute to the modulation of heat-induced AP discharges at the soma of nociceptive neurons. Given that the response patterns of type 1A and type 1B neurons were conserved in response to rectangular electrical depolarizing currents at room temperature, these data indicate that the transformation process in some DRG neurons may integrate over time. This process may control discharge rates independent of the heat stimulus. Until now, integration in the transformation process of DRG neurons has been studied extensively with electrical current injection, but not with natural nociceptive heat stimuli.

**Accelerating and decelerating heat responses**

The decelerating and accelerating heat response types were reminiscent of two different types of heat-induced AP discharges, which were found using single-fiber recording in monkeys in vivo (Treede et al. 1995, 1998). In these studies moderate noxious heat pulses induced AP bursts with a short latency and an early maximum that displayed adaptation in C-fiber mechano-heat-sensitive neurons (CMH neurons) similar to the discharges seen in A-fiber mechano-heat-sensitive neurons of type II (AMH II; Treede 1995; Treede et al. 1995, 1998). AMH type I neurons, in contrast, displayed long latencies and late peak discharges. The two heat-sensitive membrane channels TRPV1 (Caterina et al. 1997) and TRPV2 (Caterina et al. 1999) were suggested to account for those different heat response types in vivo that display low-threshold (TRPV1) and high-threshold (TRPV2) heat-evoked currents in vitro. In the present study we found no temperature difference in the heat stimulus to induce these two unequal response types, but this does not necessarily contradict the suggestion that they depended on activation of TRPV1 and TRPV2. Threshold distributions of AMH I and AMH II in vivo do
overlap and therefore the original distinction between type 1 and type 2 heat responses was based on activation latency and peak latency to rectangular heat pulses (Treede et al. 1995, 1998).

Many channels are involved in the modulation of AP trains such as voltage-gated sodium and calcium channels, several potassium channels (such as delayed rectifiers), calcium-dependent potassium channels, as well as several others (for review see, e.g., Hogg 2006). DRG neurons are very heterogeneous in expression of different membrane channels (e.g., Petruska et al. 2000) and therefore correlation of accelerating and/or decelerating responses with these channels is unknown. Moreover, channel properties of all these membrane channels are also expected to be temperature dependent.

**Effects of heat on the waveform of APs**

Cooling nonnociceptive large-diameter DRG neurons (diameter ≥50 µm) from 37 to 26°C prolonged AP duration,

![Discharge properties of heat-sensitive type 1A and type 1B neurons are conserved on electrical stimulation at room temperature. A: examples of AP discharge in a heat-sensitive type 1A neuron depolarized with rectangular current injections of 750-ms duration at room temperature (top: response to 1st suprathreshold stimulus intensity inducing at least one AP [3 nA]; bottom: 1st pulse inducing repetitive AP discharges [4 nA]). B: AP discharge in a type 1B neuron depolarized for 750 ms at room temperature (pulse intensities chosen as in A [2.0 and 2.8 nA]). C: mean discharge rates of 2 type 1A neurons in response to a rectangular depolarizing pulse of 750-ms duration. D: very similar discharge in response to rectangular depolarization was seen in an additional 8 of 12 type 2 neurons investigated with those depolarizing pulses. E: the 3 type 1B neurons also investigated with the rectangular depolarizing current pulses displayed accelerating discharge rates at room temperature. F: a similar discharge pattern was observed in the remaining 4 of the type 2 neurons.**

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**FIG. 5.** Discharge properties of heat-sensitive type 1A and type 1B neurons are conserved on electrical stimulation at room temperature. A: examples of AP discharge in a heat-sensitive type 1A neuron depolarized with rectangular current injections of 750-ms duration at room temperature (top: response to 1st suprathreshold stimulus intensity inducing at least one AP [3 nA]; bottom: 1st pulse inducing repetitive AP discharges [4 nA]). B: AP discharge in a type 1B neuron depolarized for 750 ms at room temperature (pulse intensities chosen as in A [2.0 and 2.8 nA]). C: mean discharge rates of 2 type 1A neurons in response to a rectangular depolarizing pulse of 750-ms duration. D: very similar discharge in response to rectangular depolarization was seen in an additional 8 of 12 type 2 neurons investigated with those depolarizing pulses. E: the 3 type 1B neurons also investigated with the rectangular depolarizing current pulses displayed accelerating discharge rates at room temperature. F: a similar discharge pattern was observed in the remaining 4 of the type 2 neurons.
enhanced AP amplitude, and decreased maximum repolarization rate (Li et al. 2002; see also Volgushev et al. 2000). Conversely, elevating the temperature from 20 to 37°C considerably shortened the AP duration, doubled the size of the leak current, and led to a slight increase in threshold potential in rat peripheral nerves (Schwarz and Eikhof 1987). In nociceptive neurons of the present study AP repolarization was accelerated, AP duration shortened, and AP amplitude reduced by heating from room temperature to about 34°C and further to above 42°C. These effects may be explained by an increased inactivation of sodium channels or by an increased activation of repolarizing currents. Since inactivation of voltage-gated sodium channels is enhanced with cooling and not with heating, if displaying temperature dependence at all (Chraïbi and Horisberger 2002; De Col et al. 2008; Schwarz et al. 2000; Zimmermann et al. 2007), the latter seems more probable.

