Published in final edited form as:

Pain. 2016 July ; 157(7): 1515–1524. doi:10.1097/j.pain.0000000000000556.

Agonist-dependence of functional properties for common nonsynonymous variants of human transient receptor potential vanilloid 1

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Abstract

Transient receptor potential vanilloid 1 (TRPV1) is a polymodal receptor activated by capsaicin, heat, and acid, which plays critical roles in thermosensation and pain. In addition, TRPV1 also contributes to multiple pathophysiological states in respiratory, cardiovascular, metabolic, and renal systems. These contributions are further supported by evidence that variations in the human TRPV1 (hTRPV1) gene are associated with various physiological and pathological phenotypes. However, it is not well understood how the variations in hTRPV1 affect channel functions. In this study, we examined functional consequences of amino acid variations of hTRPV1 induced by 5 nonsynonymous single-nucleotide polymorphisms (SNPs) that most commonly exist in the human population. Using electrophysiological assays in HEK293 cells, we examined 9 parameters: activation, Ca²⁺ permeation, and desensitization after activation by capsaicin, acid, and heat. Our results demonstrated that the 5 SNPs differentially affected functional properties of hTRPV1 in an agonist-dependent manner. Based upon the directionality of change of each phenotype and cumulative changes in each SNP, we classified the 5 SNPs into 3 presumptive functional categories: gain of function (hTRPV1 Q85R, P91S, and T469I), loss of function (I585V), and mixed (M315I). These results reveal a spectrum of functional variation among common hTRPV1 polymorphisms in humans and may aid mechanistic interpretation of phenotypes associated with nonsynonymous hTRPV1 SNPs under pathophysiological conditions.

Keywords

Transient receptor potential vanilloid 1; Single-nucleotide polymorphisms; Patch clamp electrophysiology; Activation; Permeation; Desensitization; Capsaicin; Heat; Acid

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Conflict of interest statement

The authors have no conflicts of interest to declare.

1. Introduction

Transient receptor potential vanilloid 1 (TRPV1) is a member of the vanilloid subfamily of transient receptor potential cation channels. Transient receptor potential vanilloid 1 is a receptor for capsaicin and multiple endogenous or natural agonists such as heat, acid, lipid ligands, and toxins. Transient receptor potential vanilloid 1 is primarily expressed in small-to-medium diameter primary sensory afferents in dorsal root ganglia and trigeminal ganglia, and its role in nociception and thermosensation is well established.⁹ Transient receptor potential vanilloid 1 is expressed not only in somatosensory afferents but also in vagal afferents, brain, and multiple nonneuronal cells.^{3,7,21,25} Accumulating evidence supports the contribution of TRPV1 to a wide variety of pathophysiological states in pulmonary, cardiovascular, digestive, and metabolic systems^{1,14,16,43} and suggests that TRPV1 may provide a therapeutic target in various pathological conditions. In humans, TRPV1 variants have been associated with multiple neurosensory, respiratory, and digestive disorders, further substantiating the contribution of TRPV1 to multiple pathophysiologies.^{2,4,5,18,31,33,34,37} However, the mechanistic link between genetic variations in TRPV1 and altered phenotypes are largely unknown because the functional impact of genetic variation at the level of channel protein is poorly understood.

Transient receptor potential vanilloid 1 is a nonselective cationic channel that is permeable to both monovalent and divalent cations such as Na⁺ and Ca²⁺.⁸ Upon activation, the channel mediates influx of cations leading to depolarization of membrane potential and resulting neuronal excitation. Influx of Ca²⁺ after activation of TRPV1 stimulates intracellular Ca²⁺-dependent pathways leading to multiple consequences such as enzyme activation, neuropeptide release, gene expression, and activation of Ca²⁺-activated ion channels.^{13,15,19,23} Activation of TRPV1 is accompanied by desensitization that decreases the extent of activation in response to the same degree of subsequent agonist stimulation.¹⁷ Such activation, permeation, and desensitization properties of TRPV1 collectively affect the activity of the channel and hence the biological consequences after TRPV1 activation. In humans, a number of nonsynonymous variations of the TRPV1 gene have been reported. Despite previous efforts to understand the functional effects of such variations,^{37,41} genetic variation-induced differences in activation, permeation, and desensitization properties of TRPV1 are far from clear. In particular, the impact of genetic variation of TRPV1 on responses to different activation modalities, such as heat and acid, is unknown. In addition, activation of TRPV1 by a specific modality could result in different consequences at the systems level. For example, an antagonist selective for proton activation of TRPV1, but not heat, is associated with hyperthermia.³⁰

In this study, we aimed to determine the functional properties of TRPV1 variants commonly expressed in human populations and performed functional analysis of 5 common, nonsynonymous, single-nucleotide variants of human TRPV1. We assessed activation, permeation, and desensitization properties of these 5 TRPV1 SNPs in response to capsaicin, heat, and acid using mutagenic and electrophysiological approaches.

2. Experimental procedures

2.1. RNA sequencing and analysis of alternative splicing

To estimate relative abundance of alternative splicing variants of human TRPV1, we performed RNA sequencing analysis using RNA samples from human dorsal root ganglia (DRG; Clontech Laboratories Inc). The University of Maryland Baltimore Institutional Review Board determined this study as a nonhuman research. A total RNA samples isolated from normal human DRG pooled from 21 male and female Caucasians was used. Illumina RNAseq libraries were prepared with the TruSeq RNA Sample Prep kit (Illumina, San Diego, CA) as per manufacturer's protocol. Adapters containing 7 nucleotide indexes were ligated to the double-stranded cDNA. The DNA was purified between enzymatic reactions, and the size selection of the library was performed with AMPure XT beads (Beckman Coulter Genomics, Danvers, MA). Libraries were assessed for concentration and fragment size using the DNA High Sensitivity Assay on the LabChip GX (Perkin Elmer, Waltham, MA). The library concentrations were also assessed by qPCR using the KAPA Library Quantification Kit (Complete, Universal) (Kapa Biosystems, Woburn, MA). The libraries were pooled and sequenced on a 100PE Illumina HiSeq 2500 run (Illumina, San Diego, CA). The sequenced reads were aligned to the Human Genome reference sequence (Ensembl version GRCh37.75) using TopHat version 1.4.1.³⁵ The alignments allowed up to 2 bp mismatches per 25 bp segment, and we removed reads that aligned to more than 20 different genomic regions. Transcript abundances and splice variant identification was done using Cufflinks version 1.3 using the BAM alignment files obtained from TopHat.³⁶ BigWig coverage files were generated from the BAM alignment files using the UCSC genome browser tools. The output from Cufflinks and the BigWig files for the known and novel splice variants were viewed and analyzed using the Integrative Genomics Viewer.

