TRPV1b: a Functional Human Vanilloid Receptor Splice Variant

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ABSTRACT

TRP (transient receptor potential) genes encode a family of related ion channel subunits. This family consists of cation-selective, calcium permeable channels that include a group of vanilloid receptor channels (TRPV) implicated in pain and inflammation. These channels are activated by diverse stimuli including capsaicin, lipids, membrane deformation, heat, and protons. Six members of the TRPV family have been identified, differing predominantly in their activation properties. However, in neurons, TRPV channels do not account for the observed diversity of responses to activators. By probing human and rat brain cDNA libraries to identify TRPV subunits we identified a novel human TRPV1 RNA splice variant, TRPV1b, which forms functional ion channels that are activated by temperature (threshold ~ 47°C), but not by capsaicin or protons. Channels with similar activation properties were found in trigeminal ganglion neurons, suggesting that TRPV1b receptors are expressed in these cells and contribute to thermal nociception.

INTRODUCTION

Mammalian homologs of the Drosophila TRP channel gene encode a family of at least 20 structurally and functionally related ion channel proteins (Benham *et al.*, 2003; Gunthorpe *et al.*, 2002; Montell *et al.*, 2002; Patapoutian *et al.*, 2003). This family consists of widely expressed cation-selective, calcium permeable, ion channels that are expressed in both excitable and non-excitable cells (Caterina and Julius, 2001; Cortright and Szallasi, 2004; Peier *et al.*, 2002; Agopyan *et al.*, 2003). Members of this family can be activated by diverse stimuli including chemical irritants, protons, lipids, membrane deformation, and noxious cold and heat. These channels appear to be structurally related to the large family of 6 transmembrane domain channels, characterized by a pore-forming loop between the 5th and 6th transmembrane domains (Caterina and Julius, 2001; MacKinnon, 1991). Functional diversity can be achieved within this gene family because proteins assemble as either homomeric or heteromeric tetramers (Kuzhikandathil *et al.*, 2001; Jung *et al.*, 2003). The functions of TRPV channels are generally unknown, but at the periphery members of the TRPV subfamily serve, in part, as thermal receptors.

The most thoroughly investigated of the TRPV receptors is TRPV1. Human, avian, murine as well as other forms have been identified, and share a marked sequence homology. When heterologously expressed in cells or *oocytes*, TRPV1 responds to capsaicin, protons, or temperatures > $43^{o}C$ with characteristics that are similar to those found in nociceptive neurons (Tominaga *et al.*, 1998; Liu and Simon, 2000). To date, TRPV1 is the only member of the TRPV family is activated by capsaicin, although at least 5 other TRPV1 variants have been described and are referred to as TRPV2 through TRPV6 (Patapoutian *et al.*, 2003; Gunthorpe *et al.*, 2002).

It is likely that further diversity of receptor properties may be achieved by the expression of alternatively spliced variants of TRPV receptors (Wang *et al.*,

2004), although this has not been previously been demonstrated for human TRPV channels (Gunthorpe *et al.,* 2002; Schumacher *et al.,* 2000a,b) . Such channel isoforms are functionally important as they provide for a diversity of cellular responses to receptor activation, and furthermore, provide mechanistic information about the location of the different binding and gating domains in TRPV channels. However, the diversity of neuronal responses to known TRPV activators is greater than can be accounted for by known TRPV receptors and receptor variants. Hence, it is likely that other TRPV receptors remain to be identified. Here we report the identification of the first human TRPV1 splice variant, which we have named hTRPV1b. This receptor differs from hTRPV1 in that it contains a 60 amino acid deletion within the N-terminus, corresponding to an alternatively RNA spiced form in which exon 7 has been deleted (Schumacher et al. 2000a). When expressed in *Xenopus* oocytes, hTRPV1b forms functional channels that are activated by noxious heat (threshold 47°C), but is not activated by capsaicin or protons. Furthermore, a channel with very similar properties is present in nociceptive neurons.

MATERIALS AND METHODS

Care of animals conformed to standards established by the National Institutes of Health. The Duke University IACUC approved all animal protocols.

Cloning of hTRPV1b fragments

Nested-PCR was used to identify novel TRPV1 variants. Initially, a number of degenerate primer pairs were created to amplify 800-900 bp fragments covering the complete coding sequence of TRPV channels. The identification of a number of N-terminal sequence variants resulted in the designing of four nested, degenerate primers to amplify either the N-terminal half of TRPV1 or the C-terminal half of the receptor. Primers were designed using GCG software (Accelrys, San Diego) based on the sequences of rat TRPV1, hTRPV2 (Caterina *et al.*, 1999) and mouse TRPV2 (Kanzaki *et al.*, 1999). Primer-1 (5'- CTG CAG AAC TCC TGG GAG ACG GCC GAC ATC -3') and primer-2 (5'- TCC AGG TCG CCC ATG CCG ATG GTG AAC TTG -3') were used as outer primers for the first round of PCR. Primer-3 (5'- AGG GAC TCG GTG GGC AAC ACG GTG CTG CAC -3') and primer-4 (5'- TGT GCG ACG TGG ACT CAG ACG GCA GGG AGT -3') were used as inner primers for the second round of PCR. The amplification program was 95°C×5min, 95°C×30s; 65°C×30s; 72°C×1 min at 30 cycles/72°C×10 min using a thermal cycler.

Cloning of the complete hTRPV1b and rTRPV1b receptor from cDNA libraries

To recover the N and C termini of both TRPV1 and TRPV1b sequences from a human brain cDNA library (Life Technologies, Inc.), or from a rat brain cDNA library created in house, primer pairs were created from within each of the amplified N- and C-terminal fragments, and from within flanking sequences of the cDNA library cloning vector (PCMV Sport). Primers 180NFO (5'- GGA GGC GGG GAG ACC ACT CTT CTC CCA CAC -3') and 180 NRO (5'- TCG GTT CAG CGG

CTC CAC CAA GAG CAT GTC -3') were used for the first round PCR of the N-terminus. Primers 180 NFI (5'- CAG CTC TCC CTT CGA GTA GCA ACC GCC TTC -3') and 180 NRI (5'- GTG GCG ATT CCC GAT CTT CCC GGT CCC AGC -3') were used for the second round of N-terminus PCR. Primers 180CFO (5'-AAC AAG AAG GGA ATG ACG CCG CTG GCT CTG -3') and 180 CRO (5'- TCA GGA GTT CGA GAC CAG CCT GGC CAA CAT -3') were used for the first round PCR of the C-terminus. Primers 180 CFI (5'- GTC GGG ACC GGG AAG ATC GGG AAT CGC CAC -3') and 180 CRI (5- TCC CAG CTA CTT GGG AAG CCG AGG CAG GAG -3) were used for the second round of PCR. The amplification program was 95°C×5min, 95°C×30s; 55°C×30s; 72°C×2 min at 30cycles/72°C×10min using a thermal cycler. The N and C terminal fragments recovered from the PCR amplification were then subcloned into the PCR2.1 vector (Invitrogen), and sequenced in both directions.