Another possible explanation is the voltage-dependent activation of the heat-sensitive TRPV1 (Ahern and Premkumar 2002; Piper et al. 1999; Schwarz et al. 2000; Vlachova et al. 2003). By this property, the channel could be activated during depolarization in the course of an AP. Voltage synergistically interacts with agonists to augment TRPV1 currents (Ahern and Premkumar 2002). Thermosensitivity of TRPV1 itself seems—at least to a substantial proportion (Matta and Ahern 2007)—to arise from a temperature-dependent shift of its voltage-dependent activation curve (Voets et al. 2004). Having a reversal potential near 0 mV, TRPV1 currents depolarize neurons from the resting potential, but repolarize them during an overshooting AP. Activation of TRPV1 during overshooting APs may therefore itself contribute to repolarization of the AP and thus shorten the AP.

Spatial buffering of intracellular calcium on heat-induced AP discharges

Intracellular calcium signals influence pain-related pathways as well as channel function (Hagenacker et al. 2007). In previous voltage-clamp mode experiments replacing EGTA with BAPTA did not change the heat-evoked generator current, its adaptation during sustained heat stimulation, nor its suppression on repetitive stimulation (cf. Schwarz et al. 2000). In current-clamp mode BAPTA neither changed the percentage of heat-responsive neurons nor the suppression induced by repetitive stimulation. However, the initial heat response was strongly reduced to one to three APs followed by sustained depolarization at around 0 mV. Different results with BAPTA and EGTA suggest that there are calcium-dependent processes involved in repetitive AP discharges that are spatially located at a distance far enough from the location of calcium influx or release to be rapidly interrupted by BAPTA but not by EGTA because it is a slower calcium chelator.

FIG. 6. Suppression of heat-induced AP discharges on repeated stimulation. A: total number of heat-sensitive type 1 neurons (including 2 type 1A and all 8 type 1B) generating AP discharges in response to repeated heat stimulation dramatically decreased when neurons were stimulated with repeated heat stimuli (interstimulus intervals about 40 s). B: the mean total number of APs during each stimulus also declined at the 2nd and 3rd stimuli (*P < 0.05, paired t-test). C: mean instantaneous discharge rate (±SE; determined in periods of 200 ms, starting with the heat stimulation and combined for all type 1A and type 1B responses) during heat stimulation (indicated as black bar) clearly decreased from the 1st stimulus (open squares) to the 2nd stimulus (open circles) and 3rd heat pulse [closed circles; "*P > 0.34, "*P < 0.07, *P < 0.05, paired t-test].

FIG. 7. AP discharges on repeated stimulation with injection of I_heat-shaped generator currents at room temperature. A: the total number of neurons generating AP discharges in response to an I_heat-shaped generator current at room temperature did not change between the stimuli. B: the mean total number of APs during each stimulus was also not significantly changed (all ns, paired t-test). C: mean instantaneous discharge rates (±SE) of 6 neurons depolarized with the heat-evoked generator current at room temperature during the 1st (open squares), 2nd (open circles), and 3rd stimuli [closed circles; (*P < 0.1, all others ns, paired t-test)].
The mean potential at the end of the sustained depolarization elicited by heat stimuli in the presence of BAPTA (about $-2.2$ mV) was close to the reversal potential of TRPV1. This behavior may be explained by a sustained opening of TRPV1. Inactivation of heat-evoked currents was observed at positive and at negative membrane potentials ($-80$ to $60$ mV), but was more pronounced for inward currents than for outward currents. This property favors a longer-lasting opening of TRPV1 induced by heat in the depolarized state (Schwarz et al. 2000). Similarly, inactivation of capsaicin-induced currents was more pronounced during depolarization (Piper et al. 1999). The instantaneous repolarization after cessation of the heat stimulus seen in all neurons recorded with intracellular BAPTA is consistent with the suggestion that the observed plateau potential was dependent on sustained activation of TRPV1.