2.2. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were cultured and transfected using lipofectamine 2000 (Invitrogen) as previously described.^{8,10} Plasmids containing cDNA-encoding TRPV1 or TRPV1 mutants were cotransfected with cDNA-encoding mCherry or GFP. Transiently transfected HEK293 cells were replated onto poly-L-ornithine-coated coverslips, kept at 32°C, and used for electrophysiological experiments after 16 to 26 hours.

2.3. cDNA and site-directed mutagenesis

The cDNA-encoding human TRPV1 in a pCMV6 vector was purchased from OriGene Technologies. Compared with the reference sequence of human TRPV1 (NM_080704.2), the hTRPV1 in pCMV6 differed in 3 nucleotides within the coding region. These variations have been reported as rs56095209, rs224534, and rs8065080. Among these, 2 missense mutations, rs224534 (c. 1406C>T, p.T469I) and rs8065080 (c. 1753A>G, p.I585V), were repaired by site-directed mutagenesis rendering the sequence consistent with the reference sequence (NM_080704). One silent mutation, rs56095209 (c. 180G>A, p. P60P), was not repaired and remained in the wild-type hTRPV1 cDNA. To tag the N-terminus of TRPV1 with a FLAG epitope, hTRPV1 cDNA without a start codon was PCR-amplified and inserted into the HindIII and NotI sites of pFlag-CMV2 (Sigma). The FLAG-tagged hTRPV1 (hTRPV1-pFlag) was used as wild type and was the template for the generation of

cDNA containing variants of hTRPV1. Site-directed mutagenesis was performed using overlap extension PCR methods or a Quick Change site-directed mutagenesis kit (Agilent Technologies). Proper mutation of each construct and lack of unintended mutation were confirmed by sequencing.

2.4. Whole-cell patch clamp

Whole-cell voltage clamp techniques were performed as described previously.^{10,39} Recording pipettes (2–3 MΩ) were pulled from borosilicate glass using a P-97 (Sutter Instrument). The pipettes were filled with standard internal solution containing 150 mM NaOH, 1 mM MgCl₂, 10 mM HEPES, and 5 mM EGTA (pH 7.4). Unless otherwise indicated, the recording bath contained solution ext A (140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, pH 7.4). In experiments involving heat-induced desensitization, Ca²⁺ was omitted and EGTA was added (ext B: 150 mM NaOH, 10 mM EGTA, 10 mM HEPES, pH 7.4). For testing capsaicin sensitivity in the absence of Ca²⁺, we used ext B containing only 5 mM EGTA. To estimate relative permeability of Ca²⁺ to Na⁺ (P_{Ca}/P_{Na}), we used an external solution containing 10 mM Ca²⁺ (ext C: 150 NaOH, 10 CaCl₂, 10 HEPES, pH 7.4). In testing the effect of acid on TRPV1 activation, permeation, and desensitization, every external solution contained MES, instead of HEPES, and 100 μM amiloride. The pH of solutions was adjusted with HCl or NaOH as necessary. Osmolarity of each solution was measured by a vapor pressure osmometer (Wescor Inc) and was adjusted with mannitol to 290 to 310 mOsm as necessary. Bath temperature was controlled using a combination of an in-line heater/cooler (Warner Instrument Inc) and in-line heater (Warner Instrument Inc). The actual temperature was recorded throughout the experiment with a thermocouple (Physitemp Instruments) that had been placed within 4 mm of the patch-clamped cell. Voltage ramps from −100 to +100 mV (200 ms duration) were applied every 2 seconds for recording capsaicin-induced and acid-induced currents or every one second for recording heat-evoked currents. Membrane potential was held at 0 mV between voltage ramps.

2.5. Data analysis

To minimize variability due to differences in individual cell size, we determined current densities (current amplitude/membrane capacitance). Unless otherwise indicated, data are expressed as the mean ± SE. Mean values were compared by unpaired Student *t* test or analysis of variance (ANOVA). The level of statistical significance was set at $\alpha = 0.05$. Concentration-dependent relationships were fitted by a logistics function: $Y = I / (1 + 10^{[\log EC_{50} - X] \times H})$, where Y = normalized current, EC_{50} = half-maximal concentration, X = log of concentration, H = Hill slope. Two concentration-dependent relationship curves derived from hTRPV1 WT and a variant were compared by extra sum-of-squares F test. P_{Ca}/P_{Na} was estimated as described previously⁸ using the equation: $P_{Ca}/P_{Na} = ([Na^+]_o / 4 [Ca^{2+}]_o) \times (\exp[-E_{rev}F/RT] - 1) \times (\exp[E_{Ca}F/RT] + 1)$, where $(X)_o$ is the activity of ion X in the bath, F is the Faraday constant, R is the gas constant, E_{Ca} is the reversal potential (E_{rev}) in ext C containing 150 Na⁺ and 10 Ca²⁺, E_{rev} is the shift in E_{rev} when ext C was replaced with ext B containing 150 Na⁺. E_{rev} was corrected for junction potentials that were calculated using pClamp software. The temperature coefficient (Q_{10}) and temperature threshold of activation were determined by following the methods

previously described.³⁸ An Arrhenius plot of the logarithm of current amplitudes against the reciprocal of the absolute temperature (K) was generated. The data points were fitted by a straight line over the temperature range where the Arrhenius plot was linear. Q_{10} was calculated from the following function: $Q_{10} = \exp(10 E_a/[RT_1 T_2])$, where R is the gas constant, E_a is an activation energy derived from the slope of the Arrhenius plot between absolute temperatures T_1 and T_2 . The thermal thresholds of activation were determined as temperatures in which the regression lines representing 2 different slopes intersect in the Arrhenius plot. The enthalpy for channel opening (ΔH°) was estimated by the following function: $\Delta H^\circ = (RT^2 \ln Q_{10})/10 \approx 20 \ln Q_{10}$ at physiological temperature (kcal/mol).¹¹ Statistical analysis, curve fitting, and plotting were performed using GraphPad Prism, Clampfit, or SigmaPlot.