Oocyte Electrophysiology: Two-Electrode Voltage Clamp

Oocyte Preparation and Electrophysiology: Female *Xenopus laevis* were obtained from Nasco (Fort Atkinson, WI). Oocytes were surgically removed and defolliculated using collagenase as described previously (Welch et al. 2001). They were extensively rinsed with Ca^{2+}-free OR2 (oocyte Ringers) solution and then with Na-105 solution consisting of (in mM): 105 NaCl; 5.0 KCl; 2.0 $CaCl_2$; 5.0 HEPES and adjusted to pH 7.6 with NaOH. Stage V-VI oocytes were separated into individual dishes and stored overnight in Na-105 solution at 17°C. Cells were injected with 0.5-50 ng cRNA, prepared from the hTRPV1b cDNA using T7 RNA polymerase (mMessage mMachine, Ambion, Austin, TX), and two-electrode voltage clamp recordings performed 7-10 days later (Welch et al., 2001). Oocytes were constantly superfused with a Ca^{2+}-free buffer containing (in mM): 90 NaCl; 1 KCl; 2.4 $NaHCO_3$; 1 $MgCl_2$; 10 HEPES; 0.1 $BaCl_2$ that was adjusted to pH 7.4 with NaOH. Recording electrodes were pulled from 1.5 mm glass (World Precision Instruments) using a programmable puller (model P-87, Sutter Instrument Company, Novato, CA). Electrodes were filled with 3 M KCl and had resistances of 1–2 MΩ. Currents were digitized at 2 kHz, filtered at 1

kHz, and were analyzed off-line using Clampfit (Axon Instruments) and Origin (Microcal) software.

Oocytes were voltage-clamped near their chloride equilibrium potential (approximately -25 mV) using an Axon 2A amplifier (Axon instruments, Forster city, CA) to reduce the contribution of endogenous Ca^{2+}-activated Cl^- channels. MES was used as a buffer for experiments that were performed at lower pH values (Welch et al., 2001). Only a single stimulus was applied to each oocyte. For experiments testing thermal responsiveness, the temperature of the recording solution in chamber was controlled with a modified in-line SH-27A heater and a TC-324B temperature controller (Warner Instruments, Hamden, CT). Threshold temperatures were evaluated by a determination of an abrupt change in current with increasing temperatures (Welch et al., 2001). All other measurements were performed at 23-24°C. For each condition, experiments were repeated in separate oocytes three to six times. All data are expressed as means ± SD.

Studies with sensory trigeminal ganglion (TG) neurons

Trigeminal ganglia were dissected aseptically and collected in modified Hank's Balanced Salt solution (mHBSS) as described previously (Liu and Simon, 2000). After washing in mHBSS, the ganglia were diced into small pieces and incubated for 30-50 min at 37 °C in 0.1% collagenase (Type XI-S) in mHBSS. Individual cells were dissociated by triturating the tissue through a fire-polished glass pipette, followed by a 10 min incubation at 37 °C in 10 μg/ml DNase I (Type IV) in F-12 medium (Life Technologies, Gaithersburg, MD). After washing three times with F-12, the cells were cultured in DMEM supplemented with 10% fetal bovine serum. The cells were plated on poly-D-lysine coated glass coverslips (15 mm diameter) and cultured overnight at 37°C in a water saturated atmosphere with 5% CO_2. Only neurons without visual processes or with short processes were used.

For whole-cell voltage-clamp experiments glass pipettes (R-6 borosilicate, Drummond Scientific Company, Broomall, PA.) exhibiting resistances between 1-

2 MΩ were used. Recordings were obtained using an Axopatch-200B patch clamp amplifier (Axon Instruments, Foster City, CA) and the output was digitized with a Digidata 1322A converter (Axon Instruments). Whole cell patch-clamp experiments in which capsaicin (1 μM), acid (pH4.9) and thermal responsiveness were investigated at a holding potential of –60 mV. Temperatures were controlled using the apparatus described above. The solutions contained (in mM): Extracellular: 145 NaCl , 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 D-glucose, 10 HEPES, pH 7.4; Intracellular 140 Cs aspartate, 1 $CaCl_2$, 2 $MgCl_2$, 10 EGTA, 10 HEPES, 5 K_2-ATP, pH 7.3.

Single-cell RT- PCR

Single-cell mRNA was harvested from TG neurons. Briefly, patch-clamp electrodes were filled with RNase-free intracellular buffer. After whole-cell electrophysiological recordings, the cytoplasm was harvested via the patch-pipette under visual control while continuously maintaining the gigaseal to prevent contamination with extracellular fluid. The pipette contents were then expelled into a sterile nuclease-free, 0.5 ml thin-walled microfuge tube, and single-cell mRNA and cDNA synthesis was performed using the Cells-to-cDNA II Kit (Ambion,TX) (Liss, 2002). For first-strand cDNA synthesis either random primers or oligo(dT) primers were used. Primer pairs for TRPV1 and TRPV1b amplification were as follows; primer-'RO': TGA GGG ATG GTC GCC TCT GCA GGA AAT ATT; primer-'FO': CGT GCA CCA ACC AGG TGG CCA TTG TGA AGT; primer-'RI': CTG ACA CAG ACA AGA TCT CTC GGG TGA CTC; primer-'RO': CTG CAG AAC TCC TGG CAG CCT GCA ATC. The amplification program was 94°C for 5 min, then 35 cycles at 94°C for 30s; 55°C for 30s; 72°C for 30s followed by 72°C for 5 min using a thermal cycler.

