

A high-threshold heat-activated channel in cultured rat dorsal root ganglion neurons resembles TRPV2 and is blocked by gadolinium

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Abstract

Heat-activated ion channels from the vanilloid-type TRP group (TRPV1–4) seem to be central for heat-sensitivity of nociceptive sensory neurons. Displaying a high-threshold (> 52 °C) for activation, TRPV2 was proposed to act as a sensor for intense noxious heat in mammalian sensory neurons. However, although TRPV2 is expressed in a distinct population of thinly myelinated primary afferents, a widespread expression in a variety of neuronal and non-neuronal tissues suggests a more diverse physiological role of TRPV2. In its role as a heat-sensor, TRPV2 has not been thoroughly characterized in terms of biophysical and pharmacological properties. In the present study, we demonstrate that the features of heterologously expressed rat TRPV2 closely resemble those of high-threshold heat-evoked currents in medium- and large-sized capsaicin-insensitive rat dorsal root ganglion (DRG) neurons. Both in TRPV2-expressing human embryonic kidney (HEK)293t cells and in DRGs, high-threshold heat-currents were sensitized by repeated activation and by the TRPV1–3 agonist, 2-aminoethoxydiphenyl borate (2-APB). In addition to a previously described block by ruthenium red, we identified the trivalent cations, lanthanum (La³⁺) and gadolinium (Gd³⁺) as potent blockers of TRPV2. Thus, we present a new pharmacological tool to distinguish between heat responses of TRPV2 and the closely related capsaicin-receptor, TRPV1, which is strongly sensitized by trivalent cations. We demonstrate that self-sensitization of heat-evoked currents through TRPV2 does not require extracellular calcium and that TRPV2 can be activated in cell-free membrane patches in the outside-out configuration. Taken together our results provide new evidence for a role of TRPV2 in mediating high-threshold heat responses in a subpopulation of mammalian sensory neurons.

Introduction

Important progress in understanding heat sensitivity in mammals has been made with the cloning of four cation channels belonging to the vanilloid group of the TRP channel superfamily (Ramsey *et al.*, 2006). These channels (TRPV1–4) are activated by various degrees of heating in heterologous expression systems (reviewed in Dhaka *et al.*, 2006), starting with the low-threshold TRPV3 (> 33 °C) and TRPV4 (27–42 °C) and entering the noxious range with TRPV1 (> 42 °C) and TRPV2 (> 52 °C). While the generation of null mutant mice has contributed to a clearer picture of the patho-physiological roles of TRPV1 (Caterina *et al.*, 2000; Davis *et al.*, 2000), TRPV3 (Moqrich *et al.*, 2005) and TRPV4 (Todaka *et al.*, 2004; Lee *et al.*, 2005), the involvement of TRPV2 (formerly VRL-1) in noxious heat sensing is still poorly understood. TRPV2 is closely related to the capsaicin receptor TRPV1, with whom it shares 49% identity and 66% similarity in primary structure (Caterina *et al.*, 1999). However, unlike TRPV1, TRPV2 is not activated or sensitized by capsaicin or protons, and requires stronger heating for activation (> 52 °C). At the mRNA level,

TRPV2 is expressed in a variety of tissues, including lung, spleen, intestine, various regions of the brain, spinal cord and sensory ganglia (Caterina *et al.*, 1999). In the nervous system, TRPV2-immunoreactivity was observed in both dorsal root (Ahluwalia *et al.*, 2002; Greffrath *et al.*, 2003) and trigeminal ganglia (Ichikawa & Sugimoto, 2000), spinal cord (Lewinter *et al.*, 2004), cortex (Liapi & Wood, 2005) and hypothalamus (Wainwright *et al.*, 2004). A recent study using real time RT-PCR reported that among TRPV channels, TRPV2 transcripts had the highest level of expression in all brain regions tested, including cerebellum, cerebrum, basal ganglia, forebrain and hippocampus (Kunert-Keil *et al.*, 2006). Such a widespread expression pattern, including tissues that are never exposed to temperatures as high as 52 °C, suggests that TRPV2 has diverse physiological functions and may be activated by a variety of stimuli other than noxious heat.

Within the dorsal root ganglia, TRPV2 is expressed in ~16% of all neurons, mainly in medium and large cells that also stain for an anti-neurofilament antibody, indicating that these are cell bodies of myelinated axons (Caterina *et al.*, 1999). A similar expression pattern was found in trigeminal ganglia (Ichikawa & Sugimoto, 2000). Interestingly, approximately 30% of TRPV2-expressing neurons also stain for the nociceptive marker, CGRP (Caterina *et al.*, 1999). This suggests that TRPV2 may be the heat transducer in cutaneous

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mechano-heat-sensitive A δ (or type I) fibres, which show a thermal threshold of ~ 50 °C (Treede *et al.*, 1995) and thus closely match the activation threshold of TRPV2 (Caterina *et al.*, 1999). A putative role of TRPV2 in mediating at least part of the noxious heat responses was proposed, especially after it was shown that TRPV1-null mutant mice display almost normal behavioural responses to noxious heat (Caterina *et al.*, 2000; Davis *et al.*, 2000). This idea was further supported by a study describing a TRPV2-like current in cultured rat DRG neurons, which displayed a thermal threshold of ~ 50 °C and was blocked by ruthenium red (Ahluwalia *et al.*, 2002). However, intracellular recordings from DRG neurons of TRPV1 $^{-/-}$ mice in an *ex vivo* skin-nerve-ganglion preparation demonstrated that $\sim 85\%$ of the heat-sensitive neurons did not immunocytochemically stain for TRPV2 (Woodbury *et al.*, 2004). Furthermore, it was proposed initially that TRPV1 and TRPV2 are expressed in distinct populations of sensory neurons (Caterina *et al.*, 1999; Ahluwalia *et al.*, 2002). However, it was recently shown that 28% of TRPV2-positive neurons also express TRPV1, raising the possibility of heteromer formation by the two receptors (Greffrath *et al.*, 2003; Liapi & Wood, 2005). Moreover, a very recent report describes high-threshold heat-activated currents in subpopulations of rat DRG neurons expressing only TRPV2 or TRPV1 and TRPV2 (Rau *et al.*, 2007).

The aim of our work was to find new evidence for functional TRPV2 expression in cultured rat DRG neurons. Analysis of the temperature dependence and sensitization of TRPV2 in a heterologous expression system, combined with a pharmacological approach using trivalent cations [lanthanum (La $^{3+}$) and gadolinium (Gd $^{3+}$)], allowed us to identify a capsaicin-insensitive neuronal subpopulation in rat DRG displaying a TRPV2-like heat-activated current. We also provide evidence for heat activation of TRPV2 in cell-free membrane patches in the outside-out configuration.

Materials and methods

Transfection procedure

Recombinant rat TRPV2 was transiently transfected into human embryonic kidney (HEK)293t cells using the calcium phosphate precipitation method. A reporter plasmid (CD8-pih3m) was cotransfected to allow the identification of successfully transfected cells using immunobeads (anti-CD8 Dynabeads, from Dynal Biotech, Oslo, Norway). HEK293t cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with penicillin/streptomycin 100 U/mL, HEPES 25 mM, Fetal Bovine Serum 10% (all Gibco-Invitrogen, Karlsruhe, Germany) and taurine 3 mM (Sigma-Aldrich, Steinheim, Germany) at 37 °C and 5% CO $_2$. After the transfection procedure cells were plated onto 35-mm Petri dishes and used for experiments within 2–3 days.