3. Results

3.1. Determination of common TRPV1 SNPs

It is reported that alternative splicing results in variant forms of human TRPV1, which causes obvious functional variations.³² To estimate the relative abundance of splice variant forms of TRPV1 in humans, we performed RNA sequencing using a library derived from mRNA pooled from human DRG. In this assay, we detected a total of 10 candidate TRPV1 splice variants (Fig. 1A). Among these, transcripts 1 to 4 were the most frequently detected forms (Fig. 1B). These 4 variants have substitutions in untranslated regions with identical coding regions; hence, they are predicted to encode an identical full-length protein of 839 amino acids. In contrast, transcripts 5 to 7 are predicted to encode proteins with altered amino acid sequences. Variants 8 to 10 are predicted not to encode protein. In our assay, the frequencies of detection of transcripts 5 to 10 were far lower than those of transcripts 1 to 4 (Fig. 1B). These results suggest that structural variations due to alternative splicing are not major determinants of functional variations in TRPV1 in the general human population. Therefore, we focused on potential functional variations caused by non-synonymous SNPs in the coding region.

To identify common nonsynonymous hTRPV1 SNPs, we performed bioinformatics searches of reported SNPs in the database: NCBI dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/), 1000 Genomes (www.1000genomes.org), and the University of California, Santa Cruz, Genome browser (genome.ucsc.edu). In these databases, we identified approximately 150 nonsynonymous SNPs of the human TRPV1 gene that were validated in multiple projects. Among these, we selected 5 SNPs whose minor allele frequency (MAF) was greater than 1% globally across all human populations tested (Fig. 1C). Based upon the high homology of human TRPV1 and rat TRPV1 and the recent crystal structure of rat TRPV1,^{20,22} we predicted that 2 SNPs, Q85R and P91S, produce amino acid variations located in the N-terminal domain before the first ankyrin repeat domain, M315I in α -helix of the fifth ankyrin repeat domain, T469I in extracellular linker between the first and second transmembrane domain, and I585V in α -helix of the fifth transmembrane domain (schematized in Fig. 1D). We performed site-directed mutagenesis to reproduce the nucleotide variation of each SNP in a human TRPV1 cDNA that contains identical sequence to the coding region shared by transcripts 1 to 4 in Fig. 1A.

3.2. Activation properties of human transient receptor potential vanilloid 1 and variants

We investigated capsaicin sensitivity of hTRPV1 WT and the 5 variants by whole-cell voltage clamp analysis. To estimate EC₅₀, we applied 4 different concentration of capsaicin (30 nM, 300 nM, 3 μM, and 30 μM) consecutively in the same cell (Fig. 2A). To prevent Ca²⁺-induced desensitization, extracellular Ca²⁺ was omitted from the bath solution. In TRPV1 WT, capsaicin activated the channels in a concentration-dependent manner with an EC₅₀ of 134 nM and Hill slope 1.3. Capsaicin concentration-dependent sensitivities of the 5 hTRPV1 variants were similar to that of WT (Figs. 2B, C), although hTRPV1 I585V showed a significant rightward shift of the relationship (I585V, 214 nM, $F_{(1,96)} = 6.57$, $P < 0.05$). Hill slope values obtained from variants were not significantly different from WT. In this analysis, maximal capsaicin-evoked current density of each variant was not significantly different from that of WT (WT, -710 ± 107 pA/pF; Q85R, -964 ± 199 pA/pF; P91S, -927 ± 174 pA/pF; M315I, -946 ± 148 pA/pF; T469I, -617 ± 140 pA/pF; I585V, -696 ± 118 pA/pF). These results suggest that hTRPV1 I585V showed attenuated capsaicin sensitivity, which could be interpreted as a loss-of-function phenotype.

To examine acid sensitivity of hTRPV1 WT and variants, we evaluated responses to increasing concentrations of protons (pH 7, pH 6, pH 5, and pH 4) in the same cell. To minimize Ca²⁺-dependent desensitization, extracellular Ca²⁺ was again omitted from bath solutions. All acidic extracellular solutions contained 100 μM amiloride to minimize contamination from endogenous acid-sensitive currents in HEK293 cells. Transient receptor potential vanilloid 1 WT was activated by acid in a concentration-dependent manner with EC₅₀ of pH 5.9 and Hill slope of 1.5 (Figs. 2D, E). Acid also activated TRPV1 variants in a concentration-dependent manner (Figs. 2E, F). Among the 5 variants, hTRPV1 P91S and T469I showed a small but significant leftward shift in their concentration–response relationships (P91S, pH 6.1, $F_{(1,108)} = 4.55$, $P < 0.05$; T469I, pH 6.1, $F_{(1,108)} = 4.46$, $P < 0.05$). Hill slope values obtained from variants were not significantly different from WT. In this analysis, maximal acid-evoked current density of each variant was not significantly different from that of WT (WT, -612 ± 83 pA/pF; Q85R, -403 ± 70 pA/pF; P91S, -330 ± 51 pA/pF; M315I, -578 ± 69 pA/pF; T469I, -400 ± 54 pA/pF; I585V, -384 ± 39 pA/pF). These results suggest that acid sensitivity of hTRPV1 P91S and T469I was higher than WT, which could be interpreted as a gain-of-function phenotype.