RESULTS

Cloning of hTRPV1b

To recover full-length genes related to TRPV1 we designed nested degenerate primers based on the sequences of rat TRPV1, human TRPV2 (Caterina *et al.*, 1999) and mouse TRPV2 (Kanzaki *et al.*, 1999). These degenerate primer sets were used to PCR amplify approximately 900 base-pair DNA fragments covering the complete coding sequence of TRPV1 by using a human brain cDNA library or a human DRG library. For N-terminal primer pairs corresponding to an N-terminal region just preceding TM1, PCR experiments consistently produced multiple amplification products from both libraries (Fig. 1A). To facilitate the rapid identification of all N-terminal TRPV1 variants the cDNA libraries were screened with a second set of primers designed to amplify either the C-terminal half or the N-terminal half of TRPV1. These experiments always resulted in the generation of multiple PCR products from the N-terminal amplification (data not shown). Sub-cloning and sequencing these N-terminal fragments revealed two different sequences, the longer of which exactly corresponded to the TRPV1 sequence (Hayes *et al.*, 2000). More interestingly, a shorter fragment consistently amplified from both human libraries is homologous to human TRPV1 except for a 180 base-pair deletion corresponding to a 60 amino acid deletion some 25 amino acids N-terminal to the putative first transmembrane domain (Figs. 1 and 3). To minimize the possibility of PCR artifacts a further series of experiments were performed in an attempt to recover the N- and C- termini corresponding to the 60 amino acid deletion in this hTRPV1 variant (which we named hTRPV1b). To this end, hTRPV1b-specific primers were generated and used to amplify the complete open reading frame under conditions that *do not* amplify TRPV1. The utilization of cDNA cloning vector flanking sequences for the second PCR primer pairs resulted in the amplification of both N-terminal and C-terminal fragments which on sequencing revealed perfect overlap with the hTRPV1b-fragment, and an open reading frame identical to that of hTRPV1 at both termini. Overlapping fragments were assembled and

sub-cloned into the expression vector-PCR2.1 (Invitrogen). To examine whether a similar TRPV1b variant can be observed in other organisms, similar 'scanning PCR' experiments were performed using a rat brain cDNA library. Amplifying the same domain that led to the identification of hTRPV1b, resulted in the cloning of a similar rat TRPV1 variant (rTRPV1b) that is truncated by 57 amino acids as compared to the 60 amino acid deletion in the human channel.

To further reduce the possibility that the human TRPV1b sequence is a PCR artifact, a human cDNA library was probed using a TRPV1 fragment immediately surrounding the deletion site. This led to the identification of a number of clones some of which corresponding to full-length TRPV1, and some to a shorter variant. Sequencing these variants resulted in the identification of an open reading frame predicting a protein of 779 amino acids identical to the previously identified hTRPV1b sequence, with the exception of the same 60 amino acid deletion within the N-terminus as observed by PCR (Figs. 1 and 3). This variant has 92.8% identity to the predicted human TRPV1 sequence, and 78.7% identity to the predicted rat TRPV1 protein (Fig. 1B). For comparison, the human and rat sequences share 85.8% identity. As expected, the predicted membrane topology is similar to that of human and rat TRPV1 receptors, and other major features such as two of the three predicted ankyrin repeats in the N-terminal region and a predicted N-linked glycosylation site in the putative pore region are retained (Fig. 2).

Functional response of human TRPV1b: capsaicin, acid and temperature

The ability to isolate the full-length TRPV1b open reading frame from a human brain cDNA library confirms that hTRPV1b is translated into mRNA, and hence likely into protein. To examine whether TRPV1b codes for a functional protein, we expressed TRPV1b in *Xenopus* oocytes along with TRPV1 from both human and rat (Welch *et al.*, 2001) and measured current responses to capsaicin, pH5, and heat (Welch *et al.*, 2001). Whereas the application of 10 μM capsaicin, or pH5 evoked currents in oocytes injected with TRPV1, they did not

evoke currents in oocytes injected with TRPV1b (Fig. 4). Furthermore, neither 50 μM capsaicin nor lowering the pH to 4.0 was able to activate TRPV1b channels (data not shown). In agreement with previous studies (Davis et al.,2000), we observed that hTRPV1 was activated by temperatures about 44 ± 1.7°C (n = 6, Fig. 4). Surprisingly, we found that TRPV1b receptors were also activated by temperature ramps by evoking inward currents with threshold temperatures of 47 ± 1.2°C (n=8, Fig. 4). Measurements of the I-V relationship, at approximately 47°C, revealed that hTRPV1b exhibits weak outward rectification, and has a reversal potential close to 0 mV (0.2 ± 0.7mV; n = 4; Fig. 5A).

To determine whether the thermal threshold temperature is altered either by the presence of 10 μM capsaicin, or by lowering the pH to 5.0 we examined the activation temperature of hTRPV1b in the presence of these other TRPV1 activators. Both rat and human TRPV1 channels are activated at lower temperatures at pH 5.0 or in the presence of 10 μM capsaicin (Tominaga *et al.*, 1998; Hayes *et al.*, 2000). For hTRPV1b channels neither agonist was able to significantly change the threshold temperature (heat: 47±1.2°C (n=8); capsaicin + heat: 45.8 ± 1.6°C (n=3); pH5.0+heat: 46.2 ±1.8°C (n=4)).

Additional experiments were performed to measure the effects of near maximal concentrations of TRPV1 agonists such as olvanil (10 μM), PPAHV (1 μM), resiniferatoxin (1 μM), and piperine (100 μM) on the activation properties of hTRPV1b (Liu et al., 2000; Cortright and Szallasi 2004). None of these compounds was able to activate hTRPV1b receptors on their own (data not shown). Further experiments were designed to examine whether the thermal response of hTRPV1b can be inhibited by the TRPV1 receptor antagonists capsazepine (CPZ) or ruthenium red (RR). We found that in both hTRPV1 and hTRPV1b 10 μM RR was able to completely inhibit the heat-activated current (Figure 5B). In contrast, CPZ (10 μM) was without effect in inhibiting the thermal responses evoked by hTRPV1 or hTRPV1b (data not shown).

Additional experiments were designed to determine whether channels with properties of TRPV1b could be identified in cultured TG neurons. Since TG neurons express a number of ion channels that respond to acidic solutions, such as ASICs, TRPV1, and some potassium channels (Berg *et al.*, 2004; Kress and Zeilhofer, 1999; Liu and Simon , 2000), it was not obvious that it would be possible to identify a sufficient number of cells that *do not* respond to either acidic solutions or to capsaicin but that *do* respond to nociceptive temperatures. Consequently, for these experiments we used suprathreshold concentrations of capsaicin (1 μM) and heat (to 50$^{\text{o}}$C) to test whether we could obtain responses similar to those seen in oocytes. In 53 experiments in which these stimuli were applied, 5 neurons were identified that were insensitive to capsaicin but were activated by temperatures with thresholds near 47$^{\text{o}}$C. One example of such a neuron is shown in Figure 6A. The remaining 48 neurons tested expressed a diversity of capsaicin and heat-activated currents (not shown).

Single cell RT-PCR experiments were performed to independently test whether TG neurons express TRPV1b transcripts, and whether such expression correlates with temperature-activated currents. Of the 53 neurons recorded using patch clamp, the messages from 21 neurons were amplified using two sets of TRPV1- and TRPV1b-specific primers. The predicted size of the PCR products is 659 bp for TRPV1 and 478 bp for TRPV1b. We found that 18 of the 21 capsaicin-responsive TG neurons probed gave rise to two PCR amplification products, one approximately 660 bp and one approximately 480 bp (see Fig. 6B). Further support for the correlation between temperature activated currents in these cells and the presence of TRPV1b transcripts is the finding that the 3 neurons that were *only* responsive to heat, but not to capsaicin were also the only 3 neurons that *only* gave rise to a single PCR amplification product of approximately 480 bp corresponding to the TRPV1b sequence (Figure 6B, lane 10).

