Heat Sensitization in Skin and Muscle Nociceptors Expressing Distinct Combinations of TRPV1 and TRPV2 Protein

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Rau KK, Jiang N, Johnson RD, Cooper BY. Heat sensitization in skin and muscle nociceptors expressing distinct combinations of TRPV1 and TRPV2 protein. J Neurophysiol 97: 2651–2662, 2007. First published February 7, 2007; doi:10.1152/jn.00840.2006. Recordings were made from small and medium diameter dorsal root ganglia (DRG) neurons that expressed transient receptor potential (TRP) proteins. Physiologically characterized skin nociceptors expressed either TRPV1 (type 2) or TRPV2 (type 4) in isolation. Other nociceptors co-expressed both TRP proteins and innervated deep tissue sites (gastrocnemius muscle, distal colon; type 5, type 8) and skin (type 8). Subpopulations of myelinated (type 8) and unmyelinated (type 5) nociceptors co-expressed both TRPs. Cells that expressed TRPV1 alone were excellent transducers of intense heat. Proportional inward currents were obtained from a threshold of ~46.5 to ~56°C. In contrast, cells expressing TRPV2 alone (52°C threshold) did not reliably transduce the intensity of thermal events. Studies were undertaken to assess the capacity of skin and deep nociceptors to exhibit sensitization to repeated intense thermal stimuli [heat-heat sensitization (HHS)]. Only nociceptors that expressed TRPV2, alone or in combination with TRPV1, exhibited HHS. HHS was shown to be Ca2+ dependent in either case. Intracellular Ca2+ dependent pathways to HHS varied with the pattern of TRP protein expression. Cells co-expressing both TRPs modulated heat reactivity through serine/ threonine phosphorylation or PLA2-dependent pathways. Cells expressing only TRPV2 may have relied on tyrosine kinases for HHS.

INTRODUCTION

Repeated application of thermal stimuli are known to sensitize nociceptive afferents. Heat-sensitization (HHS) can be shown, in vivo, in CMH (C-mechanoheat) and AMH (Aβ-mechanoheat) nociceptors in cats (Beck et al. 1974; Fitzgerald and Lynn 1977), primates (Bessou and Perl 1969; Campbell et al. 1979; Kumazawa and Perl 1977; LaMotte 1983; LaMotte et al. 1982; Perl et al. 1976), and to a lesser extent, rats (Kress et al. 1992; Lynn and Carpenter 1982; Lynn and Shakhanbeh 1988). Repetition of an intense thermal stimulus can produce de novo responding, decreased threshold, increased action potentials, decreased response latency, and increased afterdischarge. It is likely that changes in the response properties of nociceptor fibers contributes to the development of thermal allosthenia and hyperalgesia (LaMotte 1983; LaMotte et al. 1983; Meyer and Campbell 1981).

To the extent that HHS occurs without the mediation of extracellular algesics, it represents a form of autosensitization requiring distinct pain control strategies. With tests conducted in vivo, it is difficult to distinguish an HHS autosensitization from one that might occur secondary to physiological release of algesics from local cells or secondary to tissue damage. Skin keratinocytes express heat-sensitive TRPV1 (Denda et al. 2001; Inoue et al. 2002; Southall et al. 2003), TRPV3 (Pieir et al. 2002; Smith et al. 2002; Xu et al. 2002), and TRPV4 (Guler et al. 2001; Watanabe et al. 2002). These cells contain both ATP and acetylcholine (Grando et al. 1993; Wessler et al. 1998, 1999) and might release these or other agents during intense heating. Skin nociceptors express purinergic and cholinergic receptors (Jiang et al. 2006; Petruska et al. 2000a–d, 2002; Rau et al. 2005), and neurons can be activated by agents released from keratinocytes (Cook and McCleskey 2002; Kozumi et al. 2004). Sensitization might occur through amplified keratinocyte release mechanisms or after massive neuronal Ca2+ entry (Kress and Distler 2004). Numerous algesic agents associated with trauma and inflammation [N-arachidonoyl dopamine (NADA), oleoyl dopamine (OLDA), HETEs, bradykinin, NGF, ATP, protons, interleukin (IL)1β, protease activated receptor (PAR) proteinases] are able to mediate a heat sensitization in isolated skin, neurons, and host cells expressing TRPV1 (Cesare and McNaughton 1996; Chu et al. 2003; Chuang et al. 2001; Dai et al. 2004; Galoyan et al. 2003; Guenther et al. 1999; Huang et al. 2002; Hwang et al. 2000; Obreja et al. 2002; Sugiuira et al. 2002; Tominga et al. 1998, 2001). Possibly HHS reflects agent-dependent plasticity; alternately, distinct populations of nociceptors could rely on auto-sensitization and/or algesic-dependent paths to heat sensitization.

If a true autosensitization occurs, it should be possible to observe HHS in vitro, where secondary release of pro-inflammatory agents would not occur. However, it has proven difficult to show HHS in dispersed rat dorsal root ganglion (DRG) neurons, in vitro, using only heat as the instigating stimulus (Cesare and McNaughton 1996; Greffrath et al. 2001, 2002; Kirschstein et al. 1999; Kress and Guenther 1999; Nagy and Schwarz et al. 2000). Only anecdotal evidence suggests that adult mammalian nociceptors are capable of HHS (Ahlulwalia et al. 2002; Vylicky et al. 1999). It is noteworthy...
that these studies have been confined mainly to small diameter C-type neurons despite the distribution of heat transducing proteins in medium diameter cell populations. The latter are thought to include populations of myelinated afferents that are part of the Aδ family. AMH nociceptors manifest HHS, but they are typically under represented in in vitro studies; possibly because they are small in number and difficult to identify.