To investigate the effects of amino acid variations of hTRPV1 on heat activation properties, we recorded currents in response to a slow heat ramp from ~17°C to ~47°C over ~45 seconds (Figs. 3A, B). We evaluated the threshold and enthalpy of channel opening (°H) of each cell in the Arrhenius plot (Fig. 3C). Human TRPV1 WT showed robust activation in response to the heat ramp. Averaged activation threshold and °H of hTRPV1 WT calculated based upon Q₁₀ calculation was $36 \pm 0.8^\circ\text{C}$ and 88.6 ± 6.7 kcal/mol (Figs. 3D, E). Activation threshold measured in our recording was substantially lower than that reported previously.⁶ Although the source of such difference is unclear, we speculate that our recording protocol involving depolarized holding potential could contribute to the difference because it is known that depolarization decreases heat activation threshold of TRPV1.⁴² All 5 hTRPV1 SNPs were also robustly activated by the heat ramp with similar activation thresholds and °H (Figs. 3A, B). However, hTRPV1 M315I displayed small but significant

changes in heat activation properties (Figs. 3C, D). Human TRPV1 M315I exhibited a significant increase in ΔH without any change in activation threshold. Human TRPV1 I585V also exhibited a tendency toward increased threshold and ΔH but did not reach statistical significance. These results suggest that hTRPV1 M315I requires higher heat energy for activation, which could be interpreted as a loss-of-function phenotype.

3.3. Ca^{2+} permeation of human transient receptor potential vanilloid 1 and variants

To evaluate Ca^{2+} permeation properties, we estimated the relative ratio of the permeabilities of Ca^{2+} and Na^{+} ($P_{\text{Ca}}/P_{\text{Na}}$) by measuring reversal potential (E_{rev}) of currents in the presence of 10 mM extracellular Ca^{2+} followed by Ca^{2+} -free conditions in the same cell. In hTRPV1 WT, the E_{rev} of capsaicin-evoked currents in the presence of 10 mM Ca^{2+} was 8.8 ± 0.5 mV (upward arrow, Fig. 4A). The E_{rev} was shifted to -4.3 ± 0.5 mV (downward arrow, Fig. 4A), a shift of 13.1 ± 0.3 mV, after deprivation of extracellular Ca^{2+} , and $P_{\text{Ca}}/P_{\text{Na}}$ was estimated to be 12.6 ± 0.4 ($n = 14$) (Fig. 4C). Human TRPV1 variants also showed high $P_{\text{Ca}}/P_{\text{Na}}$ upon activation by capsaicin. Among the 5 variants, hTRPV1 Q85R (14.6 ± 0.7) and M315I (16.5 ± 0.9 ; Fig. 4B) showed significant increases (Fig. 4C). The greater $P_{\text{Ca}}/P_{\text{Na}}$ in hTRPV1 Q85R and M315I could be interpreted as a gain-of-function phenotype.

Permeation properties of rodent TRPV1 are known to be dynamic and are not identical when TRPV1 is activated by capsaicin vs heat.⁸ We presumed that human TRPV1 would also show distinct permeability properties and that the hTRPV1 variants may exhibit differential permeation. Therefore, we assessed $P_{\text{Ca}}/P_{\text{Na}}$ after activation by heat and acid as well. $P_{\text{Ca}}/P_{\text{Na}}$ upon heat activation was estimated as with capsaicin stimulation, by measuring E_{rev} in the presence and absence of extracellular Ca^{2+} (Fig. 4D). E_{rev} of heat-evoked currents in the presence of 10 mM Ca^{2+} was 8.4 ± 0.7 mV (upward arrow, Fig. 4D). In the presence of 0 mM extracellular Ca^{2+} , the E_{rev} was -1.2 ± 0.6 mV, a shift of 9.6 ± 0.5 mV (downward arrow, Fig. 4D). $P_{\text{Ca}}/P_{\text{Na}}$ was estimated to be 7.0 ± 0.5 . Among the 5 hTRPV1 SNPs, hTRPV1 I585V exhibited a significant ~30% lesser $P_{\text{Ca}}/P_{\text{Na}}$ than WT significantly (Figs. 4E, F). The decreased $P_{\text{Ca}}/P_{\text{Na}}$ of hTRPV1 I585V could be interpreted as a loss-of-function phenotype.

When hTRPV1 WT was activated by acid (pH 5 or 4.5) in the presence of 10 mM Ca^{2+} , the current reversed at 9.3 ± 0.6 mV (upward arrow, Fig. 5A). When the extracellular Ca^{2+} was removed, E_{rev} was -0.6 ± 0.8 mV, a shift of 9.9 ± 0.6 mV (downward arrow). $P_{\text{Ca}}/P_{\text{Na}}$ was therefore estimated to be 8.3 ± 0.6 . Among the 5 hTRPV1 SNPs, P91S (Fig. 5B) and M315I exhibited significantly greater $P_{\text{Ca}}/P_{\text{Na}}$ ratios than WT (Fig. 5C). These increased $P_{\text{Ca}}/P_{\text{Na}}$ ratios of hTRPV1 P91S and M315I could be interpreted as a gain-of-function phenotype.

The $P_{\text{Ca}}/P_{\text{Na}}$ ratios upon activation by the 3 modalities, capsaicin, acid, and heat, were significantly different from that of hTRPV1 WT ($P < 0.001$ in one-way ANOVA). Significant differences existed among the 5 SNPs as well (in one-way ANOVA, $P < 0.001$) (Fig. 5D). In hTRPV1 WT, post hoc analysis showed that the $P_{\text{Ca}}/P_{\text{Na}}$ ratio after activation by capsaicin was significantly different from $P_{\text{Ca}}/P_{\text{Na}}$ ratios after activation by either acid or heat. However, the $P_{\text{Ca}}/P_{\text{Na}}$ ratios did not differ between activation by acid vs heat. Among the 5 SNPs, 3 (Q85R, M315I, and T469I) showed the same tendency as WT. However, 2

SNPs (P91S, I585V) showed significant differences among P_{Ca}/P_{Na} ratios after activation by the 3 different agonists (Fig. 5D).

These data suggest that P_{Ca}/P_{Na} is not identical when TRPV1 is activated by different agonists and that the extent of modality-dependence of the P_{Ca}/P_{Na} ratio is altered by the amino acid variations that occur in TRPV1 SNPs.