DRG culture

Adult rats were killed by CO $_2$ inhalation. DRG from all spinal levels were removed and incubated in 0.6 mg/mL collagenase (type XI, Sigma) and 3 mg/mL protease (Sigma) for 60 min at 37 °C in DMEM. The ganglia were then gently triturated and dissociated neurons were plated onto borosilicate glass coverslips that had been treated with poly D-lysine (0.1 mg/mL for 30 min), and cultured (37 °C and 5% CO $_2$) in serum-free TNB-100 basal medium (Biochrom AG, Berlin, Germany), supplemented with penicillin/streptomycin 100 U/mL and 100 ng/mL nerve growth factor-7S (NGF, Alomone Laboratories, Jerusalem, Israel). Recordings were made after ~ 24 h in culture.

Patch-clamp electrophysiology

Whole-cell patch-clamp recordings were performed using an Axo-patch 200B amplifier and the pClamp 8.2 software (both from Axon Instruments, Union City, CA, USA) installed on a conventional PC. Patch-clamp pipettes with a final resistance of 1.5–4 M Ω were pulled from borosilicate capillary glass (GB150F-8P, Science Products GmbH, Hofheim, Germany). Currents were sampled at 2–5 kHz and filtered at 1 kHz. The holding potential was -60 mV. The standard extracellular solution contained (in mM) NaCl 140, KCl 4, CaCl $_2$ 2, MgCl $_2$ 1, HEPES 10, glucose 5, pH 7.4 (adjusted with NaOH). The pipette solution contained (in mM) KCl 140, MgCl $_2$ 2, EGTA 5, HEPES 10, pH 7.4 (adjusted with KOH). The thermal stimuli were delivered using a multichannel gravity-driven perfusion system incorporating rapid feed-back temperature control (Dittert *et al.*, 1998) and consisted in heating ramps from a holding temperature of ~ 25 °C to a maximal temperature of 60 °C. Only one cell was used for patch-clamp recording in each dish. All recordings were stored on a PC for off-line analysis using the pClamp 8.2 and Microcal Origin 7 software (OriginLab Corp, Northampton, MA, USA). Data are presented as mean \pm SEM. Two tailed Student's *t*-test (paired or unpaired) was performed using the Microcal Origin 7 software. A value of $P < 0.05$ was considered statistically significant.

Measurement of the temperature coefficient (Q_{10}) for heat-activated currents

The temperature coefficient (Q_{10}) for the heat-activated current was measured using the following formula:

$$Q_{10} = \left(\frac{I}{I_0} \right)^{\frac{10}{T-T_0}}$$

where I is the current at the temperature T , T_0 is the temperature threshold and I_0 the current at T_0 (Sauer *et al.*, 2001). From this it follows that:

$$\log I = T[(\log Q_{10})/10] + \log I_0 - T_0[(\log Q_{10})/10]$$

We have therefore used a linear fit for the $\log I$ vs. T dependence, and calculated Q_{10} from the slope factor k , according to $Q_{10} = 10^{10k}$. The temperature dependence of the heat-activated current was generally biphasic, with one phase characterized by a low Q_{10} (~ 1.7) corresponding to the non-specific effects of temperature on membrane properties, while the other phase was strongly temperature-dependent ($Q_{10} \sim 100$) and could be attributed to the thermal gating of TRPV2. In the $\log(I)$ vs. T plot each of these phases was fitted with a straight line, and the intersection of these lines was used to determine the temperature threshold of TRPV2.

Results

Properties of TRPV2-mediated heat-induced currents in HEK293t cells

Temperature and stimulus dependence of TRPV2 transiently expressed in HEK293t cells

Heat ramps of 4-s duration between ~ 25 and ~ 60 °C evoked large inward currents (-3.7 ± 0.9 nA, $n = 8$) in TRPV2-transfected HEK293t cells held at -60 mV (Fig. 1A). The temperature dependence of the current was biphasic and a linear fit of the $\log(I)$ vs. T plot was used to determine the temperature coefficients (Q_{10}) of the

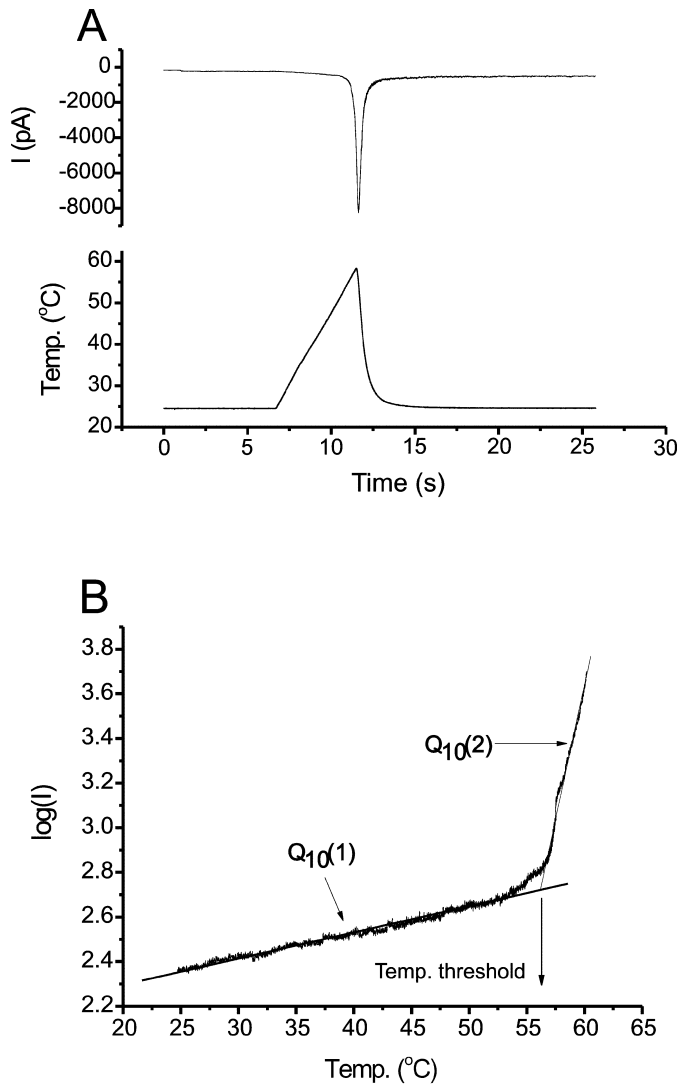


FIG. 1. Temperature and stimulus dependence of rat TRPV2 expressed in HEK293t cells. (A) Typical recording from a TRPV2-transfected HEK293t cell (upper trace) submitted to a heat ramp from $\sim 25^{\circ}\text{C}$ to $\sim 58^{\circ}\text{C}$ (lower trace). (B) The temperature dependence of the current evoked by a heat ramp similar to that in A, lower trace, was clearly biphasic, and was fitted with two straight lines yielding two distinct values for the temperature coefficient Q_{10} (see Materials and methods for the Q_{10} measurement). The temperature threshold was determined at the intersection between the two fitting lines. Note that the y-axis represents $\log(I)$.

currents and the temperature at the transition between the two phases (Fig. 1B, see also Materials and methods for the fitting procedure). The lower Q_{10} value (1.7 ± 0.3 , $n = 8$, $Q_{10}(1)$ in Fig. 1B) is in the expected range for the non-specific effect of temperature on the holding membrane current, while the higher $Q_{10}(2)$ (105 ± 33 , $n = 8$) describes the highly temperature-dependent TRPV2-mediated current. A temperature threshold of $55.3 \pm 1.1^{\circ}\text{C}$ ($n = 8$) was obtained from the intersection of the two fitting straight lines, as shown in Fig. 1B.