The cloning of transient receptor potential (TRP) family members TRPV1 (VR1; Caterina et al. 1997) and TRPV2 (VR2-1; Caterina et al. 1999) provided a molecular basis for multiple heat-transducing phenotypes in sensory afferents (for reviews, see Benham et al. 2003; Dhaka et al. 2006; Green 2004; Gunthorpe et al. 2002; Nilius and Voets 2004, 2005; Patapoutian et al. 2003). TRP proteins are widely distributed in the mammalian nervous system, including the DRG (Ahluwalia et al. 2002; Guo et al. 1999; Mezey et al. 2000; Michael and Priestley 1999; Sasamura et al. 1998). TRPV1, which also mediates capsaicin-induced currents, is highly expressed in DRG neurons that are small to medium in diameter, bind isocitcin B4, and are often associated with C-type afferents (Caterina et al. 1997). In contrast, the capsaicin-insensitive TRPV2 protein is reported to be highly expressed in rat afferent cell bodies that are medium to large in diameter, lack isocitcin B4-binding, and possibly are associated with Aδ family afferents (Caterina et al. 1999; Lewinter et al. 2004).

The functional specifics relating TRP protein distribution to heat transduction characteristics has not been fully documented. The pattern of TRP expression in skin nociceptors could provide a molecular basis for distinct transduction and plastic capacities underlying HHS.

Patterns of voltage-activated currents can be used to segregate DRG cells into individual subgroups that represent discrete classes of C and Aδ nociceptors (Cardenas et al. 1995). Thus far, our laboratory has used this technique to identify >10 distinct populations of small to medium diameter DRG cells that have nociceptive properties and differentially innervate cutaneous and other tissues (Jiang et al. 2006; Petruska et al. 2000d, 2002; Rau et al. 2005).

Given the distinct clustering of properties we have observed, cell selection could exert a major role in identifying and characterizing HHS in vitro. In studies of heat sensitization, laboratories routinely select distinct patterns of inward and outward currents that were uniquely patterned because of the differential expression of I_h and I_o in each class of cells (Cardenas et al. 1995; Petruska et al. 2000d). Recordings were made from medium (types 4, 5, and 8) and small diameter cells (type 2) using an Axopatch 200b and pClamp 8.2 software package. Signals were digitized with a Digidata 1322a. Series resistance was compensated 40–60%; a small −4-mV junction potential was not corrected.

**Drugs and solutions**

Plated cells were bathed in rat Tyrode’s solution containing (in mM) 140 NaCl, 4 KCl, 2 MgCl_2, 2 CaCl_2, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. Osmolarity was ≈300–315 mOsm. Solutions were applied through a gravity-fed pipette that was placed around 50 μm from the cell (sewer pipe). Stock solutions were prepared for capsaicin (10 mM; Sigma-Aldrich), ruthenium red (10 mM; Sigma-Aldrich), H7 (10 μM; Hidaka et al. 1984), methyllethachidonyl fluorophosphonate (MAFP) (2 μM; Lio et al. 1996), and genistein (50 μM; Geissler et al. 1990). Stocks of MAFP were kept under argon. Final concentrations were prepared fresh on the day of the experiment.

**Pharmacology and heat sensitization protocol**

Superfused heat pulses were feedback controlled by a heat probe (HPRE2, Cell Microsystems) that was positioned −50 μm from the cell. The probe fluid was maintained at the desired temperature by a second channel of the TCbip hiomtercontroller. Solutions were preheated in the probe to the desired target temperature just before application. The heat step was gravity applied to the targeted cell that had been previously classified as type 2, 4, 5, or 8. Tube volume and fluid height were strictly monitored to maintain a consistent flow rate in all tests. Sensitization was defined as a significant increase in inward current on the second application of two heat pulses (52°C; 15- or 20-s duration). Consecutive tests were separated by a 4-min interval. A ratio was formed from the peak amplitude of TEST2 and TEST1. This sensitization ratio (SR) was used as an index of sensitization; however, statistical assessment of HHS was made on the actual TEST1 and TEST2 peak currents that were measured at a point 15 or 20 s after the pulse. In some cases, heat thresholds were determined before and after the HHS protocol. Changes in threshold after HHS were assessed at 2 min after TEST2 using a 6-s pulse applied at consecutive 2° steps separated by 2-min intervals. Currents typically achieved steady state within 6 s. Threshold was defined as a current exceeding 1 pA/pF. To examine the intracellular pathways of HHS, several agents (H7, genistein, or MAFP) were administered in independent experiments. Each agent applied for 2 min before the TEST1 heat pulse, during the 4-min interval that followed TEST1, and during TEST2. To examine whether extracellular Ca^{2+} was...
needed for heat sensitization, the above sensitization protocol was repeated using a modified Tyrode’s solution containing 4 mM Mg\(^{2+}\) and 0 mM Ca\(^{2+}\).