3.4. Desensitization of human transient receptor potential vanilloid 1 and variants

To investigate capsaicin-induced desensitization, we measured responses to 2 consecutive applications of 1 μ M capsaicin (Fig. 6A). In rat TRPV1, the second response was approximately half of the first response in the presence of 0.3 mM extracellular Ca^{2+} .¹⁷ We tested capsaicin-induced desensitization of hTRPV1 and variants under this condition, so that we could detect both increases and decreases in the extent of desensitization. In the presence of 0.3 mM extracellular Ca^{2+} , the ratio of the second/first response of hTRPV1 to 1 μ M capsaicin was $55.2 \pm 6.7\%$ (Fig. 6B). Human TRPV1 variants also showed desensitization upon repeated application of capsaicin (Figs. 6A, B). Among the 5 variants, hTRPV1 M315I showed a significantly smaller ratio of second/first response ($31.7 \pm 6.1\%$) than WT. Current density of the response evoked by the first application of capsaicin was not significantly different between WT and each variant (WT, -546 ± 77 pA/pF; Q85R, -509 ± 50 pA/pF; P91S, -783 ± 86 pA/pF; M315I, -749 ± 135 pA/pF; T469I, -491 ± 97 pA/pF; I585V, -407 ± 256 pA/pF; $P > 0.05$). The greater extent of capsaicin-induced desensitization of hTRPV1 M315I could be interpreted as a loss-of-function phenotype.

The extent of acid-induced desensitization was assessed by 2 consecutive applications of acid (pH 4.5) in the presence of 2 mM Ca^{2+} (Fig. 6C). Under this condition, the second response was $35.5 \pm 6.2\%$ in WT (Fig. 6D). Repeated exposure of hTRPV1 variants to acid (pH 4.5) also induced desensitization. Among the 5 variants, hTRPV1 Q85R (Fig. 6C) showed a significantly greater ratio of second/first response ($53.6 \pm 4.9\%$) (Fig. 6D). In this analysis, current density of the response evoked by first application of acid was not significantly different between hTRPV1 WT and SNPs (Q85R, -199 ± 32 pA/pF; P91S, -251 ± 39 pA/pF; M315I, -251 ± 26 pA/pF; T469I, -159 ± 20 pA/pF; I585V, -301 ± 60 pA/pF; $P > 0.05$). Therefore, the reduced extent of acid-induced desensitization of hTRPV1 Q85R cannot be explained by reduced Ca^{2+} influx after activation. The reduced acid-induced desensitization of hTRPV1 Q85R could be interpreted as a gain-of-function phenotype.

Heat-induced desensitization of TRPV1 is not dependent on Ca^{2+} and occurs through different mechanisms from those involved in capsaicin-induced desensitization.¹⁷ Therefore, the extent of heat-induced desensitization of hTRPV1 WT and variants was evaluated in the absence of extracellular Ca^{2+} . The ratio of second/first response after the application of 2 consecutive heat stimuli to $\sim 45^{\circ}\text{C}$ was measured. Human TRPV1 WT showed a robust response to the first application of heat, whereas the following application of heat evoked only a modest amount of current activation. The ratio of second/first response was $30.1 \pm 4.0\%$ in WT ($n = 18$). The extent of heat-induced desensitization was not significantly different between hTRPV1 WT and SNPs (Q85R, $34.0 \pm 7.8\%$, $n = 11$; P91S, $30 \pm 6.6\%$, n

=9; M315I, $36.3 \pm 4.6\%$, $n = 15$; T469I, $25.7 \pm 3.7\%$, $n = 14$; I585V, $27.9 \pm 4.0\%$, $n = 12$; $P > 0.3$ vs WT).

4. Discussion

In this study, we investigated functional properties of 5 non-synonymous SNPs of hTRPV1 by electrophysiological assays of recombinant TRPV1 in HEK293 cells. Despite the recent information regarding TRPV1 structure,²⁰ mechanistic prediction of the direction or the extent of functional alteration due to nonsynonymous SNPs of TRPV1 remains difficult. The crystal structure and most structure–functional relationship studies have been based on rodent TRPV1, which makes it even more difficult to infer functional changes resulting from amino acid variation in human TRPV1. Moreover, unbiased, random mutagenesis revealed that multiple residues throughout TRPV1 alter its activation properties.²⁷ In addition, substitution mutations of a single amino acid residue result in completely different consequences.²⁸ Therefore, nonsynonymous variations in any region of TRPV1 could potentially induce significant functional alterations, and as the nature of such changes is unpredictable, should be experimentally determined.

There are few reports regarding functional properties of hTRPV1 SNPs. The functional effects of 6 hTRPV1 SNPs have been reported previously.⁴¹ But, Xu et al.⁴¹ regarded hTRPV1 Ile³¹⁵ allele as WT, and therefore, the rest of the SNPs happened to simultaneously carry the Ile³¹⁵ allele. In the current study, the reference hTRPV1 was the Met³¹⁵ allele based on allele frequency obtained from databases. Therefore, our study represents phenotypes of SNPs that are different from those presented previously.⁴¹ Consequently, we could compare our results with those of the previous study only between hTRPV1 Met³¹⁵ and Ile³¹⁵ alleles. Previously, hTRPV1 Met³¹⁵ was reported to show greater maximal response to capsaicin than the Ile³¹⁵ allele.⁴¹ However, our data indicated that maximal density of capsaicin-evoked currents in hTRPV1 Met³¹⁵ and Ile³¹⁵ alleles was not different. Furthermore, hTRPV1 Ile³¹⁵ exhibited greater P_{Ca}/P_{Na} than Met³¹⁵ in our data. Although the source of such difference is unclear, our electrophysiological assay could have yielded a more clear difference in the extent of Ca^{2+} permeability of TRPV1, compared to the Ca^{2+} imaging assay used previously.⁴¹ In contrast, the phenotypes of hTRPV1 I585V were consistent with previously reported properties.⁴ The intracellular Ca^{2+} changes after the application of capsaicin or heat was smaller in I585V than in WT. In our electrophysiological analysis, hTRPV1 I585V showed slightly reduced capsaicin sensitivity but had P_{Ca}/P_{Na} identical to WT upon activation by capsaicin. Although the threshold and θ_H of hTRPV1 I585V were not different from WT in response to activation by heat, the P_{Ca}/P_{Na} ratio was significantly reduced. These phenotypes are consistent with attenuation of intracellular Ca^{2+} changes evoked by heat and capsaicin.⁴ Therefore, our data support previously reported activation phenotypes of hTRPV1 I585V, but not M315I.