DISCUSSION

hTRPV1b is a novel TRPV receptor

Here we report the cloning and functional characterization of a new human TRPV receptor, TRPV1b. Sequence analysis, and data from a TRPV1 genomic analysis (Xue *et al.*, 2001), indicates that hTRPV1b is an alternatively spliced isoform of hTRPV1. The boundaries marking the 60 amino acid deletion are aligned with previously identified exon-intron junctions (Fig. 3), the first of these at the 3' end of exon 6, and the second at the 5' end of exon 8 (Xue *et al.*, 2001). Hence, the 60 amino acid deletion in TRPV1b represents an alternatively spliced form in which exon 7 is removed. This exon corresponds to a protein domain between the N-terminus and the first transmembrane domain (TM1), thus the predicted transmembrane arrangement is unlikely to be altered (Fig. 3).

Examining the functional properties of hTRPV1b receptors revealed that, in contrast to the unspliced TRPV1 channel (Caterina *et al.*, 1997; Davis, 2000; Hayes *et al.*, 2000), these channels are not activated by capsaicin, acidification, or several other standard TRPV1 agonists. Although these channels are functionally similar to other thermally-sensitive TRPV receptors: TRPV3 (22-36°C), TRPV4 (27-42 ^{o}C), and TRPV2 (~52°C) (Caterina *et al.*, 1999; Smith *et al.*, 2002; Jordt et al., 2003), the protein sequence of hTRPV1b is only 32.8%, 40.3%, and 36.1% homologous to TRPV3, TRPV4, and TRPV2, respectively. The defined spectrum of threshold temperatures that activate TRPV channels, and the very steep temperature dependence of activation (Patapoutian *et al.*, 2003; Nagy and Rang, 1999) allows the generation of an threshold temperature activation series - TRPV3<TRPV4<TRPV1 (44°C)<TRPV1b (47°C)<TRPV2. It therefore follows that, a possible physiological role for TRPV1b is that of a transducer for noxious temperatures greater than the temperature at which TRPV1 receptors are activated.

Structure/function implications of hTRPV1 properties

For TRPV1 the proton binding site is extracellular, whereas the binding site(s) for capsaicin (RTX) are intracellular (Welch *et al.*, 2001; Jordt and Julius, 2000; Jordt et al., 2002). It is instructive that the removal of an intracellular stretch of amino acids at the N-terminal renders hTRPV1b receptors insensitive to both protons and capsaicin. The RTX binding site (resides within the TM2-TM3 domain (Jordt et al., 2002). Truncation and mutation strategies using rTRPV1 revealed that truncations at either the N- or C-terminus abolish RTX binding and/or responses to capsaicin (Jung *et al.*1999; Liu *et al.*, 2004). Single amino acid deletions or mutations at N-terminal or C-terminal sites were also found to reduce capsaicin-activated currents, RTX binding, decrease proton-induced currents, but not alter responses to noxious heat (Kuzhikandathil *et al.*, 2001; Jung *et al.*, 2003). Since hTRPV1b receptors have identical TM2-TM3 domains as hTRPV1, these N- and C-terminal sites are necessary, but not sufficient, for the generation of capsaicin or proton-induced channel gating. Overall, these data show that several regions of the protein contribute to agonist binding and the intramolecular transduction of this signal resulting in an activated channel. In the absence of structural information about TRPV1 channels it is not possible to differentiate between models in which hTRPV1b cannot be activated by capsaicin or protons due to an alteration in the ligand-binding domain, or models in which ligand binding is unchanged, but binding can no longer be transduced into channel activation.

Properties of TRPV splice variants

To date there are no reports of human TRPV variants, however, in rats, an N-terminal deletion splice variant of TRPV1, VR.5'sv, has been identified (Schumacher *et al.*, 2000a). This variant lacks the majority of the N-terminus (amino acids 1-308 and 345-404) and does not form functional channels. The smaller of the two deletion domains corresponds to the human exon 7 sequence (Schumacher *et al.*, 2000a; Xue *et al.*, 2001), indicating that exon 7 is

replaceable in both rats and humans. More recently, two mouse splice variants, mTRPV1α and mTRPV1β, were identified; mTRPV1β arising from an alternative use of an intron recognition site within exon 7 (Wang *et al.*, 2004). When it is heterologously expressed in HEK293 cells or *Xenopus* oocytes mTRPV1α (but not mTRPV1β) could be activated by capsaicin, acid, as well as other TRPV1 agonists. This suggests that mTRPV1α is activated in a similar manner to other TRPV1 channels. In contrast, the mTRPV1β subunit that is characterized by a 10 amino acid deletion near the N-terminus, acts as a naturally occurring dominant negative regulator. This is evidenced by the reduced responses that are evoked when it is co-expressed with functional TRPV1 subunits. Finally, a TRPV1 splice variant was identified in rat taste receptor cells that is responsible for the amiloride-insensitive salt taste (Lyall et al ., 2004).

Capsaicin or acid do not sensitize thermal responses of hTRP1b

The thermal responses of TRPV1 receptors may be decreased by capsaicin, protons and intracellular signaling molecules (Tominaga *et al.*, 1998; Prescott and Julius, 2003). Such data indicates that protons and capsaicin are activators of gating, and also enhance receptor sensitivity to heat, vanilloids, or protons (Tominaga *et al.*, 1998; Liu and Simon, 2000). This latter effect is consistent with a model in which each of the agonists can destabilize channel closed states, biasing dose-response curves towards open states (Hui *et al.*, 2003). It is evident that the differences in amino acid composition between hTRPV1 and hTRPV1b (Fig. 2) are sufficient to prevent this conformational change. Our data imply that during inflammation, where the pH is reduced, hTRPV1b may not be sensitized (as is TRPV1) through this pathway, and for this reason may serve predominantly as a thermal receptor for nociceptive heat stimuli. We of course cannot eliminate the possibility that other endogenous proinflammatory compounds could sensitize the receptor so that it may be important in inflammatory processes.