In another group of cells, the molecular basis of the current was determined. Before application of any inhibitor, a heat pulse (52°C) was applied for ~6 s. Two minutes later, an inhibitor (capsazepine, ruthenium red) was applied for an additional 2 min. The 6-s heat pulse was reapplied with the inhibitor. As before, the peak current amplitude was measured 6 s after application of the pulse. Cells that did not exhibit ≥100 pA of current on TEST1 or during pharmacological testing were excluded from the study (17 of 206 cases).

Afferent tracing

Under aseptic conditions, the fluorescent tracer FastDiI oil (1,1'-dilinoleyl-3,3',3'-tetramethylindocarbocyanine perchlorate; 25 mg FastDiI dissolved in 0.5 ml methanol; Molecular Probes) was injected into either the distal colon (n = 15) or gastrocneumius muscles (n = 12) of young adult male rats (80–100 g). The rats were anesthetized with a mixture of ketamine and xylazine (80 mg/kg ketamine; 10 mg/kg xylazine). The following signs were monitored during surgery: heart rate, respiratory rate, ventilatory status (end-expired P\(_{CO_2}\)), and body temperature. Anesthetic depth was assessed by corneal, palpebral, and pinna reflexes. The animals were placed on a heating pad to maintain ideal body temperature (36–37°C). Injections of DiI were made with a 33-gauge needle coupled to a Hamilton microsyringe.

For injections into the distal colon, a small longitudinal incision was made through the hairy skin and linea alba into the peritoneal cavity. The bladder was aseptically evacuated, and intestine and mesentery were gently pushed aside to expose the distal colon. Intramuscular injections (16-μl volume per animal divided into 8 injections per limb of 1 μl each) were made into the lining of the distal colon, ~1–3 cm rostral to the anus. After injections were completed, the surgical incision was closed in layers. The linea alba was closed with suture, and the overlying hairy skin was closed with cyanoacrylate and surgical staples.

For injections into the gastrocnemius muscle, a small longitudinal incision was made through the hairy skin overlying the muscle. Intramuscular injections (16-μl volume per animal divided into 8 injections per limb of 1 μl each) were performed, and subsequently the incision was closed with cyanoacrylate and surgical staples.

Rats were monitored daily and allowed to recover for 7 days. They were killed for in vitro electrophysiological studies. Cells were plated in the usual manner but protected from ambient light. Dishes were mounted on a Nikon TE 2000 inverted microscope with an epifluorescence attachment. Tracer-labeled cells were viewed with the appropriate Vivid filter set (XF102, Omega Optical), and UV light exposure to all fields was limited to 1 min in duration. Only intensely fluorescent cells were considered positive. Only one cell was recorded per dish. After a recording was completed, digital images of the bright-field and fluorescent fields of view were captured using a Dage MTI RC300 camera coupled to a PC running Scion Image 4.0.2.

Care was taken not to contaminate tissues adjacent to the target tissue (i.e., mesocolon and subumbilical skeletal muscles near the distal colon; skin fascia overlying the gastrocnemius muscle). After each injection, the needle was slowly removed, any leakage was controlled by cotton-tipped applicators, and the site was rapidly sealed with n-butyl cyanoacrylate monomer glue (either Nexaband Liquid or SteriTac-B). To assess the possible spread of DiI from injection sites, injected tissue and adjacent tissues were harvested before plating the DRG cells. The tissues were placed in vials containing 4% paraformaldehyde in phosphate-buffered saline (PBS) for a 24-h period. Subsequently, this fixative solution was replaced by 30% sucrose in PBS for cryoprotection. Once the tissue equilibrated it was embedded in TBS Tissue Freezing Medium (Triangle Biomedical Sciences), and 10-μm sections were cut on a cryostat (HM 550, Microm). Sections were thaw-mounted onto slides and placed in a −20°C freezer until viewed under fluorescent microscopy. Cases in which DiI had leaked into overlying skin tissue were not included.

Immunocytochemistry

The immunoreactivity of neurofilament-m (NFm), TRPV1, and TRPV2, and binding of IB4 isolectin (from *Griffonia simplicifolia*) was examined in dishes that contained recorded cells from the afferent tracing studies (described above). For immunocytochemical (ICC) processing, dishes were thoroughly rinsed with PBS to remove any residual paraformaldehyde. Before ICC procedures, tissue sections were encircled with hydrophobic resin (PAP Pen, The Binding Site). The slide-mounted sections were incubated at room temperature for 1 h in a solution of 1:30 normal goat serum (Jackson ImmunoResearch Laboratories) in PBS with 0.4% Triton X-100 (Sigma; GS-PBS-T) to block nonspecific antibody binding. Sections were incubated over sequential evenings in solutions of primary antisera and species-specific secondary antibodies conjugated to fluorophores. All steps were followed by multiple rinses with 1% GS-PBS-T. Primary antibodies were diluted in PBS in the following manner: 1:500 mouse anti-NFm (RBI), 1:1,000 guinea pig anti-TRPV1 and rabbit anti-TRPV2 (Chemicon), and 5 μg/ml IB4 (Sigma). Incubations in primary antisera or IB4 were overnight (14–18 h). The use of DiI (Molecular Probes) limited the number of fluorescent channels that could be examined during any immunocytochemical study (i.e., Alexafluor 594 was not used). However multiple molecular features could still be detected from the same cell using other secondary conjugates that fluoresce at different wavelengths. Goat-conjugated secondary antibodies Pacific Blue (Molecular Probes), AlexaFluor 488 (Molecular Probes), and Cascade Yellow (Molecular Probes) allowed for multilabel fluorescent histochemical phenotyping for each cell examined (although not all of the markers were examined for each recorded cell). All secondary antibodies were diluted 1:100 in PBS, and incubations were 3 h in duration. Anti-mouse secondary antisera were preadsorbed before use for 1 h with normal rat serum (Jackson ImmunoResearch Laboratories).