TRPV2-transfected HEK293t cells were subjected to three consecutive identical heat ramps at 1-min intervals. As previously reported (Caterina *et al.*, 1999), the response to the second stimulus was sensitized (4.7 ± 0.8 nA, compared to 3.7 ± 0.9 nA during the first heat ramp, $P = 0.01$, Student's paired *t*-test, $n = 8$), and again to the third stimulus (5.6 ± 1.0 nA, compared to the second heat ramp, $P = 0.04$, $n = 8$, Fig. 2A and B). Interestingly, the current during the

second ramp appeared triphasic, at least, and three regions with different temperature coefficients were operationally defined (Fig. 2C) $Q_{10}(1) = 1.5 \pm 0.1$; $Q_{10}(2) = 3.0 \pm 0.6$; $Q_{10}(3) = 333 \pm 249$ ($n = 8$). While the first phase with the lowest Q_{10} could still be assigned to the non-specific effects of temperature on the membrane holding current, the proper TRPV2-mediated current was divided into two phases, starting with the second stimulation. The temperature threshold was then measured as shown in Fig. 2C (T_2), at the transition to the first phase of the TRPV2 current, and it was significantly lower than the threshold during the first heat ramp ($38.2 \pm 1.6^{\circ}\text{C}$, compared to $55.3 \pm 1.1^{\circ}\text{C}$, $P < 0.001$, $n = 8$, Fig. 2D). The temperature threshold during the third heat ramp was also much lower compared to the first stimulation, but not significantly different from the second threshold (Fig. 2D). In conclusion, TRPV2 expressed in HEK293t cells undergoes a self-sensitizing process, in which a second supra-threshold stimulation leads to an increased current amplitude and a decreased temperature threshold. Similar experiments were also carried out in mock-transfected HEK293t cells. When submitted to 4-s long heat ramps from 25°C to $\sim 60^{\circ}\text{C}$, these cells responded with small, linear inward currents (61.0 ± 9.8 pA, $n = 7$). These heat-evoked responses displayed no clear threshold and were not sensitized by consecutive heat stimuli (second heat ramp -57.0 ± 9.7 pA; third heat ramp -61.4 ± 9.1 pA, $n = 7$). Moreover, the temperature coefficients (Q_{10}) of the heat-evoked currents in mock-transfected HEK293t cells were low (1.5 ± 0.1 , $n = 7$) and did not change significantly with subsequent heat stimulation.

The self-sensitization to heat of TRPV2 is not calcium-dependent

The role of extracellular calcium in the self-sensitizing response to heat of heterologously expressed TRPV2 was investigated by applying consecutive heat ramps in Ca^{2+} -free conditions. The self-sensitization of heat-activated currents mediated by TRPV2 was still present in the absence of external calcium (Fig. 2E and F). A statistical comparison revealed significantly increased current amplitudes evoked by the second heat stimulation (5.3 ± 2.0 nA) compared the first stimulation (4.0 ± 1.7 nA, $n = 6$, $P = 0.01$). Furthermore, repeated activation resulted in a shift of the threshold for activation to lower temperatures (first stimulus $57.1 \pm 1.1^{\circ}\text{C}$; 2nd stimulus $36.4 \pm 1.6^{\circ}\text{C}$, $n = 6$, $P < 0.001$). It can be thus inferred that a calcium-influx is not required for the self-sensitization to heat of TRPV2.

TRPV2 expressed in HEK293t cells is inhibited by the trivalent cations lanthanum (La^{3+}) and gadolinium (Gd^{3+}) and is sensitized by 2-aminoethoxydiphenyl borate (2-APB)

Heat-activated currents evoked from TRPV2-HEK293t cells were reversibly blocked by $20 \mu\text{M}$ ruthenium red ($\sim 80\%$ inhibition, $n = 3$, data not shown), as described in a previous study (Caterina *et al.*, 1999). In a separate group of cells, following upon at least three control heat stimuli (in order to allow for the self-sensitizing effect to occur and the heat-activated current to reach a constant amplitude), a further stimulus was applied in the presence of $100 \mu\text{M}$ La^{3+} . La^{3+} reversibly inhibited heat-evoked TRPV2-currents by 30% (from 7.0 ± 1.0 nA to 4.9 ± 1.2 nA, $P < 0.01$, Student's paired *t*-test, $n = 4$, Fig. 3A). An even stronger inhibitory effect was produced by $100 \mu\text{M}$ Gd^{3+} which blocked TRPV2-mediated currents by 57% (from 2.7 ± 2.2 nA to 1.1 ± 0.3 nA, $P < 0.01$, $n = 9$, Fig. 3B). The corresponding IC_{50} -value of Gd^{3+} was $\sim 40 \mu\text{M}$ (calculated using the Hill equation, Fig. 3C).

In cells generating very large heat-evoked TRPV2-currents, the current following the peak of the heat ramp did not completely recover to the prestimulus level but instead remained elevated (Fig. 3D). The