Heat activated hTRPV1 and hTRPV1b currents are inhibited by RR but not by capsazepine

The TRPV1 antagonist, capsazepine, has been shown to inhibit responses to acid and capsaicin both in sensory neurons and in TRPV1 expressing cells (Liu and Simon, 2000; Tominaga *et al.*, 1998; Caterina *et al.*, 1997). Since protons activate the channel from the outside and capsaicin from the inside, it is unlikely that capsazepine acts only as a competitive inhibitor of capsaicin binding to its receptor site, but rather that capsazepine is acting at other site(s) stabilizing a closed state of the channel (Hui *et al.*, 2003). There is disagreement as to whether capsazepine inhibits thermal responses in neurons and in cells expressing TRPV1 receptors. In COS and HEK293 cells with expressed TRPV1 receptors, 10 μM capsazepine inhibited 34% (Savidge *et al.*, 2001; McIntyre *et al.*, 2001) and 90% (Tominaga *et al.*, 1998) of the heat response, respectively. In two investigations with sensory neurons, capsazepine was shown to be a partial antagonist (~ 30%) of heat-evoked currents (Liu and Simon, 2000; Savidge *et al.*, 2001; Kirschstein *et al.*, 1999), but, in a third stud, it did not inhibit heat-evoked currents (Nagy and Rang, 1999). Here we found that 10 μM capsazepine did not inhibit the thermally-activated currents in oocytes evoked by either hTRPV1 or hTRPV1b receptors. The differences in the reported effects of capsazepine in the different cells and conditions are not understood.

In contrast, there is uniform agreement regarding the inhibition heat-activated currents by ruthenium red (Tominaga *et al.*, 1998; Savidge *et al.*, 2001; Jerman *et al.*, 2000). In this report we also show that this pore-blocking antagonist inhibits heat responses in both channel variants (Fig. 5).

Evidence for hTRPV1b type channels in sensory neurons

Previous studies in sensory neurons support the presence of functional TRPV1b-type channels. For example, it was found that approximately 8% (4/49)

of DRG neurons containing heat-activated currents were insensitive to capsaicin (Greffrath *et al.*, 2001). In a different study, approximately 14% (5/37) of TRPV1-expressing cells were found to be only activated by heat (Savidge *et al.*, 2001). Finally, in a single channel patch-clamp study of rat DRGs neurons, responses were found that were activated at nociceptive temperatures but were capsaicin-insensitive (Nagy and Rang, 1999). Consistent with these results, we have found current responses in TG neurons that closely resemble those of TRPV1b in that they are not activated by capsaicin (5/53 cells), but are activated by noxious temperatures (Fig. 6A).

Furthermore, when PCR was used to amplify TRPV1 and TRPV1b channel sequences from individual TG neurons expressing only capsaicin-insensitive, heat-activated channels, it was found that they all expressed the TRPV1b isoform (e.g., Fig. 6B). These findings suggest that subsets of nociceptors may selectively express TRPV1 channel variants. It also suggests that the TRPV1b variant alone can form functional channels with the expected properties. The majority of capsaicin-responsive TG neurons that were probed were found to express both TRPV1 and TRPV1b subunits (Fig. 6B). We do not presently know whether TRPV1 and TRPV1b subunits combine to form functional channels.

In summary, we have presented evidence for the existence of a novel splice variant of human TRPV1 channels that appear to be expressed in a significant population of sensory neurons. This study emphasizes the existence of additional human TRPV1 isoforms in neurons arising from alternative RNA splicing.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. PCR identification of novel TRPV1-related sequences.   A. TRPV1-related sequences were amplified in two stages from a human cDNA library. Two nested, degenerate primer sets were generated from TRPV sequences and used to amplify ~900 bp fragments. Lane 2 shows the major product from the first round of PCR amplification is approximately 1100 bp. The reaction products were used as a template for a second round of amplification to generate the DNA fragments shown in lane 3. **B.** The human vanilloid receptor family phylogenetic tree. A phylogram showing human members of the vanilloid receptor family that was based on a CLUSTAL X alignment. The scale bar is a function of amino acid substitution scores based on the PAM series substitution weight matrix.

Figure 2. Alignment of cDNA sequences of hTRPV1b, hTRPV1, rat TRPV1 and rat TRPV2. Ankyrin-repeat domains (dashed blue lines), putative transmembrane (TM) domains (solid red lines) and the pore-loop region (dotted green line) are indicated. Conserved residues for all TRP variants are shaded yellow.

Figure 3. (A) Transmembrane domain cartoon for hTRPV1 and hTRPV1b. In humans the two TRPV1 variants differ in that hTRPV1b contains a 60-amino acid deletion in the N-terminus preceding TM1 (yellow highlight). Blue shading indicates six putative transmembrane domains (TM1-TM6).

Figure 4. Responses of hTRPV1 and hTRPV1b to capsaicin, acid, and heat. The left hand panels show the inward currents produced by 10 μM capsaicin, pH 5 and the normalized currents to heat in hTRPV1 expressing oocytes. The right hand panels show the absence of responses to 10 μM capsaicin and pH 5 and the normalized response to heat in hTRPV1b expressing oocytes. In both cases the heat responses were normalized to the maximum current. Holding potential = -25 mV.

Figure 5. Properties of hTRPV1 and hTRPV1b A. Current-voltage relationship of heat-evoked currents in hTRPV1 and hTRPV1b expressing oocytes**. B**. Ruthenium red (10 μM) inhibits heat-evoked currents in hTRPV1 and hTRPV1b expressing oocytes. Holding potential = -25 mV.

Figure 6. hTRPV1b-like currents in TG neurons.   A. Responses to 1 μM capsaicin and heat. The current–heat trace shows that the threshold temperature is 44°C and the maximum current was –3.6 nA. Holding potential = -60 mV. **B.** Single-cell RT-PCR identification of TRPV1 and TRPV1b in TG neurons. TRPV1 and TRPV1b specific DNA fragments were amplified by two step RT-PCR using cytoplasm obtained from TG neurons after the completion of whole-cell patch clamp experiments. Two nested, degenerate primer sets were constructed from published TRPV1 sequences and used to amplify a 659 bp (TRPV1) and a 478 bp (TRPV1b) fragment, respectively. Lanes 1-9 shows the two-step RT-PCR amplification products (approximately 659 bp and 478 bp) from 9 of 48 TG neurons that were responsive to both capsaicin and heat. The PCR amplification product (approximately 478 bp) from the neuron in **A** is shown in lane 10. The leftmost lane contains a 100 bp DNA ladder; the 600 bp fragment is highlighted (asterisk).
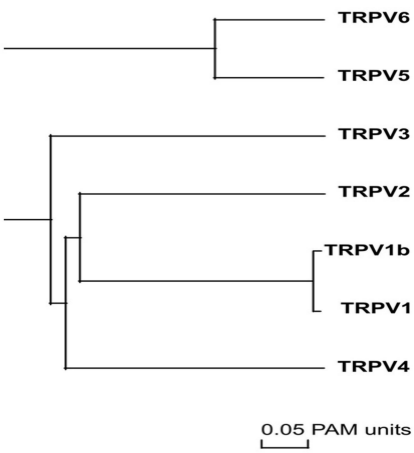
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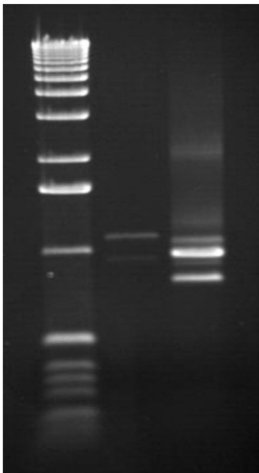
1) 1Kb DNAladder
2) Outside Primers
3) Inside Primers