It was necessary to amplify the signal for TRPV2. Secondary antisera for TRPV2 (1:500 biotinylated goat anti-rabbit IgG, Jackson ImmunoResearch Laboratories) was applied to the cells for 3 h, followed by an incubation with the VectaStain Elite ABC reagent kit (ABC kit, Vector Laboratories) for 30 min. Subsequently, the cells were incubated in a 1:100 dilution of a tyramide signal-amplification (TSA) kit conjugated to biotin (Perkin Elmer) for 5 min and a 1:100 dilution of avidin-Alexa 488 (Molecular Probes) for 40 min. After adding ProLong Gold Antifade Reagent (Molecular Probes) and coveringslip, the cells were viewed with a Zeiss Axiopt microscope equipped with the appropriate fluorescence filters (Omega Optical) and a Photometrics CoolSnap HQ 12-bit camera (Roper Scientific). Cells were considered positive if they were distinctly brighter than the background and other negative cells.

Before testing dishes that contained physiologically categorized cells, a dilution series for each primary antibody was run in control dishes to determine optimum concentrations for identifying positive versus negative cells. As a negative control, competitive inhibition of the primary antibodies was performed by preadsorption of the antibody with excess synthetic blocking peptides (1 μg of peptide per 1 μg of antibody), which were supplied by Chemicon. This procedure completely prevented the immunofluorescence in all cases. Additionally, control dishes to ensure antibody specificity were tested in which the full protocols were run with the omission of applying the primary antibody, secondary antibody, or TSA kit. When testing dishes that contained subclassified cells, a control dish was also always run with the omission of the primary antibody.

Statistics

Heat-activated currents were quantified as the difference between the baseline current recorded immediately before heat pulse

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application and current peak that occurred at the end of the heat pulse (6, 15, or 20 s; Clampfit 8.2). Unless otherwise noted, data for groups of cells under various conditions are expressed as the means and SE. Tests of sensitization and the molecular basis of heat reactivity were evaluated by repeated measures on the same cell. Paired t-tests were used to evaluate the abrogation of current by TRPV1 antagonist capsazepine or TRPV1/TRPV2 antagonist ruthenium red. Tests of intracellular pathways to sensitization were made by comparing SRs calculated for treated (MAFP, H7, genistein) and untreated (DMSO/water or water vehicle) applications of the sensitization protocol. Independent t-tests were used to examine the statistical significance of comparisons. The α level was set at 0.05.

Heat current response curves were formed from peak currents evoked during a 6-s application of heat pulses from 44–58°C. Peak currents were normalized by the maximum evoked current for the subclassified cell being studied. Normalized responses were averaged across all cells in the class and plotted against the applied temperature. A sigmoidal curve was fit to the resulting distribution \[ Y = \frac{I_{\text{max}} - I_{\text{min}}}{1 + e^{(T-H50)/k}} \]. The temperature corresponding to the half-maximal response (H50) was determined from this Boltzman equation. The H50s determined from curves fit to individual cells representing nociceptor types 2, 5, and 8 were used to make statistical comparisons between cell classes.

RESULTS

Distribution of TRP proteins

We previously identified, in vitro, a number of unique classes of capsaicin sensitive cells among both small diameter (types 1, 2, and 7) and medium diameter DRG neurons (types 5, 8a, 8b, and 9). These capsaicin-sensitive groups were likely to represent distinct classes of heat sensitive Aδ and C fiber nociceptors (Petruska et al. 2000d, 2002). Because of the divergence between heat-gated and capsaicin-sensitive proteins (Benham et al. 2003; Nilius and Voets 2004; Tominaga and Caterina 2004), heat-sensitive nociceptors might also be found among the capsaicin insensitive populations that we have identified (types 4 and 6). After whole cell recordings and physiological classification, we used ICC methods to determine the expression pattern of TRPV1 and TRPV2 protein in physiologically classified DRG afferents (Fig. 1).

Consistent with the known pattern of capsaicin reactivity, capsaicin-sensitive TRPV1 was expressed in capsaicin-sensitive cell types. These included types 2 (19/20 cases; positive/total cases), 5 (23/24), 7 (3/13), and 8 (11/13). TRPV1 protein was absent in known capsaicin-insensitive type 3 and type 4
neurons. Capsaicin-insensitive TRPV2 was expressed in all type 4 neurons and was co-expressed with TRPV1 in types 5 (15/17) and 8 (10/11). Co-expression was prominent and consistent in these medium diameter cell groups. We did not identify TRPV2 in small diameter type 2, type 3, or type 7. In these labeling studies, we did not distinguish between types 8a and 8b (subphenotypes with respect to ASIC expression; Jiang et al. 2006); however, it is notable that >80% of type 8 cells examined exhibited the same pattern of dual TRPV1/TRPV2 expression. With the exception of type 7, all known capsaicin-sensitive cell types consistently expressed TRPV1. Previously, very weak capsaicin reactivity had been noted in type 7 cells (Petruska et al. 2000d).