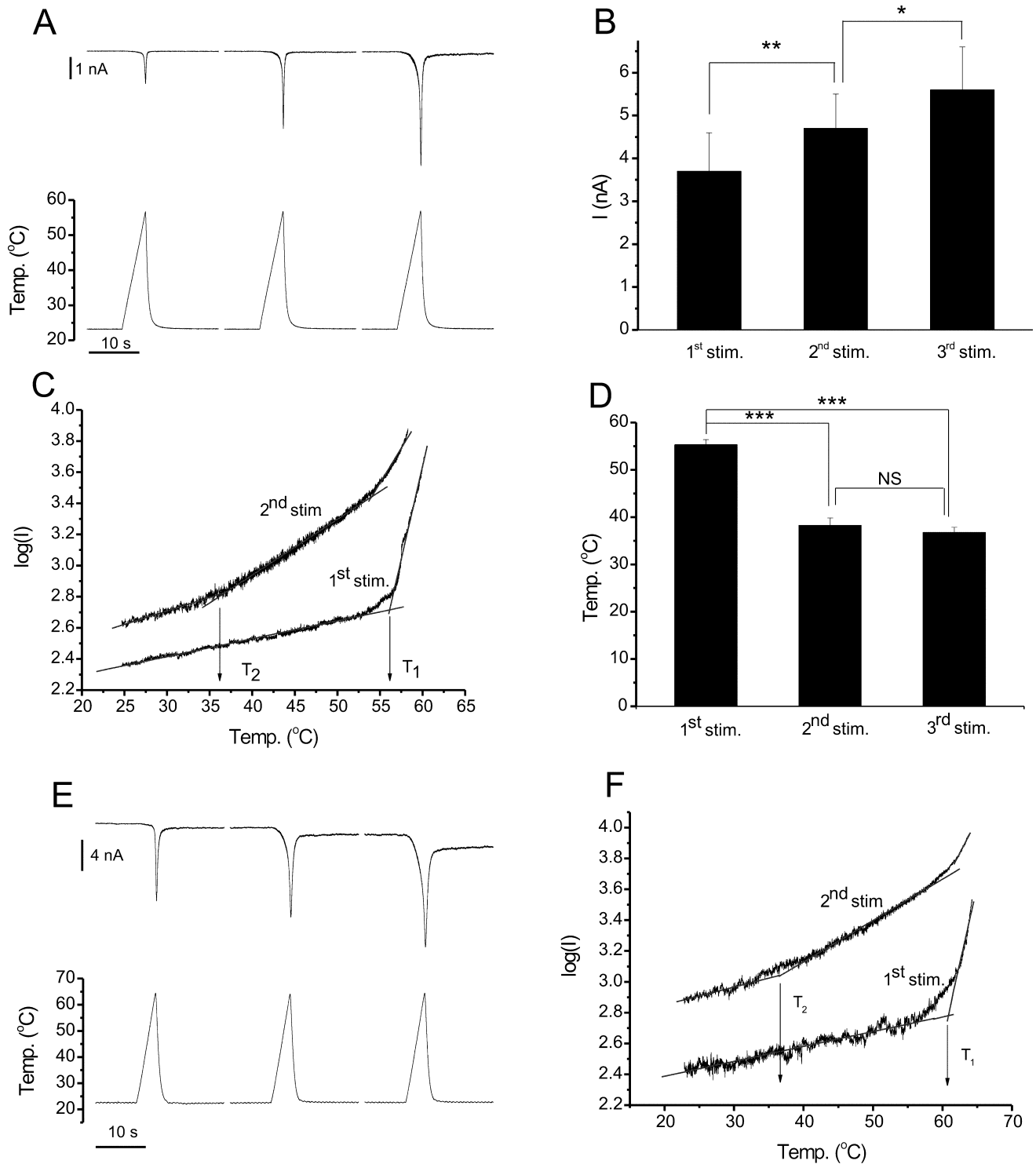


FIG. 2. Self-sensitization to heat of TRPV2 expressed in HEK293t cells. (A) Typical trace showing that the TRPV2-mediated heat-induced current (upper trace) was substantially increased during repetitive stimulation (lower trace). The heat stimuli were separated by 1-min intervals. (B) Statistical summary for the experiments illustrated in panel A. The amplitudes of the responses to the first three heat stimuli are represented as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; Student's paired t -test, $n = 8$. (C) Comparison of the temperature dependence of the TRPV2 currents evoked by the first two heat ramps. T_1 and T_2 indicate the temperature threshold for the heat current during the first and the second ramp, respectively. Note that the current during the second stimulus required fitting with three straight lines. (D) Statistical summary of the measurements illustrated in panel C. The temperature thresholds of the responses to the first three heat stimuli are represented as mean \pm SEM. *** $P < 0.001$, NS, not significantly different; Student's paired t -test, $n = 8$. (E) Representative recording of TRPV2-mediated heat currents (upper trace) in Ca^{2+} -free conditions, during repetitive heat stimulation (lower trace). Note the remaining sensitization to heat in the absence of extracellular calcium. (F). Comparison of the temperature dependence of the TRPV2 currents evoked in Ca^{2+} -free conditions by the first two heat ramps (same cell as in E). T_1 and T_2 indicate the temperature threshold for the heat current during the first and the second ramp, respectively.

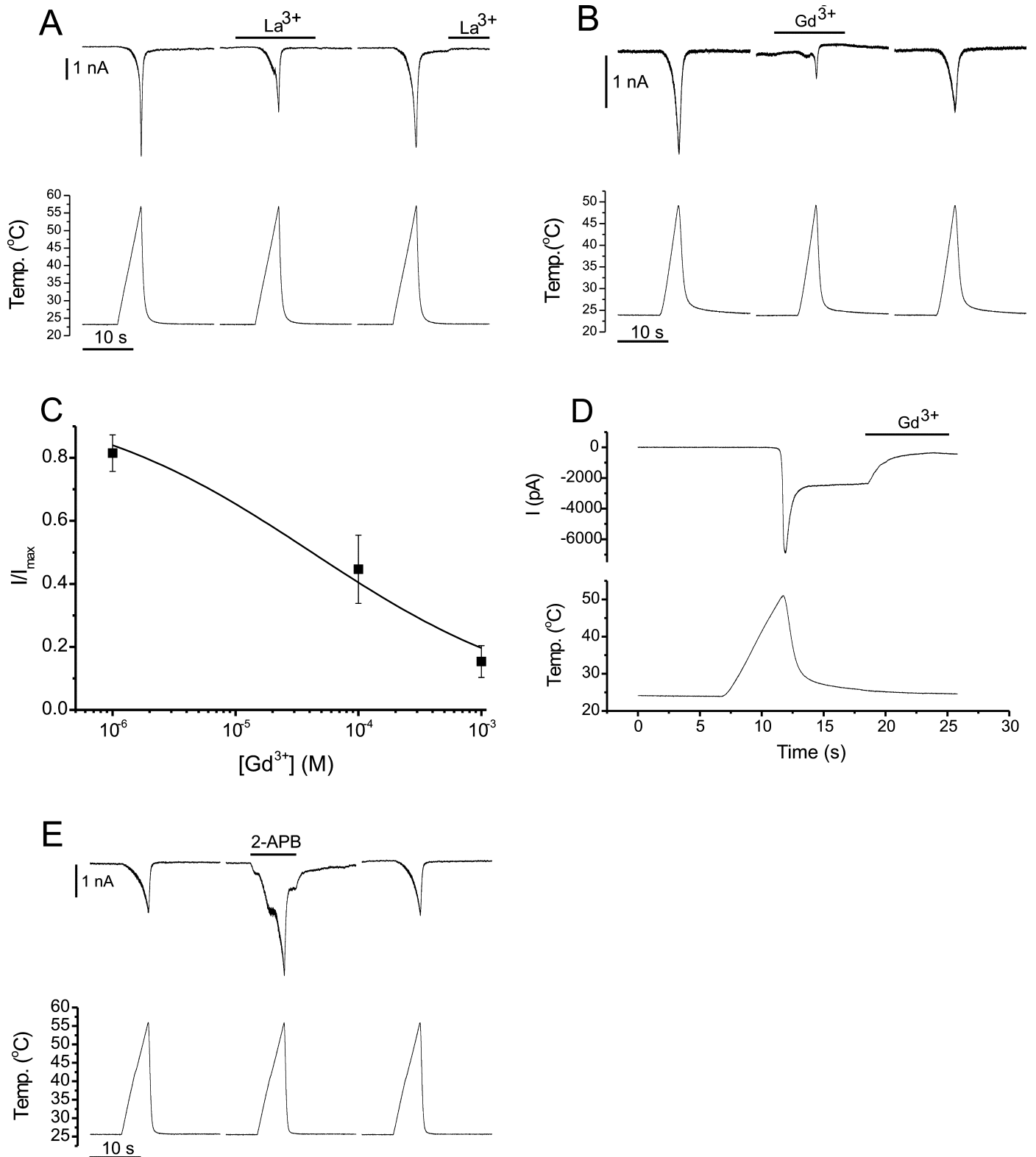


FIG. 3. Pharmacological properties of TRPV2 expressed in HEK293t cells, (A and B) Upper traces, heat-activated currents before, during and after application of $100 \mu\text{M}$ La^{3+} (A) and Gd^{3+} (B). Lower traces, temperature profile of the heat ramps applied at 1-min intervals. (C) Dose-dependence of the gadolinium-mediated inhibition of heat-activated currents through TRPV2. Current amplitudes were normalized for each cell to the current values previous to the application of gadolinium. Mean values (\pm SEM, $n = 5-7$ cells for each data point) were fitted with the Hill equation, yielding a half-maximal inhibitory concentration of $\sim 40 \mu\text{M}$. (D) Representative example of a cell in which the heat-activated current did not return to baseline after the stimulus but instead remained elevated on a plateau. This residual current was blocked by $100 \mu\text{M}$ Gd^{3+} . (E) Upper trace, heat-activated currents before, during and after application of $100 \mu\text{M}$ 2-APB. Lower traces, temperature profile of the heat ramps applied at 1-min intervals.

effects of Gd^{3+} , La^{3+} or ruthenium red on this residual current were investigated to determine whether this was due to a persistent activation of TRPV2 or simply to a non-specific increase in the leak current. As shown in Fig. 3D, Gd^{3+} applied after the heat stimulus strongly reduced the residual current ($\sim 90\%$ inhibition, from 2.7 ± 0.6 nA to 0.3 ± 0.09 nA, $P < 0.01$, $n = 7$). The same blocking effect was observed for the other two TRPV2 antagonists, La^{3+} and ruthenium red (data not shown).

A sensitization of the heat-activated TRPV2-currents was obtained in the presence of 2-APB, a compound which was previously shown to activate TRPV1, 2 and 3 in heterologous expression systems (Hu *et al.*, 2004). Application of $100 \mu M$ 2-APB elicited an inward current at the holding temperature of $\sim 25^\circ C$, and sensitized the heat-activated current through TRPV2 (from 1.4 ± 0.6 nA to 3.0 ± 1.0 nA, a 2.1-fold increase, $P = 0.03$, $n = 4$, Fig. 3E).