B

```
              1                                                                  70
rTRPV1    (1) MEQRASLD SEESESPP QENSCLD PPDRDPNCKPPPVKPH IFTTRSRTRL FGKGD SEE---- ASPLDCP YE
hTRPV1b   (1) MKKWSSTDL GAAADPL QKDTCPDPLDGDPNSRPPPAKPQLST AKSRTRL FGKGDSEE---- AFPVDCP HE
hTRPV1    (1) MKKWSSTDL GAAADPL QKDTCPDPLDGDPNSRPPPAKPQLST AKSRTRL FGKGDSEE---- AFPVDCP HE
hTRPV2    (1) ------------------------MTSPS SSPVFRLETLDGGQEDGSEADRGKLDFGSGLPPMESQFQ

              71                                                                 140
rTRPV1   (67) EGGLASCP I ITVSSULT IQRPGDGPASURPS SQDSUSAG-EKPPRLYDRRS IFD AUAQSNC QELESLLP F
hTRPV1b  (67) EGELDSCPT ITVSPVIT IQRPGDGPTGARLL SQDSUAASTEKTLRL YDRRS IFE AUAQNNC QDLESLLL F
hTRPV1   (67) EGELDSCPT ITVSPVIT IQRPGDGPTGARLL SQDSUAASTEKTLRL YDRRS IFE AUAQNNC QDLESLLL F
hTRPV2   (45) GEDRKFASQ IRUVNLNYRKGTG------------------ASQPDPNRFDRDRLFNUUSRGUPEDLAGLPEY

              141                                                              ---210
rTRPV1  (136) LQRSKKRLTDSEFKDPETGKTCLL KAMLNLHNGQNDT IALLLDUARKTDSLKQFUNASYTD SYYKGQTAL
hTRPV1b (137) LQKSKKHLTDNEFKDPETGKTCLL KAMLNLHDGQNTT IPLLLE IARQTDSLKEUUNASYTD SYYKGQTAL
hTRPV1  (137) LQKSKKHLTDNEFKDPETGKTCLL KAMLNLHDGQNTT IPLLLE IARQTDSLKEUUNASYTD SYYKGQTAL
hTRPV2   (98) LSKTSKYLTDSEYTEGSTGKTCLMKAULNLKDGUNAC ILPLLQ IDRDSGNPQPLUNAQCTDDYYRGHSAL
              211----------A1----------           -------------A2----------280
rTRPV1  (206) HIAIERRNMTLUTLLUENGADUQA AANGDFFKKTKGRPGFYFGELPLSLAACTNQLAIUKFLLQNSWQP A
hTRPV1b (207) HIAIERRNM ALUTLLUENGADUQA AAHGDFFKKTKGRPGFYFGELPLSLAACTNQLG IUKFLLQNSWQT A
hTRPV1  (207) HIAIERRNM ALUTLLUENGADUQA AAHGDFFKKTKGRPGFYFGELPLSLAACTNQLG IUKFLLQNSWQT A
hTRPV2  (168) HIAIEKRSL QCUKLLUENGANUHARACGRFFQNGQG-TCFYFGELPLSLAACTKQWDUUSYLLENPHQP A
              281-                                     -----A3-----350
rTRPV1  (276) D ISARDSUGNTULHAL UEUADNTUDNTKFUT SMYNE IL ILGAKLHPTLKLEE ITNRKGLTPLALAASSGK
hTRPV1b (277) D ISARDSUGNTULHAL UEUADNTADNTKFUT SMYNE IL ILGAKLHPTLKLEELTNKKGMTPLALAAGTGK
hTRPV1  (277) D ISARDSUGNTULHAL UEUADNTADNTKFUT SMYNE IL ILGAKLHPTLKLEELTNKKGMTPLALAAGTGK
hTRPV2  (237) SLQATDSQGNTULHAL UMISDNSAEN IALUT SMYDGLLQAGARLCPTUQLED IRNLQDLTPLKLAAKEGK
              351----------------                                            420
rTRPV1  (346) IGULAYIL QRE IHEPE CRHLSRK FTEWAYGP VHSSLYD LSC IDTCEKNSULEU IAYSSSETPNRHDMLLU
hTRPV1b (347) IG------------------------------------------------------NRHDMLLU
hTRPV1  (347) IGULAYIL QRE IQEPE CRHLSRK FTEWAYGP VHSSLYD LSC IDTCEKNSULEU IAYSSSETPNRHDMLLU
hTRPV2  (307) IE IFRHIL QREFSG--LSHLSRK FTEWCYGP VRUSLYD LASUDSCEENSULE IIAFHCK-SPHRHRMUUL
              421                         TM1                                   490