Peripheral fields of TRP-expressing nociceptors

Previously we used DiI tracing methods to identify the tissue sites innervated by subclassified DRG neurons (Jiang et al. 2006; Rau et al. 2005). Using similar methods, we made multiple DiI injections (16 μl) into the gastrocnemius muscle and distal colon. One week later, we identified, in gastrocnemius (GM)- and distal colon (DC)-injected subjects, highly fluorescent neurons with signatures corresponding to types 5 (n = 29 and 7; Fig. 2) and type 8 (n = 9 and 33; GS and DC, respectively; Fig. 2). A number of traced neurons did not conform to any known subclassified nociceptor population. Other neurons, traced from the DC, could be classified into at least two new afferent subclasses. The properties of these newly classified neurons will be detailed in a future publication.

In limited testing, we determined that histochemical features of GM and DC type 5 neurons were consistent with those of nontraced, subclassified type 5 nociceptors we had previously characterized (Fig. 3). In both tissue sites, type 5 neurons were uniformly IB4+ and NFm+ (IB4+: 21/21 and 7/7; NFm+: 6/8 and 3/3; GM and DC, respectively) (Petruska et al. 2002). Type 8 cells exhibited a different pattern, with negligible IB4 labeling (2/7 and 0/31; GM and DC), but relatively consistent NFm+ label (2/2 and 8/13) in both tissue sites.

Heat reactivity

Signature-identified populations were examined for heat reactivity (n = 194). Potentially, a distinct physiology and pharmacology would be observed in nociceptors that expressed TRP heteromers from those that expressed homomeric TRPV1 or TRPV2. Stepped heat pulses were applied by close superfusion from a pipette positioned ~50 μm from the leading edge of the cell. Pulses were preheated to the designated temperature by a servo-controlled heater that was integral to the pipette. The heating element was located 40 mm from the pipette opening. Because the temperature of the preheated fluid declined as it flowed the final 40 mm to the cell, the exact temperature was reduced at the point of contact. During preliminary calibration experiments, we used a miniature fast thermocouple, placed at the physical location of a target cell, to gauge the mismatch between nominal and delivered temperature. Our best estimates indicated that the actual temperature of the heat pulse was reduced by about 2°C relative to the preset temperature.

Heat thresholds were defined as the applied temperature that produced >1 pA/pF of inward current during the heat step from the baseline temperature of 32°C. At the initial step from 32 to 44°C, small subthreshold heat-induced currents were observed in type 2 (0.48 ± 0.23 pA/pF; n = 8), type 5 (0.83 ± 0.34 pA/pF; n = 6), and type 8 neurons (0.93 ± 0.26 pA/pF; n = 8). Type 4 neurons exhibited weak currents at 46°C (0.28 ± 0.15 pA/pF). The heat threshold for TRPV2-expressing type 4 nociceptors was significantly higher than those of classes expressing TRPV1/TRPV2 and/or TRPV1 alone (Fig. 4C).

To characterize the range of heat reactivity of each identified TRP-expressing cell class, we presented relatively precise heat steps ranging from 44 to 58°C. In these tests, bath temperature was maintained at 32°C by an indium oxide–coated plate. Each stepped heat pulse was applied for ~6 s with subsequent pulses presented at 2-min intervals in an ascending series (2°C/step). Four cell classes, corresponding to major skin and muscle nociceptor populations, with distinct TRP expression patterns were examined extensively in this manner (types 2, 4, 5, and

FIG. 2. Cell types identified in gastrocnemius muscle and distal colon. Current signature patterns identified these cells as type 5 (A and C) or type 8 (B and D). Each panel shows overlaid current signature patterns evoked by CP1 (top trace), CP2 (bottom left trace), and CP3 (bottom right trace). Vertical scale bars are 500 pA for CP1; 20,000 pA for CP2; and 5,000 pA for CP3. Horizontal scale bars are 100 ms for CP1; 100 ms for CP2; and either 1 (A and C) or 2 ms (B and D) for CP3. Brightfield (top) and fluorescent (bottom) images are shown for each cell type. Arrow indicates DiI-positive cell that was electrophysiologically subclassified. Scale bar (D) indicates 100 μm.

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8). Heat-response curves were constructed from the normalized steady-state responses and nominal temperature of the heat pulse applied.

As indicated in Fig. 4A, there was little difference in heat-response curves in classes that expressed TRPV1 (either singly or in combination). Heat-activated currents increased steadily with temperature. There was no evidence of bimodal or distinct high-threshold heat reactivity in nociceptors co-expressing both TRP proteins. Distinct high-threshold responding would have been consistent with two independently functioning homomeric channels. In cell types 2, 5, and 8, curve fits were reliable and characterized by similar H50 (the temperature at which half-maximal ionic flux was observed; 52.4 ± 0.03, 52.4 ± 0.11, and 51.9 ± 0.44°C for types 2, 5, and 8, respectively). Although maximal heat-evoked current density trended higher in type 8, maximal currents were not significantly different in cells that expressed TRPV1 or combinations of TRPV1 and TRPV2 proteins (21.6 ± 4.2, 24.0 ± 4.2, and 34.3 ± 4.8 pA/pF for types 2, 5, and 8, respectively).