Our results demonstrated that activation, permeation, and desensitization properties of 5 nonsynonymous TRPV1 SNPs were differentially altered after modality-dependent activation (Table 1). Significant changes could be interpreted as gain-of-function (red arrows) or loss-of-function (blue arrows). Although the extent of differences in each individual analysis is modest, the cumulative consequences of multiple functional effects

could be impactful, especially in cases where these accumulate in the same direction to positively or negatively affect channel function. Based upon the directionality of each phenotype and ensemble of multiple phenotypes, we predicted the overall nature of the altered functions conferred by SNP-induced amino acid variations into 3 categories. The reduced calcium entry or agonist sensitivity, as compared to wild-type TRPV1, was considered a loss-of-function variant because reduced calcium entry would cause a reduction in signaling initiated by TRPV1 for the same dose of an agonist. In contrast, the reduced desensitization was considered a gain-of-function because the decreased extent of desensitization would increase the subsequent signaling of TRPV1 in response to repeated agonist applications. Human TRPV1 I585V showed reduced capsaicin sensitivity and reduced P_{Ca}/P_{Na} upon heat activation, both of which result in loss-of-function phenotypes. In contrast, hTRPV1 Q85R, P91S, and T469I showed gain-of-function phenotypes. Human TRPV1 M315I showed both gain-of-function and loss-of-function changes and could be classified as mixed phenotypes. Such functional classification of hTRPV1 SNPs may provide a mechanistic basis for phenotypes associated with the SNPs in human population as discussed below. We acknowledge, however, that corroboration of in vitro phenotypes with human phenotypes would not be straightforward. Although our data clearly define functional properties of single SNP, we do not know the consequences of the simultaneous occurrence of 2 or more SNPs. Furthermore, it is unclear how each SNP might interact with WT TRPV1 when the SNP is expressed in only one allele. When we compared P_{Ca}/P_{Na} in WT, M315I and coexpression of both, coexpression showed intermediate phenotype of WT and M315I alone (unpublished observation). Although this observation was a predictable outcome, potential specific interactions between WT and any particular SNPs remain elusive and need to be determined independently.

TRPV1 is implicated in pathophysiological conditions involving the neurosensory, cardiovascular, respiratory, metabolic, and renal systems.^{1,9,14,16,40,43} In addition to accumulating evidence in preclinical studies, the contribution of TRPV1 to multiple pathophysiological conditions in humans has been further supported by association studies of TRPV1 variants with human phenotypes.^{2,4,18,31,33,34,37} The positive associations of human phenotypes and nonsynonymous TRPV1 SNPs are summarized in Table 2. Among 5 SNPs investigated in this study, positive associations with human phenotypes have been reported only for hTRPV1 M315I and I585V. Since the allelic frequencies of these 2 SNPs are relatively high compared to other common SNPs (Fig. 1), it is not surprising that positive associations have been found more often with these. However, positive associations of these 2 SNPs cannot be entirely attributed to the relatively high minor allele frequency since hTRPV1 T469I SNP was not reported to be significantly associated with any human phenotypes despite its high minor allele frequency. We presume that functional effects conferred by the amino acid variations due to allelic variations within the TRPV1 gene contribute to altered human phenotypes. This is supported by the comparison of cumulative functional phenotypes of hTRPV1 I585V and M315I and the human phenotypes associated with the SNPs.

Human TRPV1 I585V SNP has been associated with multiple neurosensory phenotypes in healthy subjects and patients experiencing neuropathic injury or osteoarthritis.^{2,18,37} Although difficult to provide a mechanistic interpretation related to TRPV1 function,

hTRPV1 Val⁵⁸⁵ allele is associated with an enhanced pain threshold and decreased sensitivity to noxious and innocuous stimuli. In addition, TRPV1 contributes to the perception of salt taste in rodents,²⁴ and hTRPV1 Val⁵⁸⁵ allele is associated with reduced salt taste perception.¹² These associated phenotypes suggest that hTRPV1 Val⁵⁸⁵ allele would negatively alter the functions of TRPV1. Furthermore, hTRPV1 Val⁵⁸⁵ allele was reported to be associated with a lower risk of asthma and cough.^{4,33} Because it is well known that TRPV1 in vagal sensory afferents contributes to cough and airway disorders,¹⁴ the negative correlation also suggests reduced functions of TRPV1 may be conferred by hTRPV1 I585V SNP. Indeed, our data indicated that hTRPV1 Val⁵⁸⁵ allele conferred loss-of-function phenotypes, consistent with predictions from human association studies. Therefore, it is possible that loss-of-function of hTRPV1 I585V SNP could contribute to the multiple neurosensory and respiratory phenotypes in subjects carrying hTRPV1 Val⁵⁸⁵.

In contrast to hTRPV1 I585V, associations of hTRPV1 M315I SNP with human phenotypes are less straightforward. Human TRPV1 I³¹⁵ allele was associated with decreased sensitivity to innocuous cold and reduced gastric pain or discomfort,^{1,2} both of which suggest loss of function. Furthermore, hTRPV1 I³¹⁵ allele was reported to be associated with increased risk of type I diabetes.³¹ This phenotype may also be interpreted as a loss-of-function of hTRPV1 I³¹⁵ allele because hypofunctional TRPV1 mutation was suggested to be correlated with diabetes in diabetes-prone NOD mice.²⁹ In contrast, hTRPV1 I³¹⁵ allele was associated with greater cortical excitability after transcranial magnetic stimulation.²⁶ Although the role of TRPV1 in the human cortex is unclear, this result suggests a gain-of-function phenotype of hTRPV1 I³¹⁵ allele. Therefore, the associations of hTRPV1 M315I SNP with human phenotypes suggest both gain-of-function and loss-of-function consequences. Consistently, our in vitro analysis identified potential gain-of-function and loss-of-function phenotypes of hTRPV1 I³¹⁵ allele (Table 1). It is difficult to predict the most dominant phenotype in vivo or the net consequence of such mixed phenotypes. We speculate that TRPV1 might affect neuronal functions differentially in different environments, eg, sensory neurons vs cortex.