TRPV2 expressed in HEK293t cells is activated by heat in excised outside-out patches

Heat-activated currents were evoked in nine patches excised from TRPV2-transfected HEK293t cells (Fig. 4A). The recordings were performed in the outside-out configuration. The heat-activated current was inhibited in all five patches in which Gd^{3+} ($100 \mu M$) was applied during the heat stimulus (64% inhibition, from 97 ± 19 pA to 35 ± 9 pA, $P = 0.016$, $n = 5$, Fig. 4B). As in the whole-cell mode, in three patches we observed residual TRPV2 activity after the heat stimulus, and this residual current was almost completely blocked by $100 \mu M$ Gd^{3+} (Fig. 4B).

A TRPV2-like heat-activated current in rat DRG neurons: properties and comparison with TRPV1-mediated heat-activated currents

In order to investigate the properties of putative TRPV2 channels in sensory neurons we investigated medium- and large-sized DRG neurons with the whole-cell patch-clamp technique as performed on TRPV2-HEK293t cells. According to their response to capsaicin ($1 \mu M$), the cells were divided into capsaicin-sensitive (and thus TRPV1-expressing) and capsaicin-insensitive. Heat-sensitive but capsaicin-insensitive DRG neurons have been observed in the past (Nagy & Rang, 1999a; Caterina *et al.*, 2000; Ahluwalia *et al.*, 2002), but the involvement of TRPV2 in their response to heat is still not established. Using heat ramps between $\sim 25^\circ C$ and $\sim 60^\circ C$ and the pharmacological tools described above (Gd^{3+} , La^{3+} , 2-APB), we investigated the role of TRPV2 in mediating the responses to heat in rat DRG neurons.

Capsaicin discriminates two distinct types of heat transducers in DRG neurons

A comparison between the properties of the heat-activated currents in capsaicin-sensitive and capsaicin-insensitive medium and large rat DRG neurons revealed two distinct patterns of heat responses, illustrated in Fig. 5A and B. For analysis we only used neurons with a capacitance of 30 pF or more, corresponding to a diameter larger than $\sim 30 \mu m$. Of 78 medium and large DRG neurons, 56 (72%) responded to heat with inward currents larger than 100 pA and showing a clearly defined temperature threshold. Of all heat-sensitive neurons, 27% (21 out of 56) were capsaicin-sensitive ($1 \mu M$) and thus expressed TRPV1. The remaining 35 (45% of all neurons tested) were heat-sensitive but capsaicin-insensitive. In capsaicin-sensitive neurons the heat-activated currents displayed pronounced tachyphylaxis during three consecutive heat stimuli (Fig. 5A and C). In contrast, much like TRPV2-mediated currents in transfected HEK293t cells, heat-activated currents in

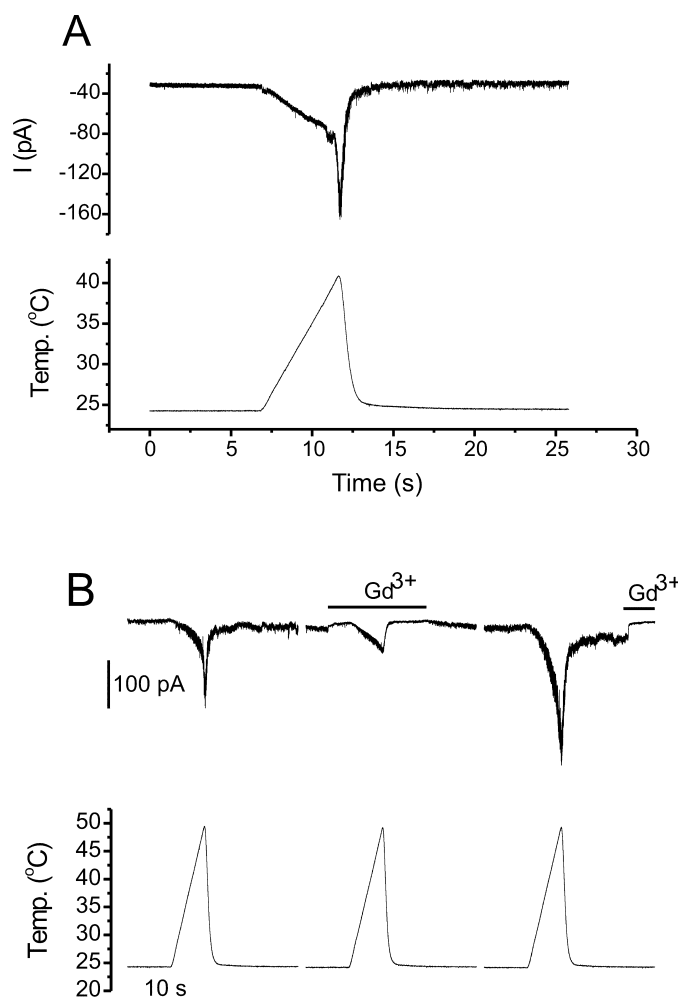


FIG. 4. Activation of TRPV2 by heat in cell-free outside-out patches. (A) Typical recording of a heat-activated current in a multichannel outside-out membrane patch excised from a TRPV2-expressing HEK293t cell. (B) Gd^{3+} blocks heat-activated currents in cell-free outside-out patches from TRPV2-expressing HEK293t cells. Upper trace, heat-activated currents before, during and after application of $100 \mu M$ Gd^{3+} . Lower trace, temperature profile of the heat ramps applied at 1 min interval. Note that Gd^{3+} also blocked the residual current after the third thermal stimulus.

capsaicin-insensitive neurons were significantly larger during the second heat ramp compared to the first, while the third response was still larger than the first but not significantly different from the second (Fig. 5B and D). In terms of the temperature thresholds for activation, the difference between capsaicin-sensitive and capsaicin-insensitive neurons was highly significant ($P < 0.001$, Student's unpaired *t*-test). The former ($42.1 \pm 0.6^\circ C$, $n = 8$) were almost identical to the reported value of $\sim 42^\circ C$ at which TRPV1 is activated in heterologous expression systems. In contrast, heat-evoked currents in capsaicin-insensitive neurons were first activated at $\sim 51^\circ C$ ($50.8 \pm 1.5^\circ C$, $n = 7$), and this was in good agreement with the thermal threshold of $\sim 52^\circ C$ for TRPV2 reported by Caterina *et al.* (1999) and with our own measurements of the TRPV2-mediated heat current in HEK293t cells ($55.3 \pm 1.1^\circ C$, see above). Thus, it is very likely that the heat transducers in the capsaicin-sensitive and capsaicin-insensitive medium and large rat DRG neurons are TRPV1 and TRPV2, respectively. Interestingly, both types of heat-sensing neurons displayed a reduction in temperature threshold between the first and the second heat stimulus, compatible with a self-sensitizing mechanism,