Using antagonists of TRPV1 (capsazepine, 10 µM) or TRPV2 (ruthenium red, 10–20 µM; Tominaga et al. 1998), we were able to reduce heat-evoked currents in a manner consistent with the identified TRP expression pattern (Fig. 4, D and E). After preliminary cell characterization, 52°C heat pulses were applied for 6 s. Subsequently, the antagonist was applied for 2 min by close superfusion, followed by a test heat step application that contained the antagonist. Heat currents in cells that expressed TRPV1 were reduced ~75% (type 2, P < 0.02). Cell types that co-expressed TRPV1 and TRPV2 were powerfully inhibited by either capsazepine (type 5, P < 0.002; type 8, P < 0.02) or ruthenium red (type 5, P < 0.02; type 8, P < 0.03).

Heat-response curves from individual neurons that expressed only TRPV2 (type 4) were erratic. Although we could form a reasonable curve from averaged cases (Fig. 4A), sigmoidal relationships could not be shown for individual neurons (see Benham et al. 2003). At 52°C, heat-evoked currents were weak but increased in amplitude rapidly above this temperature. Maximal currents in type 4 neurons were very large (56.1 ± 5.8 pA/pF; n = 4) and significantly exceeded those of type 2 neurons expressing only TRPV1 or in type 5 and 8 cells co-expressing both TRPs (types 2, 5, and 8; P < 0.003, 0.008, and 0.02, respectively). Consistent with TRPV2 physiology and pharmacology, heat-activated currents in type 4 cells reversed at −6.5 ± 3.0 mV (n = 3) (Bender et al. 2005; Caterina et al. 1999), were insensitive to capsazepine (n = 5), but were significantly reduced 40% by ruthenium red (10–20 µM, P < 0.003).

Heat sensitization

Sensitization of nociceptors by heat is a common property of skin nociceptors in vivo, but it has been rarely shown in vitro. We examined the capacity of physiologically classified myelinated and unmyelinated skin and muscle nociceptors to sensitize to repeated thermal events. Using methods similar to those above, classified neurons were subjected to repeated presentations of stepped heat pulses at 52°C (15- or 20-s duration). Tests were repeated at 4-min intervals. HHS was defined by a sensitization ratio (SR = Test2/Test1; measures at 15 or 20 s after application).

The small diameter type 2 hairy skin nociceptor, shown to express TRPV1 but not TRPV2 (Fig. 1), failed to sensitize to repeated heat pulses (Fig. 5A). Heat-induced currents remained vigorous and stable over the 4-min test–retest interval (656 ± 170 and 598 ± 159 pA). Because 15-s exposures could have been insufficient, we extended heat application tests to 20-s duration; however, we were still unable to detect any significant HHS in this small diameter, IB4 positive, C type class. In contrast, all nociceptive neurons that expressed or co-expressed TRPV2 could be sensitized by repeated heat application.

Hairy skin, type 4 nociceptors exhibited dramatic HHS (52°C test pulses; Fig. 5A). The ratio of posttest to pretest currents exceeded 8:1 (P < 0.03). HHS was manifested both as an increase in peak current at the test temperature and a decrease in heat response threshold. Using the 1-pA/pF criterion, threshold shifts of −4.0 ± 2.82°C (n = 8; P < 0.0001) were observed 2–4 min after the second heat test. Peak current sensitization was shown to be dependent on extracellular Ca2+. Under identical test conditions, sensitization could be completely blocked in 0 Ca2+ external solution. It should be noted.
that the exceptionally large increase of SR in type 4 cells was partly caused by the selection of test temperature. Because 52°C was at threshold for type 4 cells, the ratio was calculated against a relatively small denominator current (236 ± 23 pA). Regardless, the absolute increases in current were still impressive in this myelinated, presumptive Aδ cell class (2,001 ± 409 pA) and greatly exceeded that observed in other sensitized nociceptor classes.

Heat sensitization could also be observed in cells in both unmyelinated and myelinated nociceptors that co-expressed TRPV2 with TRPV1 (types 5 and 8). Using identical methods, IB4-positive type 5 cells consistently sensitized to repeated heat application (15-s test pulse). Sensitized currents were accompanied by modest decreases in heat thresholds (2°C; n = 3). Although we were unable to observe sensitization in the presumptive A6 type 8 cells using the 15-s heat application procedure, 20-s duration heat steps consistently produced a high SR in these nociceptors. Again, HHS was prevented by removal of Ca²⁺ from the external solution (Figs. 5B and 6C).

Pathways to heat sensitization

Several intracellular pathways have been linked to TRPV1 modulation (PKA, PKC, PLC, SRC). Intracellular pathways for TRPV2 sensitization have not been identified (but see Penna et al. 2006). We examined whether intracellular pathways were distinct in neurons expressing various combinations of TRP proteins.