rTRPV1  (416) EPLNRLLQDKWDRIFYFNFFUYCLYMI IFTAAAYYRPUEGLPPYKLKNTUG-DYFRUTGE ILSUS G
hTRPV1b (357) EPLNRLLQDKWDRFUKR IFYFNFLUYCLYMI IFTMAAYYRPUDGLPPFKMEKTG--DYFRUTGE ILSUL G
hTRPV1  (417) EPLNRLLQDKWDRFUKR IFYFNFLUYCLYMI IFTMAAYYRPUDGLPPFKMEKTG--DYFRUTGE ILSUL G
hTRPV2  (374) EPLNKLLQAKWDLL IPK-FFLNFLCNL IYMF IFTAUAYHQPTLKKQ AAPHLKAEUGNSMLLTGHIL ILL G
              491   TM2                         TM3                            560
rTRPV1  (485) GUYFFFRG IQYFLQRRPSLKSLFUDSYSE IL FFUQSLFMLUSUULYFSQRKEYUASMUFSLAMGWTNMLY
hTRPV1b (425) GUYFFFRG IQYFLQRRPSMKTLFUDSYSEML FFLQSLFMLATUULYFSHLKEYUASMUFSLALGWTNMLY
hTRPV1  (485) GUYFFFRG IQYFLQRRPSMKTLFUDSYSEML FFLQSLFMLATUULYFSHLKEYUASMUFSLALGWTNMLY
hTRPV2  (443) G IYLLUGQLWYFWRRHLFIWISYTDSYFE IL FLFHSLLTUUSLULCFLUIEWYLPLLUSALULGWLNLLY
              561 TM4              TM5                                         630
rTRPV1  (555) YTRGFQQMG IYAUMIEKMILRDLCRFMFUYLUFLFGFST AUUTL IEDGKNNSLPMESTPHKCRGSACKP-
hTRPV1b (495) YTRGFQQMG IYAUMIEKMILRDLCRFMFUYUUFLFGFST AUUTL IEDGKNDSLP SESTSHRWRGPACRPP
hTRPV1  (555) YTRGFQQMG IYAUMIEKMILRDLCRFMFUYUUFLFGFST AUUTL IEDGKNDSLP SESTSHRWRGPACRPP
hTRPV2  (513) YTRGFQHTG IYSUMIQKUILRDMURFLUIYLUFLFGFAU ALUSLSQEAWRPEAPTGPNATE SUQPMEGQE
              631 ???????Pore Loop??????                 TM6                  700
rTRPV1  (624) --- GNSYNSLYSTCLELFKFT IGMGDLEFTENYDFKAUFI ILLLAYUILTYILLLNML IALMGETUNKI
hTRPV1b (565) ----DSSYNSLYSTCLELFKFT IGMGDLEFTENYDFKAUFI ILLLAYUILTYILLLNML IALMGETUNKI
hTRPV1  (625) ----DSSYNSLYSTCLELFKFT IGMGDLEFTENYDFKAUFI ILLLAYUILTYILLLNML IALMGETUNKI
hTRPV2  (583) DEGNGAQYRGILEASLELFKFT IGMGELAFQEQLHFRGMULLLLLAYULLTYILLLNML IALMSETUNSU
              701                                                              770
rTRPV1  (690) AQESKN IWKLQRAIT ILDTEKSFLKCMRKAFRSGKLLQUGFTPDGKDDYRWCFRUDEUNWTTWNTNUG II
hTRPV1b (631) AQESKN IWKLQRAIT ILDTEKSFLKCMRKAFRSGKLLQUGYTPDGKDDYRWCFRUDEUNWTTWNTNUG II
hTRPV1  (691) AQESKN IWKLQRAIT ILDTEKSFLKCMRKAFRSGKLLQUGYTPDGKDDYRWCFRUDEUNWTTWNTNUG II
hTRPV2  (653) ATDSWS IWKLQKAIXULEMENGYWUC-RKKQ RAGUMLTUGTKPDGSPDERWCFRUEEUNWASWEQTLPTL
              771                                                              840
rTRPV1  (760) NEDPGNCE GUKRTLSFSLRSGRUS GRNWKNF ALUPLLRG ASTRDRH ATQQEEUQLKHYTGSLKPEDAEUF
hTRPV1b (701) NEDPGNCE GUKRTLSFSLRSSRUS GRHWKNF ALUPLLRE AS ARDRQ SAQPEEUYLRQFSGSLKPEDAEUF
hTRPV1  (761) NEDPGNCE GUKRTLSFSLRSSRUS GRHWKNF ALUPLLRE AS ARDRQ SAQPEEUYLRQFSGSLKPEDAEUF
hTRPV2  (722) CEDPSGAG----------UPRTLENPULAS PPKEDEDG ASEENYUPUQLLQSN----------------
              841
rTRPV1  (830) KDSMUPGEK
hTRPV1b (771) KSPAASGEK
hTRPV1  (831) KSPAASGEK
hTRPV2  (765) ---------
```
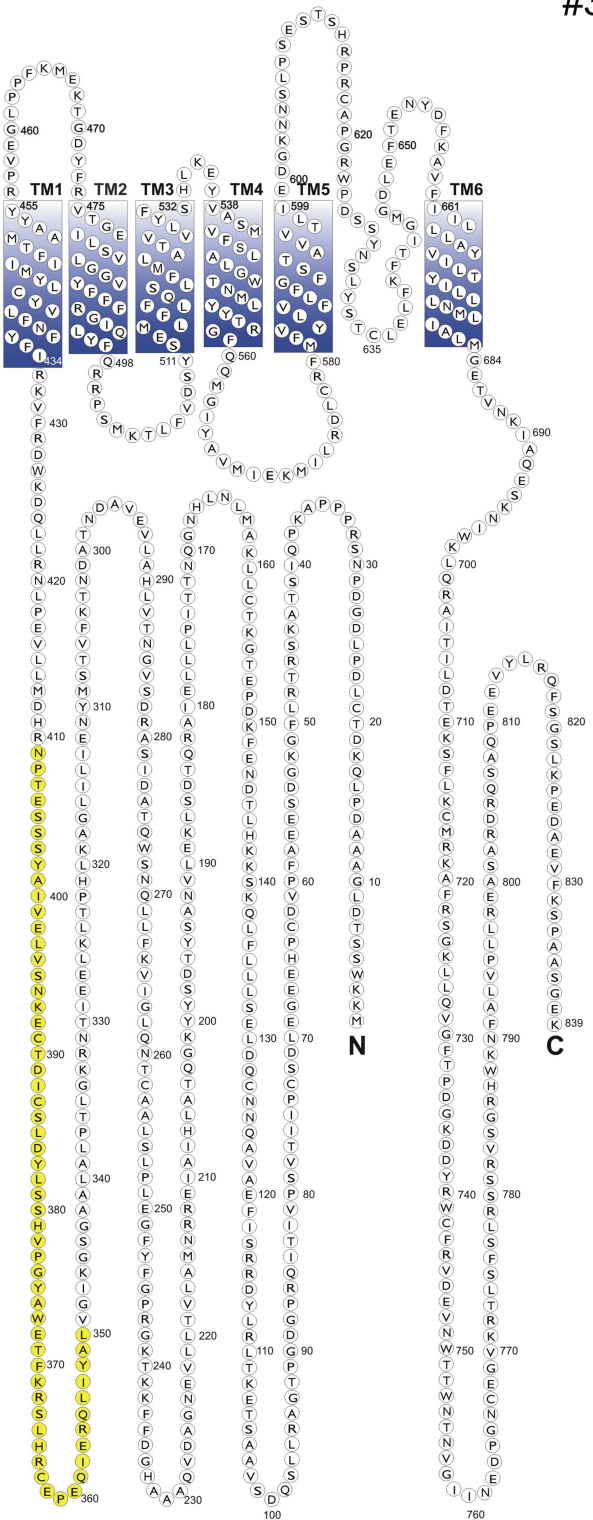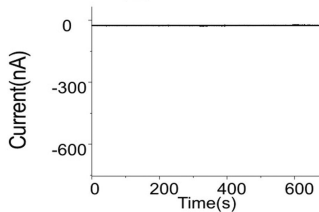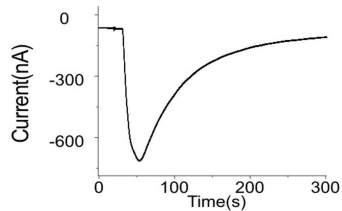
#2