Despite these apparent correlations of in vitro phenotypes of hTRPV1 I585V and M315I and human association studies, it is unclear whether the altered phenotypes observed in this study are sufficient to cause changes of phenotypes in humans. Similarly, we do not presume that the altered functions of TRPV1 conferred by SNPs exclusively contribute to the associated phenotypes in humans. Multiple genes and haplotype-associated genes may be involved in determining the phenotypes in humans. Our assay did not exclude a potential contribution of variants affecting the expression of TRPV1 to human phenotypes.

In conclusion, we demonstrated that 5 common nonsynonymous SNPs of human TRPV1 distinctly alter functional properties upon activation by distinct agonists. The data reveal a spectrum of functional variation among common hTRPV1 polymorphisms and provide information useful for studying mechanistic linkages between TRPV1 SNPs and multiple pathophysiological conditions in humans.

Acknowledgments

The authors thank Dr. Michael Caterina for helpful suggestions and Jamila Asgar for technical assistance. This study was supported by the National Institutes of Health grant R01 DE023846 (M.-K.C.) and DE16062 (J.Y.R.). S. Wang and J. Joseph contributed equally to this study.

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Figure 1.

Common nonsynonymous single-nucleotide polymorphism (SNPs) of human transient receptor potential vanilloid 1 (TRPV1) examined in this study. (A) RNA-sequencing of human DRG RNA samples pooled from 19 subjects revealed 10 splice variant forms of human TRPV1 (1–10). (B) FPKM (Fragments Per Kilobase of Exon Per Million Fragments Mapped) values of variants are plotted. (C) Identity and minor allele frequency (MAF) of 5 nonsynonymous TRPV1 SNPs. Minor allele frequency was obtained from the University of California Santa Cruz Genome browser (<http://genome.ucsc.edu>). (D) Schematics of TRPV1 structure and the location of amino acid variations induced by 5 SNPs. Human TRPV1 Q85, P91, M315I are located in amino terminal domain. Human TRPV1 T469I is located in extracellular linker between transmembrane domains 1 and 2. Human TRPV1 I585V is located in the fifth transmembrane domain.

Figure 2.

Effects of amino acid variations of human transient receptor potential vanilloid 1 (hTRPV1) on capsaicin-induced and acid-induced activation (A) Representative current traces recorded from HEK293 cells transfected with TRPV1 WT. Currents were evoked by the consecutive application of 4 progressively increasing concentration of capsaicin. Bath solution did not contain Ca^{2+} (ext. B with 5 mM EGTA). Current densities were evaluated at -60 mV. Scale bar, 200 pA/pF and 1 min. (B and C) Capsaicin concentration-dependent relationships of hTRPV1 WT and variants. The 5 SNPs were arbitrarily divided into 2 groups and presented along with WT. Current amplitudes in each capsaicin concentration were normalized to the maximal response in each cell. Numbers in parenthesis within label represent the number of observations. The lines were derived from data fitting using sigmoidal logistics equation with fixed minimum and maximum value of 0 and 1, respectively. $*P < 0.05$ in extra sum-of-squares F test vs WT. Log(EC_{50}) and Hill slope of WT, -6.87 ± 0.05 and 1.34 ± 0.15 ; Q85R, -6.79 ± 0.06 and 1.99 ± 0.38 ; P91S, -6.82 ± 0.05 and 1.3 ± 0.18 ; M315I, -6.73 ± 0.06 and 1.43 ± 0.27 ; T469I, -6.87 ± 0.06 and 1.27 ± 0.16 ; I585V, -6.67 ± 0.06 and 1.31 ± 0.27 . (D) Representative current traces recorded from HEK293 cells transfected with TRPV1 WT (top) or TRPV1 A401T (bottom). Currents were evoked by the consecutive application of 4 progressively increasing concentrations of proton. Bath solution did not contain Ca^{2+} (ext. B with 5 mM EGTA). $V_m = -60$ mV. Scale bar, 200 pA/pF and 1 min. (E and F) Proton concentration-dependent relationships of hTRPV1 WT and variants. The 5 SNPs were arbitrarily divided into 3 groups and presented along with WT. Numbers in parenthesis within label represent the number of observations. $*P < 0.05$ in extra sum-of-squares F test vs WT. EC_{50} and hill slope of WT, pH 5.91 ± 0.04 and 1.49 ± 0.33 ; Q85R, pH 6.03 ± 0.07 and

1.51 ± 0.60 ; P91S, pH 6.05 ± 0.05 and 1.55 ± 0.46 ; M315I, pH 5.97 ± 0.04 and 1.80 ± 0.69 ; A401T, pH 5.71 ± 0.08 and 2.44 ± 0.65 ; D412N, pH 5.94 ± 0.06 and 2.09 ± 1.65 ; T469I, pH 6.05 ± 0.05 and 1.51 ± 0.43 ; I585V, pH 5.80 ± 0.07 and 1.86 ± 0.55 .

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Figure 3.

Effects of amino acid variations of human transient receptor potential vanilloid 1 (hTRPV1) on heat-induced activation. (A and B) Superimposed representative current-temperature relationships of hTRPV1 WT (black) and hTRPV1 SNPs in response to a heat ramp. Currents are relative to the respective maximum response of each cell. $V_m = -60$ mV. (C) Representative Arrhenius plot of hTRPV1 WT showing a threshold temperature. (D and E) Plots of threshold temperature (D) and $\Delta^{\circ}\text{H}$ (E) of hTRPV1 WT and TRPV1 SNPs. Symbols represent scatter dot plot of raw data and the superimposed horizontal lines represent mean $\pm$ SEM. * $P < 0.05$; # $P < 0.005$ vs TRPV1 WT, Student t test. Numbers of observations are indicated within bars.