After cell classification, the HHS procedure was repeated in the presence of inhibitors of serine/threonine kinases (H7, 10 μM), TRK kinases (genistein, 50 μM), or PLA2 (MAFP; 1–2 μM). Inhibitors were in all test solutions and preapplied for 2 min. In TRPV2-expressing type 4 skin nociceptors, sensitization was unaffected by H7 and MAFP, but was strongly reduced, although not prevented, by the wide spectrum TRK kinase inhibitor genistein (Fig. 6A). It is noteworthy, however, that genistein may also influence proteins unrelated to TRK kinase pathways (Hwang et al. 2003). We did not attempt to further clarify the sensitization pathway in type 4 cells. In contrast, inhibition of either PLA2 or serine/threonine kinases completely abrogated sensitization in cells co-expressing TRPV1 and TRPV2 (types 5 and 8; Fig. 6, B and C).
Discussion

Physiological signatures were used to classify small and medium diameter capsaicin-sensitive and -insensitive nociceptors of rat DRG. The molecular basis and plasticity of heat reactivity was determined for C and putative Aδ nociceptors that innervated skin, muscle, and colon. We observed that 1) distinct classes of nociceptors differentially expressed TRPV1 and/or TRPV2 protein; 2) TRPV2 was co-expressed with TRPV1 in some medium-sized cells and the presence of TRPV2 was required for HHS; 3) molecular pathways to heat sensitization were Ca²⁺ dependent; and 4) downstream effectors of HHS (serine/threonine kinases, phospholipase A2, and possibly protein tyrosine kinases) varied with the pattern of TRP expression.

Heat sensitization in TRP-expressing nociceptors

Using traditional crushed end recordings, nociceptor HHS can be shown in several species (see Introduction). There are clear parallels to heat hyperalgesia in humans (LaMotte et al. 1991, 1992; Schmelz and Kress 1996; Simone et al. 1987). Although most demonstrations of HHS are in cat and monkey, attempts to study the molecular basis of HHS have been exclusively conducted in dissociated rat DRG. Rat skin is innervated by both C fiber and Aδ mechanoeheat nociceptors (Leem et al. 1993; Lynn and Carpenter 1982; Lynn and Shahanbeh 1988; Seno and Dray 1993; Szolcsanyi 1987; Szolcsanyi et al. 1988; Yeomans and Proudfit 1996), and both families have exhibited forms of heat sensitization (Kress et al. 1992; Lynn and Carpenter 1982; Lynn and Shahanbeh 1988). Although observations of HHS in Aδ nociceptors are very rare, skin heating methods may have contributed to a selection bias against Aδ populations (Yeomans and Proudfit 1996).

Initial reports suggested that both of the major heat-gated TRP proteins of the rat would sensitize with repeated heat application in host cells (Caterina et al. 1999), yet numerous laboratories, examining small- and medium-sized capsaicin-sensitive and -insensitive cells, have been unable to reproduce HHS in adult rat nociceptors in vitro (Cesare and McNaughton 1996; Greffrath et al. 2001, 2002; Kirschstein et al. 1999; Nagy and Rang 1999; Schwarz et al. 2000; Vyklicky et al. 1999). One exception is a reported instance of HHS in neonatal rat DRG, where outcomes were attributed to high temperature membrane/protein damage (Lyfenko et al. 2002). A second report of HHS, in medium-sized cells, was limited to only three cases. Moderate threshold shifts were observed in “high heat threshold” capsaicin-insensitive cells (Ahluwalia et al. 2002) that may have been Aδ nociceptors. These outcomes lend credence to the notion that HHS, in vivo, requires the release of algescics from surrounding tissue. Direct application of pro-inflammatory agents readily produces HHS in vitro (see Introduction). Despite these negative findings, there is evidence that ionophore-induced Ca²⁺ entry can produce HHS in isolated capsaicin-sensitive neurons (Kress and Distler 2004).

Given that cell selection methods may vary from laboratory to laboratory, we examined well-defined populations of known skin nociceptors for the capacity to display HHS. We were unable to produce heat sensitization in a small diameter C nociceptive class that innervated superficial tissue. Although we did not quantify Ca²⁺ entry, the extended heat test intervals (≥20 s) ensured massive increase in intracellular Ca²⁺. We did observe that other C fiber nociceptors could exhibit HHS (type 5), but these were medium-sized cells that innervated muscle or colon and were not associated with skin. Although our experiments, using physiological stimuli, were unable to show an HHS in C type skin nociceptors, exclusive agent mediation
of the phenomenon in skin is not certain. Because classification of skin nociceptors is not comprehensive, the possibility remains that additional C type skin nociceptors will be identified and that these will exhibit HHS, in vitro, independent of the intervention of algesic substances.