TM1 **TM2** **TM3** **TM4** **TM5** **TM6**
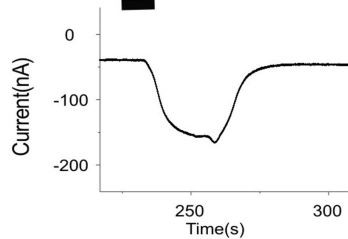
N

C

#4

TRPV1   **TRPV1b**

A
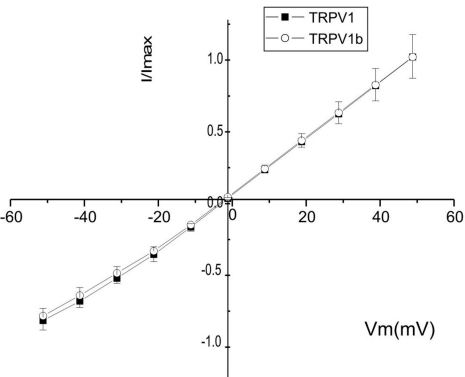

Figure 5A

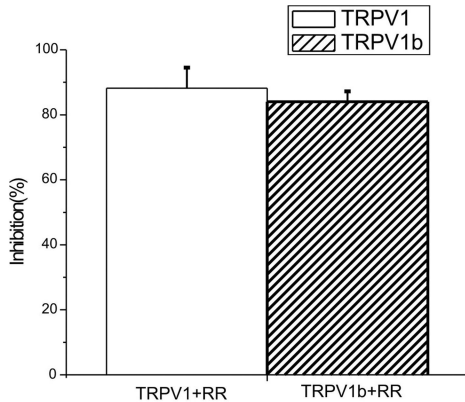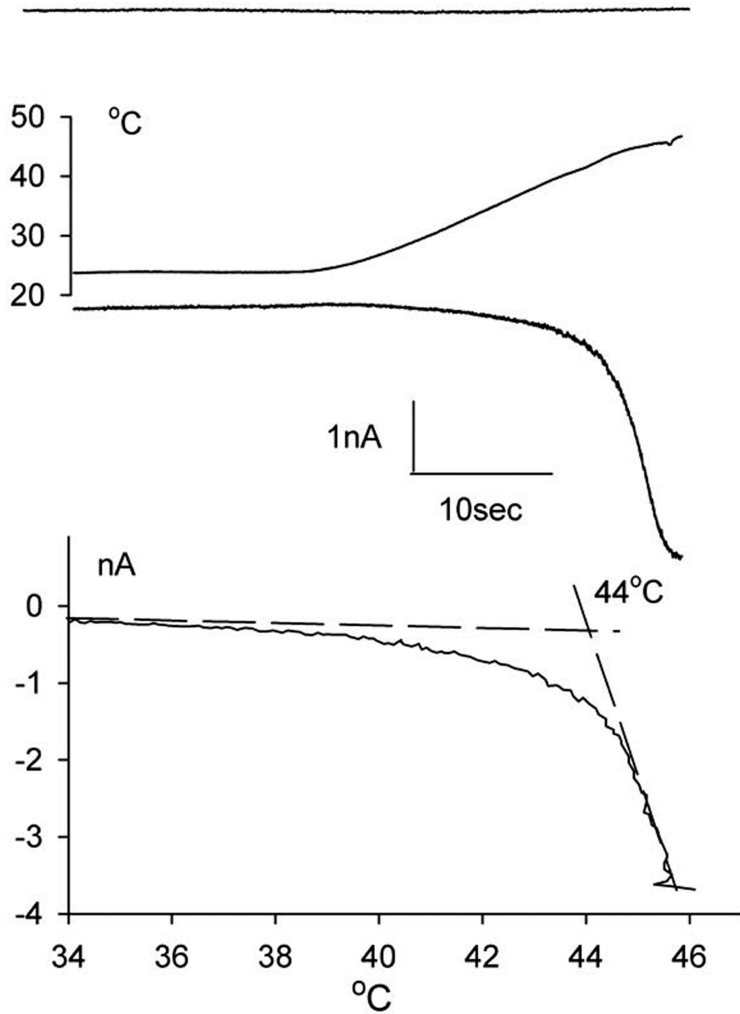B   #5
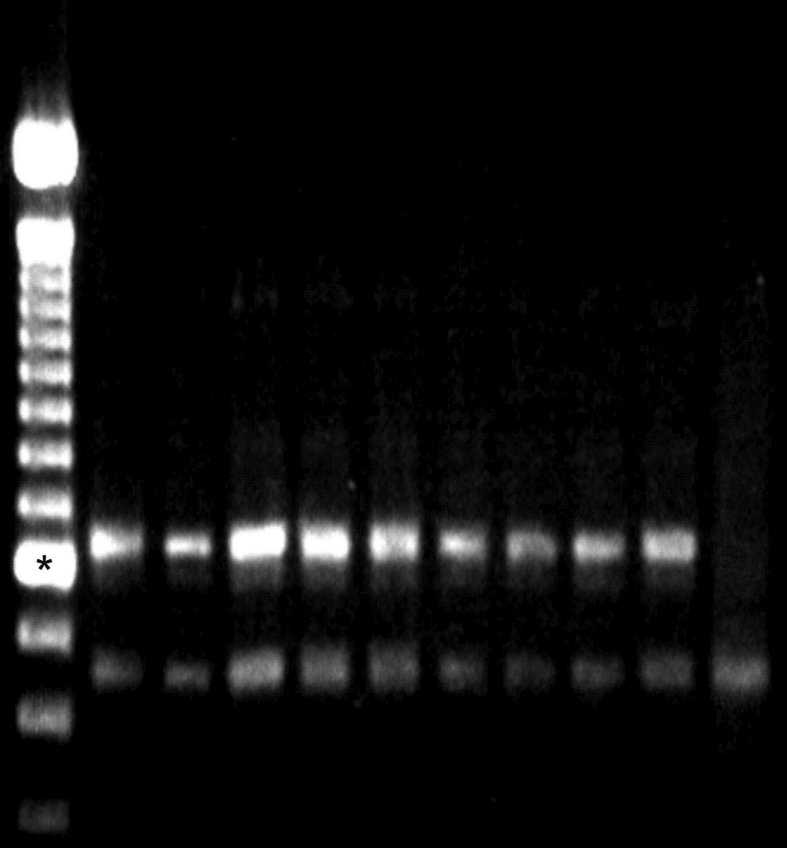

Figure 5B

A

Capsaicin, 1μM