An alternative explanation is that DRG neurons express repolarizing channels that are gated by heat, calcium, and voltage. Existence of a heat- and voltage-activated, calcium-dependent potassium channel may explain the blockage of the repolarization phase in the presence of BAPTA. Several voltage-dependent potassium channels of the two-pore domain family, like TREK-1, TREK-2, and TRAAK, display pronounced temperature dependence (Alloui et al. 2006; Kang et al. 2005; Maingret et al. 2000). However, thermosensitivity of those channels did not depend on calcium (Kang et al. 2005), making them rather unlikely to account for the lack of repolarization in neurons investigated with BAPTA. Calcium-dependent potassium channels ($K_{Ca}$) of the SK-, BK-, and IK-type were also functionally and/or immunocytochemically identified in DRG neurons (Boettger et al. 2002; Gold et al. 1996b; Scholz et al. 1998b). To our knowledge, however, nothing is known about a specific temperature dependence of $K_{Ca}$ channels; thus it remains to be determined whether these channels specifically support heat-induced AP discharges.

**Suppression of heat-induced AP discharges during repetitive stimulation**

Repetitive heat stimulation of DRG neurons led to suppression (a pronounced decrease of the APs discharged during successive stimulation) as well as to a decrease of the number of neurons responding to heat stimuli. This response pattern resembles that of heat-evoked currents (Greffrath et al. 2002; Schwarz et al. 2000; Vyklický et al. 1999). Decreasing depolarizing generator current $I_{heat}$ leads in current-clamp mode to a reduction of membrane depolarization. It is conceivable that membrane depolarization, which declined from stimulus to stimulus, eventually failed to reach AP threshold potential. This phenomenon may explain the stronger suppression of heat-sensitive neurons with repetitive stimulation in current-clamp (Fig. 6A) than that in voltage-clamp mode (Fig. 8A, left).

Repetitive heat stimulation of the receptive field of a nociceptor in vivo also induces a reduction of AP discharges (“fatigue”) in both Aδ-fiber and C-fiber nociceptive afferents (LaMotte and Campbell 1978; Peng et al. 2003; Treede 1995).

**FIG. 8.** Rapidly buffering intracellular calcium with BAPTA does not reduce $I_{heat}$ but induces a blockade of the repetitive heat-induced AP discharge. A: when analyzing the heat-evoked generator current $I_{heat}$ under control conditions during repeated heat stimulation in voltage-clamp mode using EGTA-containing intracellular solution the absolute number of heat-sensitive neurons decreased only slightly (left). The area under the curve (AUC), as a measure of excitation with time, significantly decreased from stimulus to stimulus (right). B: rapidly buffering free intracellular calcium with BAPTA in the recording pipette neither changed the number of heat-sensitive neurons (left) nor affected the degree of adaptation observed (right). *$P < 0.05$ and **$P < 0.01$ vs. 1st stimulus; **$P < 0.05$ and ***$P < 0.01$ vs. 2nd stimulus, paired $t$-test. C: the total number of heat-sensitive type 1 neurons generating AP discharges in response to repeated heat stimulation when rapidly chelating free intracellular calcium using $10$ mM BAPTA in the intracellular solution still decreased, as shown in the left column. Right column shows that, in contrast, the mean total number of APs during each stimulus was low and did not change substantially. D: representative example of a neuron stimulated with a heat pulse while spatially buffering free intracellular calcium with BAPTA, leading to a blockade of the repolarization phase above $0$ mV (marked as a dotted line). Membrane potential, however, immediately recovered after the end of the heat stimulus. A magnification of the AP is given in the right panel.
Human heat pain perception and contact-heat-evoked potentials display signs of rapid habituation when the stimulation is restricted to a fixed receptive field and thus exactly reflect tachyphylaxis of $I_{\text{heat}}$ in vitro and fatigue of peripheral nociceptive neurons seen in vivo (Greffrath et al. 2007). Similar to tachyphylaxis of $I_{\text{heat}}$ (Schwarz et al. 2000), suppression of heat-induced AP discharges in the present study persisted when rapidly chelating free intracellular calcium. Suppression was not observed in response to repeated electrical stimulation with $I_{\text{heat}}$-shaped currents, indicating that this calcium-independent phenomenon is a characteristic feature of the transduction process for noxious heat.