Medium and large diameter DRG cells contain important nociceptor populations (Djouhri and Lawson 2004; Fang et al. 2005), and all HHS capable cells in our studies were from the medium diameter pool. Consistent with the observations of Ahluwalia et al. (2002), we identified medium-sized neurons that exhibited HHS (type 4 and type 8). Using a variety of evidence, we identified these classes as presumptive Aδ skin nociceptors that depended on Ca²⁺ entry and TRPV2 to initiate sensitization (see also Petruska et al. 2000d, 2002). It is likely that the failure to observe HHS in vitro has been caused, in part, by cell selection procedures that focused experiments mainly on small diameter cells presumed to represent C type nociceptors. It is also possible that relatively low temperatures or short application times were not potent enough to produce Ca²⁺ entry sufficient to initiate phosphorylation of suitable substrates if that substrate was present in cells targeted for study (TRPV2). We found that Aδ populations seemed to be more prone to this form of plastic behavior because of the presence of TRPV2 in Aδ phenotypes. However, the simple presence of TRPV2 was not sufficient to guarantee HHS. Some nociceptor phenotypes that expressed TRPV2 would not sensitize to a 52°C stimulus shorter than 20 s in duration (type 8); other phenotypes had the capacity to sensitize with a 15-s long stimulus (type 4 and 5). The molecular basis for this distinction was not apparent but certainly suggested that cell selection had important implications for the success of a given thermal test procedure when a suitable TRPV2-expressing cell was chosen for study.

Our evidence suggests that, regardless of myelination status, the capacity to develop HHS is dependent on the expression of TRPV2. Histochemical and other forms of evidence have indicated that the major heat reactive TRP proteins co-localize in rat DRG and are capable of forming functional heteromeric channels (Greffrath et al. 2003; Hellwig et al. 2005; Liapi and Wood 2005; Rutter et al. 2005). The physiological consequences of co-localization have not been determined in detail (Caterina et al. 1999). Assuming that functional TRP heteromerization occurred in cell types 5 and 8, we conclude that the basal physiology and pharmacology of such channels resembled the physiology and pharmacology of TRPV1 homomers. Although homomeric TRPV2 is not antagonized by capsaicin (Caterina et al. 1999), either capsazepine or ruthenium red effectively antagonized heat-activated currents of cells expressing putative TRP heteromers. Heat-evoked currents in type 2 (TRPV1) and types 5 and 8 (TRPV1 + TRPV2) had...
thresholds similar to those reported for heterologously expressed TRPV1 (Caterina et al. 1997; Tominaga et al. 1998). Heat-response curves for TRPV1 expressing type 2 cells were indistinguishable from those of types 5 and 8 (Fig. 4A). The important contribution of the TRPV2 subunit seemed to be the capacity to confer HHS. High heat application (15 or 20 s) could not sensitize capsaicin sensitive nociceptors to subsequent heat pulses in the absence of TRPV2 expression (type 2 cells). Other capsaicin sensitive C family nociceptors that expressed TRPV2 could exhibit HHS.

Multiple intracellular pathways have been linked to algesic-initiated heat sensitization. Few mechanisms for TRPV2 regulation have been identified (Penna et al. 2006). In contrast, a wide variety of intracellular paths up-regulate TRPV1 activity. These include phosphorylation by PKC (Cesare et al. 1999; Chuang et al. 2001; Dai et al. 2004;olah et al. 2002; Sugiyama et al. 2002; Tominaga et al. 2001), PLK (Bhave et al. 2002; De Petrocellis et al. 2001; Rathee et al. 2002), and protein tyrosine kinases (Jin et al. 2004; Obreja et al. 2002; Zhang et al. 2005), as well as the binding of downstream metabolites of PLA2 (Carr et al. 2003; Hwang et al. 2000; Shin et al. 2002). Synergism between multiple convergent pathways has been implicated in some reports (Chuang et al. 2001; Premkumar et al. 2004). It was noteworthy that blockade of either serine/threonine or the PLA2 path completely abrogated sensitization in TRPV2 co-expressing cell classes. It is possible that both pathways must be engaged to produce HHS through a Ca2+-dependent autosensitization. The influx of extracellular Ca2+ can induce a heat sensitization indirectly through activation of Ca2+-dependent protein kinases and lipases (Ahluwalia et al. 2003; Distler et al. 2003; Firmer et al. 2006; Guenther et al. 1999; Kress and Distler 2004; Kress and Guenther 1999; Vellani et al. 2001). This is the most likely path for HHS in cells that co-expressed TRPV1 and TRPV2. Skin nociceptor phenotypes, expressing only TRPV2, sensitized through a distinct pathway that might have depended on protein tyrosine kinases. Although we did not examine this pathway in detail, inhibition of PKA and PKC did not impair HHS in type 4 cells. Regardless, the route to HHS had a distinct molecular basis in this nociceptive class.

Preparations of isolated peripheral afferent neurons offer ideal opportunities to study the molecular basis of thermal and chemical transduction and their plasticity. In patch-clamp experiments, stimulus control is excellent, and outstanding signal-to-noise ratios are routinely achieved. Dissociated neuron preparations can provide a powerful means to test hypotheses related to the development and maintenance of chronic inflammatory and neuropathic pain conditions. However, it is often difficult to interpret experimental outcomes between laboratories with otherwise skillful experimental designs. Nociceptors are a highly complex family of afferents, and little effort is made to standardize methods by which cells are chosen for study. Laboratories practice inhered cell selection methods that cannot be communicated as a specific procedure. A given cell chosen for study may be C type, Aβ, or Aδβ, may innervate deep or superficial tissues, and may express highly diverse sets of receptors and distinct intracellular regulatory pathways that will be reflected in observed capacities. The failure to form known sets of well-defined nociceptor phenotypes with known capacities substantially impairs testing of well-defined hypotheses and the generalization of experimental outcomes.

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REFERENCES