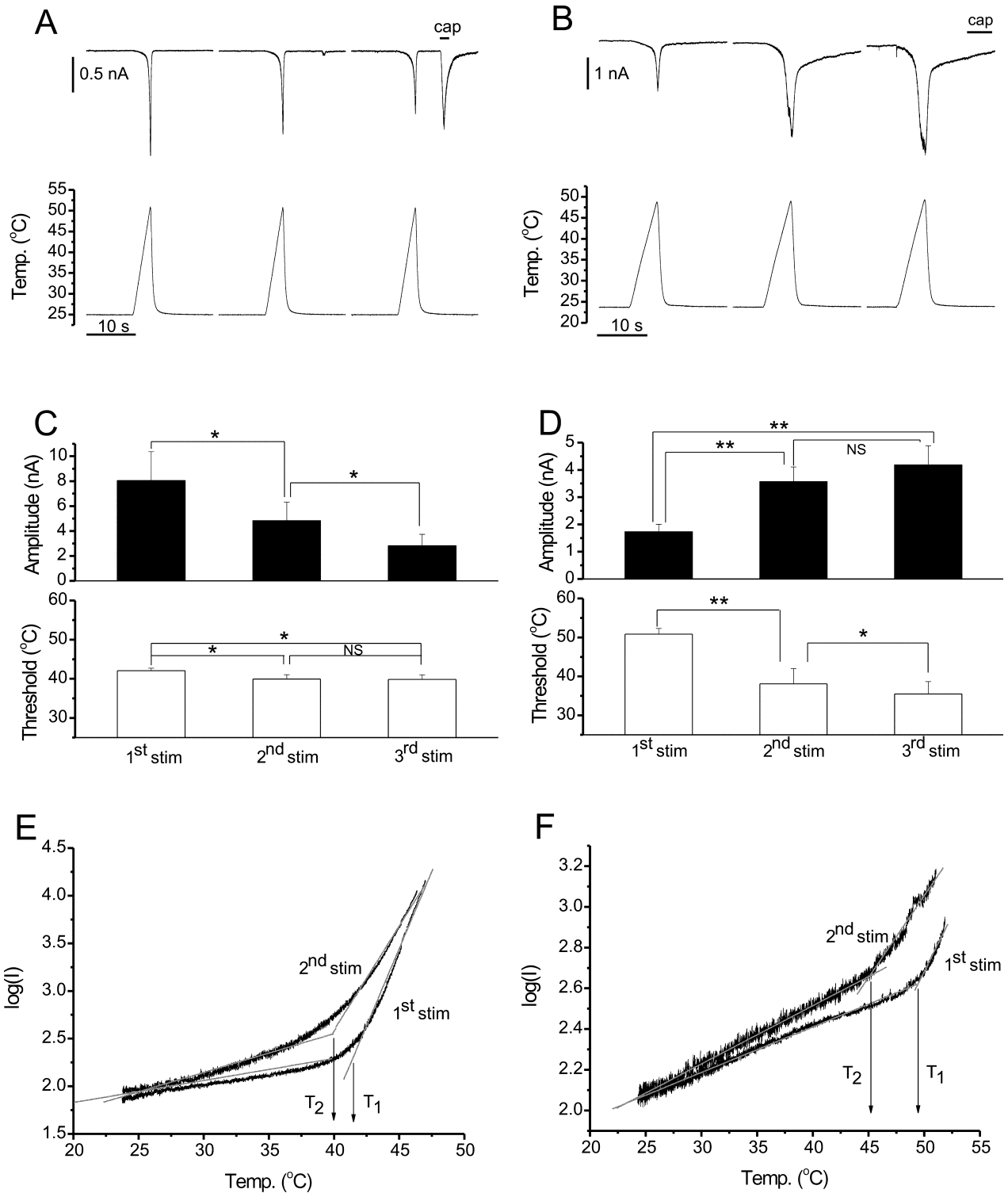


FIG. 5. Comparison of heat responses in capsaicin-sensitive (left column) and capsaicin-insensitive (right column) cultured rat DRG neurons. (A and B) Upper trace, heat-activated currents recorded in response to three consecutive heat ramps applied at 1-min intervals (lower trace) in a capsaicin-sensitive neuron (A) and a capsaicin-insensitive neuron (B). Note that capsaicin was applied at 1 μM after the third thermal stimulus. (C and D) Statistical summary of the experiments shown in panels A and B. Upper plots, the amplitudes of the currents activated by the three consecutive thermal stimuli shown in A and B are represented as mean ± SEM (black columns). * $P < 0.05$; ** $P < 0.01$; NS, not significantly different; Student's paired t -test, $n = 8$ for capsaicin-sensitive neurons (C) and $n = 7$ for capsaicin-insensitive neurons (D). Lower plots, the temperature thresholds of the currents activated by the three consecutive thermal stimuli shown in A and B are represented as mean ± SEM (white columns). * $P < 0.05$; ** $P < 0.01$; NS, not significantly different; Student's paired t -test, $n = 8$ for capsaicin-sensitive neurons (C) and $n = 7$ for capsaicin-insensitive neurons (D). (E and F) Temperature dependence of the currents evoked by the first and the second heat stimulus in a capsaicin-sensitive neuron (E), and a capsaicin-insensitive neuron (F). T_1 and T_2 represent the temperature thresholds during the first and the second stimulus, respectively.

but the effect was much more pronounced in the capsaicin-insensitive, very likely TRPV2-expressing cells (Fig. 5E and F). In these cells the mean threshold for activation was reduced from 50.8 ± 1.5 °C to 38.1 ± 3.9 °C ($P < 0.01$, $n = 7$) and further to 35.5 ± 3.2 °C ($P < 0.05$ compared to the second threshold, $n = 7$) during the third heat stimulation. For comparison, the threshold in capsaicin-sensitive neurons was decreased by just ~ 2 °C (from 42.1 ± 0.6 °C to 40.0 ± 1.0 °C, $P = 0.04$, $n = 8$), but this occurred in spite of decreased peak current amplitudes.

Pharmacological evidence for a role of TRPV2 as a heat transducer in capsaicin-insensitive heat-sensitive rat DRG neurons

In capsaicin-insensitive neurons the effects of La^{3+} , Gd^{3+} , and 2-APB on the heat-activated currents were very similar to the effects of the same compounds on the cloned TRPV2. Thus, in the presence of $100 \mu\text{M}$ La^{3+} the heat-activated current was reduced by 54% (from 4.8 ± 1.0 nA to 2.2 ± 0.6 nA, $P < 0.01$, $n = 6$, Fig. 6A), while $100 \mu\text{M}$ Gd^{3+} had a stronger inhibitory effect (63% inhibition, from 4.1 ± 0.8 nA to 1.5 ± 0.3 nA, $P < 0.01$, $n = 8$, Fig. 6C). Application of $100 \mu\text{M}$ 2-APB induced a small inward current at 25 °C and sensitized the heat-activated current by 1.8-fold (from 2.7 ± 1.2 nA to 5.0 ± 0.9 nA, $P = 0.04$, $n = 4$, Fig. 6E). For comparison, we investigated the effects of the same agents on capsaicin-sensitive neurons. La^{3+} and Gd^{3+} (both $100 \mu\text{M}$) strongly sensitized the heat-activated current in these neurons. La^{3+} increased the heat-evoked current by 2.1-fold (from 2.2 ± 0.6 nA to 4.6 ± 0.9 nA, $P = 0.01$, $n = 5$, Fig. 6B), while Gd^{3+} induced a 2.3-fold increase (from 3.1 ± 1.1 nA to 7.3 ± 2.0 nA, $P < 0.01$, $n = 7$, Fig. 6D). This effect of gadolinium is in good agreement with a recent report showing sensitization of TRPV1 by Gd^{3+} in a heterologous expression system (Tousova *et al.*, 2005). It is quite clear that the two trivalent cations have opposite effects on the heat-activated currents in capsaicin-sensitive and capsaicin-insensitive neurons, and these effects support the concept of TRPV2 mediating the heat response in the latter subpopulation.

2-APB was recently reported to activate TRPV1, 2 and 3 in heterologous expression systems (Hu *et al.*, 2004). When applied at $100 \mu\text{M}$, 2-APB induced a very small inward current in capsaicin-sensitive neurons at 25 °C, but strongly sensitized their response to heat (3.8-fold, from 1.5 ± 0.5 nA to 5.6 ± 1.3 nA, $P < 0.01$, $n = 7$, Fig. 6F). Thus, 2-APB was much more effective in capsaicin-sensitive than in capsaicin-insensitive DRG neurons.

Similar to what we observed in the case of TRPV2 expressed in HEK293t cells, in some capsaicin-insensitive neurons the current following a heat ramp did not return to baseline but remained at an elevated plateau. This residual current was almost entirely blocked by $100 \mu\text{M}$ Gd^{3+} (83% inhibition, from 2.2 ± 0.6 nA to 0.4 ± 0.2 nA, $P = 0.02$, $n = 7$, Fig. 6G).