**Technical considerations**

In our study using an Axopatch200A patch-clamp amplifier purchased in 1996, series resistance was compensated in the voltage-clamp mode (values documented in Table 1), but could technically not be compensated in the current-clamp mode. Therefore a voltage drop across the series resistance may have affected our recordings whenever current was applied through the pipette. This resistive voltage error $V_R$ explains the relatively high strengths of the currents that had to be injected for AP generation but is irrelevant to the majority of our findings, for example, discharge rates or relative height of the AP in response both to electrical and to thermal stimulation. Moreover, neither serial resistance nor pipette resistance differed between the three groups of neurons and thus may also not account for any differences observed between heat response types.

Even under nominal zero-current conditions, however, inaccuracies in the recorded AP waveforms may have occurred in our study by using a patch-clamp amplifier for recording in current-clamp mode instead of a specialized voltage follower circuit-based microelectrode amplifier (Magistretti et al. 1996). Traditional patch-clamp amplifiers are not as fast and stable when functioning in the current-clamp mode and may transiently draw currents from the neuron during rapid signaling events. These error currents account for distortions in the recorded rapid events such as APs due to an additional capacitive voltage error $V_C$. As a consequence, the raw values of the following parameters determined in our study may differ from recordings obtained with a classical microelectrode amplifier: 1) the AP amplitude, 2) the hyperpolarizing afterpotentials, and 3) the depolarizing and repolarizing slopes (Magistretti et al. 1996). However, the relative changes of those parameters in response to different stimulus temperatures should also not be appreciably affected by the amplifier.

**Conclusions**

Heat-induced AP discharges can be elicited in the soma of a subgroup of DRG neurons. Heat-induced AP discharges at the DRG neuron soma reflect 1) induction of heat-activated generator currents, 2) temperature dependence of background potassium currents, and 3) calcium-dependent repolarization currents. Adaptation of heat-induced AP discharges was seen in about half of the type 1 neurons (decelerating responses, type 1A) faithfully encoding the inactivation properties of the generator currents, which could thus be attributed directly to the heat transduction process. The other half of the neurons, however, showed accelerating discharges reminiscent of that of a subclass of A-fiber nociceptors in vivo. The AP generation of these type 1B responses could therefore involve additional integrating processes at the stage of transformation. In conclusion, heat-activated membrane channels do induce AP discharges and probably contribute to shaping and forming of resulting heat-induced APs. On repeated stimulation, AP discharge exhibited fatigue consistent with tachyphylaxis of $I_{\text{heat}}$ and consistent with habituation of the psychophysical correlate heat pain. This is the first study to describe in a systematic fashion the transduction process for noxious heat stimuli into AP discharges in DRG neurons. Future studies should reveal more detail on waveforms of heat-induced APs, membrane channels contributing to heat-induced AP discharges and their respective heat sensitivity, and intracellular signaling cascades involved in decelerating and accelerating discharges.

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Gold MS, Shuster MJ, Levine JD. Temperature dependence of background potassium currents, and 3) calcium-dependent repolarization currents. Adaptation of heat-induced AP discharges was seen in about half of the type 1 neurons (decelerating responses, type 1A) faithfully encoding the inactivation properties of the generator currents, which could thus be attributed directly to the heat transduction process. The other half of the neurons, however, showed accelerating discharges reminiscent of that of a subclass of A-fiber nociceptors in vivo. The AP generation of these type 1B responses could therefore involve additional integrating processes at the stage of transformation. In conclusion, heat-activated membrane channels do induce AP discharges and probably contribute to shaping and forming of resulting heat-induced APs. On repeated stimulation, AP discharge exhibited fatigue consistent with tachyphylaxis of $I_{\text{heat}}$ and consistent with habituation of the psychophysical correlate heat pain. This is the first study to describe in a systematic fashion the transduction process for noxious heat stimuli into AP discharges in DRG neurons. Future studies should reveal more detail on waveforms of heat-induced APs, membrane channels contributing to heat-induced AP discharges and their respective heat sensitivity, and intracellular signaling cascades involved in decelerating and accelerating discharges.

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