Discussion

Following its cloning by Caterina *et al.* (1999), the evidence for a physiological role of TRPV2 as a transducer of noxious heat in sensory neurons has remained poor. Heat-sensitivity of the recombinant TRPV2 could not be reproduced by several laboratories (Benham *et al.*, 2003). A very recent report described increases in intracellular Ca^{2+} evoked by heat and 2-APB in HEK293 cells expressing rat and mouse TRPV2, but not human TRPV2 (Neeper *et al.*, 2007), suggesting that interspecies differences may account for the lack of consensus concerning the role of TRPV2 in noxious heat sensing. Heterogeneous responses to noxious heat in DRG cultures, as well as

the existence of heat-sensitive but capsaicin-insensitive DRG neurons had already been described by Nagy & Rang (1999a, b). Another paper from the same group presented evidence in favour of TRPV2 mediating high-threshold heat-induced currents in medium and large DRG neurons; the current was blocked by ruthenium red but not by the competitive TRPV1 antagonist capsazepine, and it was activated at a temperature that was very close to the threshold of TRPV2 in heterologous expression systems (Ahluwalia *et al.*, 2002). Moreover, the biophysical properties of the high threshold heat-activated currents in DRG neurons and those of TRPV2 in expression systems were similar; both were non-selective cation currents with inward rectification at negative membrane potentials and in both cases the temperature threshold was reduced with repetitive stimulation (Caterina *et al.*, 1999; Ahluwalia *et al.*, 2002). High-threshold heat-activated currents were also reported by Greffrath *et al.* (2003) and very recently by Rau *et al.* (2007) in rat DRG neurons and by Bender *et al.* (2005) in an F-11 cell line (a hybridoma derived from rat DRG and mouse neuroblastoma cells). In spite of these results, the characterization of TRPV2 in terms of pharmacology and temperature dependence is still incomplete and its involvement in noxious heat sensing remains controversial. Firstly, intracellular recordings performed in TRPV1 $^{-/-}$ mouse DRG neurons with their cutaneous projections intact showed that the overwhelming majority (85%) of heat-sensitive C-fibre neurons did not stain with an antibody against TRPV2 (Woodbury *et al.*, 2004). Secondly, earlier work from our laboratory showed that the sensitization of heat responses in mouse C-fibre cutaneous nociceptors by 2-aminoethoxydiphenylborate (2-APB, an activator of TRPV1, 2 and 3) was abolished in TRPV1 $^{-/-}$ animals, suggesting that TRPV2 is not responsible for the retained heat sensitivity in the absence of TRPV1 (Zimmermann *et al.*, 2005). However, these apparently conflicting results could be reconciled if TRPV2 was primarily or exclusively expressed in the cutaneous terminals of A δ nociceptors, which is indeed indicated by its expression in medium and large DRG neurons (Ma, 2001). Moreover, the heat stimuli used by Woodbury *et al.* (2004) and by Zimmermann *et al.* (2005) of 52 °C and 48 °C, respectively, may have been just below the activation threshold for TRPV2 in the native tissue. Finally, the discrepancy could well arise from a mouse (Woodbury *et al.*, 2004; Zimmerman *et al.*, 2005) vs. rat (Ahluwalia *et al.*, 2002) species difference, as it has been shown recently that rodent and human TRPV2 have different biophysical properties (Neeper *et al.*, 2007). In any case, taking into account the scarcity of published data regarding heat activation of TRPV2, we considered that additional evidence for functional TRPV2 expression in cultured DRG neurons together with a detailed analysis of its temperature dependence in comparison to the heterologous expression system would shed new light on the molecular mechanisms of noxious heat sensing in mammals.

As ruthenium red is a non-selective TRPV channel blocker, we sought a more specific TRPV2 antagonist, in particular one that would clearly distinguish between TRPV1 and TRPV2. In a recent report it was shown that a growth factor-dependent, Ca^{2+} -permeable, non-selective cation channel blocked by ruthenium red (thus considerably TRPV2-like) in striated muscle fibres was also blocked by the trivalent cation gadolinium (Gd^{3+} Rolland *et al.*, 2006). On the other hand it is known that gadolinium activates and sensitizes TRPV1 (Tousova *et al.*, 2005). It was therefore conceivable that Gd^{3+} might be a useful tool in distinguishing between TRPV1- and TRPV2-mediated heat-induced currents in cultured sensory neurons.

Large high-threshold heat-activated currents were recorded from HEK293t cells transfected with the rat TRPV2 clone. The properties of this TRPV2-mediated current were very similar to those reported by

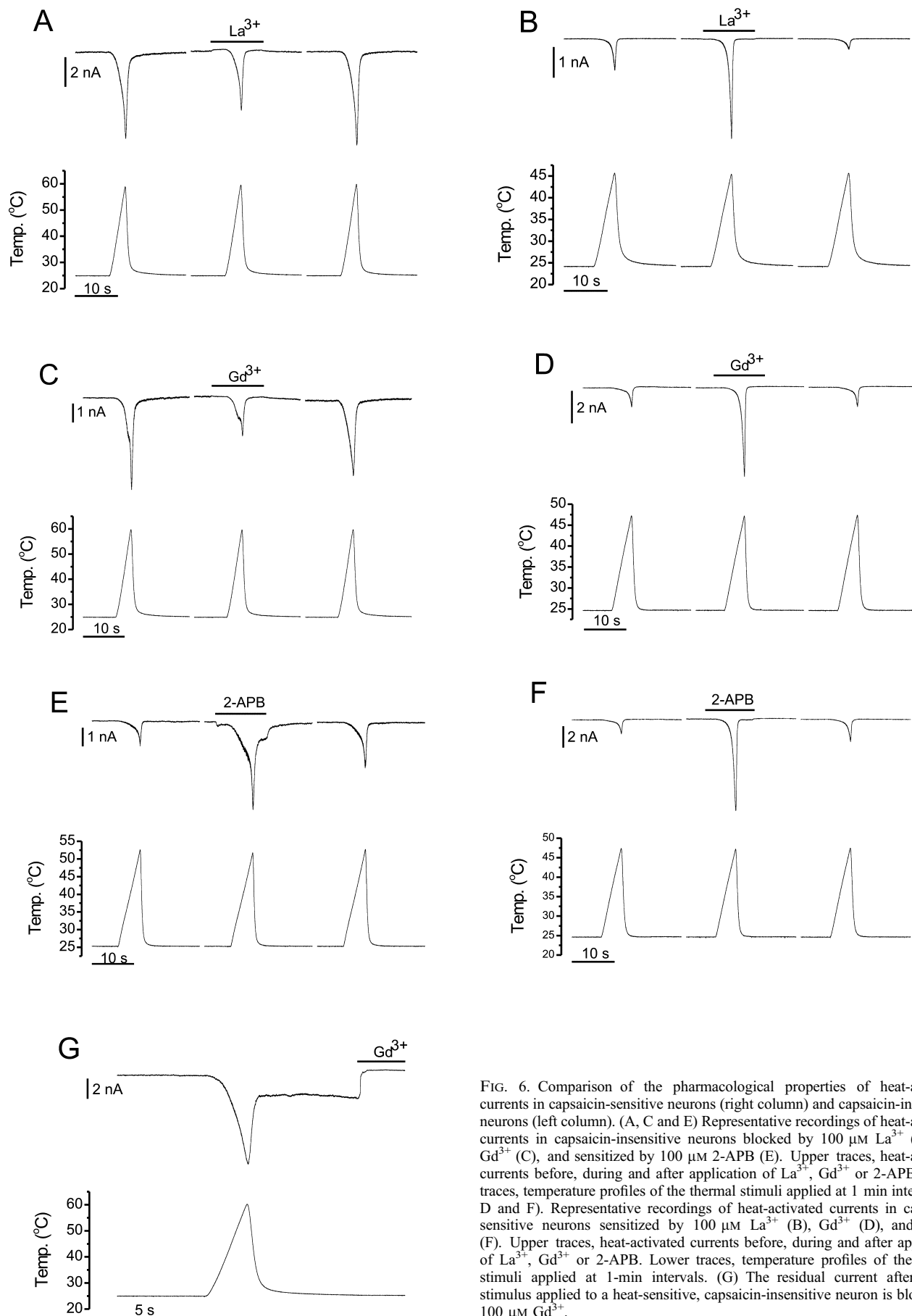


FIG. 6. Comparison of the pharmacological properties of heat-activated currents in capsaicin-sensitive neurons (right column) and capsaicin-insensitive neurons (left column). (A, C and E) Representative recordings of heat-activated currents in capsaicin-insensitive neurons blocked by 100 μM La^{3+} (A), and Gd^{3+} (C), and sensitized by 100 μM 2-APB (E). Upper traces, heat-activated currents before, during and after application of La^{3+} , Gd^{3+} or 2-APB. Lower traces, temperature profiles of the thermal stimuli applied at 1-min interval. (B, D and F). Representative recordings of heat-activated currents in capsaicin-sensitive neurons sensitized by 100 μM La^{3+} (B), Gd^{3+} (D), and 2-APB (F). Upper traces, heat-activated currents before, during and after application of La^{3+} , Gd^{3+} or 2-APB. Lower traces, temperature profiles of the thermal stimuli applied at 1-min intervals. (G) The residual current after a heat stimulus applied to a heat-sensitive, capsaicin-insensitive neuron is blocked by 100 μM Gd^{3+} .

Caterina *et al.* (1999); a temperature threshold of ~ 55 °C, inhibition by ruthenium red, self-sensitizing responses to repetitive stimulation. Furthermore, we discovered an inhibitory effect of the trivalent cations La^{3+} and Gd^{3+} on TRPV2, with Gd^{3+} being the more potent antagonist. As expected (Hu *et al.*, 2004), 2-APB acted as an activator of TRPV2, inducing small inward currents at -60 mV, but it also strongly augmented the response to heat (by more than 100%). We observed that in some cells, following ramps to ~ 55 – 60 °C, the heat-activated current did not return to baseline, but instead remained at an elevated value. This feature was present in both TRPV2-expressing HEK293t cells and in capsaicin-insensitive heat-sensitive DRG neurons. However, we were able to block this residual current almost completely with the TRPV2 antagonist Gd^{3+} (and also with ruthenium red and La^{3+} in TRPV2-expressing HEK293t cells), which indicates that persistent opening of TRPV2 rather than non-specific membrane breakdown is responsible for this residual activity.

We completed our analysis of TRPV2 expressed in HEK293t cells by recording heat-induced channel activity in excised multichannel membrane patches in the outside-out configuration. Inward currents blocked by Gd^{3+} could be elicited in cell-free patches held at -60 mV, indicating that heat activation of TRPV2 is a localized event restricted to the cell membrane and does not require a second messenger cascade.

The temperature dependence of TRPV2-mediated currents in HEK293t cells was analysed by measuring the temperature coefficients (Q_{10}) and temperature thresholds (T). During the first exposure to a heat ramp, TRPV2 activated with a steep temperature dependence ($Q_{10} \sim 100$) and a high temperature threshold ($T \sim 55$ °C). With the second and the third stimulation, TRPV2 activated in a biphasic manner, with a highly temperature-dependent phase triggered close to the initial threshold ($Q_{10} \sim 300$, $T \sim 56$ °C in the second response) and a low Q_{10} phase activating at a much lower threshold ($Q_{10} \sim 3.0$, $T \sim 38$ °C for the second response). Such a self-sensitizing phenomenon has been reported for both TRPV2 in *Xenopus* oocytes (Caterina *et al.*, 1999) and for the TRPV2-like currents in rat DRG neurons (Ahluwalia *et al.*, 2002; Rau *et al.*, 2007). Interestingly, in a very recent report the heat-heat sensitization in subpopulations of TRPV2-expressing DRG neurons from the rat was shown to be dependent on extracellular calcium (Rau *et al.*, 2007). In contrast, self-sensitization to heat of TRPV2 expressed in HEK293t cells measured in this study was not changed upon removal of external calcium. The reason for this discrepancy is not yet clear.

Heat-sensitive neurons in rat DRG cultures were classified according to their responses to $1 \mu\text{M}$ capsaicin into capsaicin-sensitive and capsaicin-insensitive. We focused our investigation on medium and large neurons, as TRPV2 immunoreactivity was associated mainly with such cells (Ahluwalia *et al.*, 2002). The two groups of neurons displayed distinct types of responses when challenged with heat (~ 55 – 60 °C). Two main features distinguished heat-activated currents in capsaicin-insensitive neurons from those in capsaicin-sensitive neurons; a significantly higher temperature threshold and an increase in amplitude with repetitive stimulation (in contrast, capsaicin-sensitive neurons showed pronounced tachyphylaxis to supra-threshold heat stimuli). In addition, the decrease in threshold with repetitive stimulation was much more pronounced in capsaicin-insensitive neurons (~ 13 °C), compared to capsaicin-sensitive cells (~ 2 °C). The temperature and sequence dependence of the heat-activated current in capsaicin-insensitive DRG neurons closely matched the features of TRPV2-mediated currents in HEK293t cells; the temperature threshold, the change in threshold between first and second stimulation and the sensitizing effect of repetitive stimulation on current amplitude, all revealed an uncanny similarity. Moreover, both the heat current in capsaicin-insensitive neurons and the TRPV2

current in HEK293t cells were inhibited to a similar degree by the trivalent cations La^{3+} and Gd^{3+} , and both were sensitized in the presence of 2-APB. In contrast, heat-activated currents in capsaicin-sensitive neurons were substantially enhanced by trivalent cations.

Neutralization of two extracellular acidic residues, E600 and E648, seems to be responsible for the sensitizing effect of Gd^{3+} on TRPV1 (Tousova *et al.*, 2005), the same residues that are involved in the sensitizing and, respectively, activating effects of protons on the same channel (Jordt *et al.*, 2000). These residues are not conserved in TRPV2 (Caterina *et al.*, 1999). In rat TRPV2, E600 is replaced by an arginine (R560) and E648 by an alanine (A611) at the equivalent positions. The absence of these two key acidic residues may explain the lack of a sensitizing effect of Gd^{3+} on TRPV2.

In conclusion we have identified new pharmacological tools for distinguishing between TRPV1 and TRPV2-mediated heat-induced currents, based on their differential sensitivity to trivalent cations. We have provided evidence for heat activation of TRPV2 in cell-free membrane patches excised from TRPV2-expressing HEK293t cells. We have shown that prolonged activation of native TRPV2 and not membrane damage is likely to be at least partly responsible for the residual current following the application of intense heat in capsaicin-insensitive DRG neurons, which could be blocked to a large extent by gadolinium. However, as it is known that certain mechano-sensitive channels from sensory neurons (Cho *et al.*, 2006), as well as other TRP channels expressed in the DRG, such as TRPV4 (Becker *et al.*, 2005) and TRPA1 (Nagata *et al.*, 2005), are also blocked by gadolinium, it may be that other channels contribute to this residual current. Finally, the temperature and sequence dependence of the heat-activated currents in capsaicin-insensitive neurons together with their pharmacology indicate that TRPV2 is involved in mediating their response to noxious heat. Taken together, our results support a concept in which TRPV1 and TRPV2 are both involved in mediating heat responses in non-overlapping subpopulations of medium and large rat DRG neurons.

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Abbreviations

2-APB, 2-aminoethoxydiphenyl borate; DRG, dorsal root ganglion; Gd^{3+} , gadolinium; HEK, human embryonic kidney; La^{3+} , lanthanum.

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