Figure 4.

Effects of amino acid variations of human transient receptor potential vanilloid 1 (hTRPV1) on the relative permeability of Ca^{2+} to Na^{+} ($P_{\text{Ca}}/P_{\text{Na}}$) after capsaicin-induced and heat-induced activation. (A and B) Representative current traces recorded from HEK293 cells transfected with TRPV1 WT (A) or TRPV1 M315I (B). Currents were evoked by $0.3 \mu\text{M}$ capsaicin in the presence of 10 mM extracellular Ca^{2+} (ext C) followed by 0 mM Ca^{2+} (ext B). The reversal potential (E_{rev}) was measured at the potential where the capsaicin-evoked current traces intersected with the baseline in the presence (upward arrow) or absence (downward arrow) of Ca^{2+} . (C) Relative permeability of Ca^{2+} to Na^{+} ($P_{\text{Ca}}/P_{\text{Na}}$) upon activation by capsaicin calculated using the Equation described in Methods. $*P < 0.05$; $\#P < 0.0005$, Student t test. (D and E) Representative current traces recorded from HEK293 cells transfected with TRPV1 WT (A) or TRPV1 I585V (B). Currents were evoked by the application of heat ($\sim 43\text{--}46^{\circ}\text{C}$) in the presence of 10 mM extracellular Ca^{2+} (ext C) followed by 0 mM Ca^{2+} (ext B). E_{rev} was measured at the potential where the heat-evoked current traces intersected the baseline in the presence (upward arrow) or absence (downward arrow) of Ca^{2+} . (F) $P_{\text{Ca}}/P_{\text{Na}}$ upon activation by heat. $*P < 0.05$; $**P < 0.01$, Student t test.

Figure 5.

Effects of amino acid variations of human transient receptor potential vanilloid 1 (hTRPV1) on P_{Ca}/P_{Na} following acid-induced activation of hTRPV1 SNPs and comparison of modality-dependence of P_{Ca}/P_{Na} . (A and B) Representative current traces recorded from HEK293 cells transfected with TRPV1 WT (A) or TRPV1 P91S (B). Currents were evoked by the application of acid (pH 4.5) in the presence of 10 mM extracellular Ca^{2+} (ext C) followed by 0 mM Ca^{2+} (ext B). E_{rev} was measured at the potential where the acid-evoked current traces intersected with the baseline in the presence (upward arrow) or absence (downward arrow) of Ca^{2+} . (C) P_{Ca}/P_{Na} ratio upon activation by acid. * $P < 0.05$; *** $P < 0.005$, Student t test. (D) Comparison of P_{Ca}/P_{Na} ratio upon activation by capsaicin (black), acid (blue), or heat (red) in hTRPV1 WT and SNPs. Data (mean $\pm$ SEM) are derived from Figs. 4C, 4F, and 5C. Three groups of P_{Ca}/P_{Na} values upon activation by capsaicin, acid, and heat were significantly different in hTRPV1 WT and each SNP ($P < 0.005$ in one-way analysis of variance) except A401T ($P > 0.05$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; # $P < 0.001$, Holm–Sidak post hoc analysis.

Figure 6.

Effects of amino acid variations of hTRPV1 on capsaicin-induced and acid-induced desensitization (A) Representative current traces recorded from HEK293 cells transfected with TRPV1 WT (upper panel) or TRPV1 M315I (lower panel). Currents were evoked by 2 consecutive applications of capsaicin (1 μ M) in the presence of 0.3 mM extracellular Ca^{2+} (ext C with 0.3 mM Ca^{2+}). Membrane potential (V_m) = -60 mV. Scale bar, 200 pA/pF and 1 min. (B) Ratios of second response/first response after 2 consecutive applications of capsaicin. * P < 0.05, Student t test. (C) Representative current traces recorded from HEK293 cells transfected with TRPV1 WT (upper panel) or TRPV1 Q85R (lower panel). Currents were evoked by 2 consecutive applications of acid (pH 4.5) in the presence of 2 mM extracellular Ca^{2+} (ext A with MES and 100 μ M amiloride). V_m = -60 mV. Scale bar, 100 pA/pF and 1 min; * P < 0.05. (D) The ratio of second response/first response following 2 consecutive applications of acid. * P < 0.05, Student t test.

Summary of electrophysiological phenotypes of human transient receptor potential vanilloid 1 single-nucleotide polymorphisms.

Table 1

	Modality	Q85R	P91S	M315I	T469I	I585V
Activation	Capsaicin					↓
	Heat			↓		
	Acid		↑		↑	
Ca ²⁺ permeation	Capsaicin	↑		↑		
	Heat					↓
	Acid		↑	↑		
Desensitization	Capsaicin			↑		
	Heat					
	Acid	↓				

Red, predicted gain-of-function; blue, predicted loss-of-function.

Table 2

Summary of association studies of nonsynonymous variants of transient receptor potential vanilloid 1 in humans.

		M315I	I585V	Ref
Neurosensory phenotypes	Cold pain threshold		↑ *	2, 18
	Heat pain threshold		↑ †	2
	Mechanical pain sensitivity		↓ ‡	2
	Innocuous mechanical detection		↓ §	2
	Innocuous cold detection	↓ *		2
	Development of painful type of osteoarthritis		↓ *//	37
	Salt taste		↓ ‡	12
	Short-interval intracortical facilitation	↑ ¶		26
Respiratory phenotypes	Risk of active childhood asthma/lower risk of wheeze and cough in children with asthma		↓ ¶	4
	Risk of nocturnal cough		↓ ¶#	33
Other phenotypes	Risk of type I diabetes	↑ ¶		31
	Functional dyspepsia	↓ ¶		34

P values are derived from the references.

* $P < 0.005$.

† $P < 0.001$.

‡ $P < 0.01$.

§ $P < 0.0001$.

// A group with cc genotype.

¶ $P < 0.05$.

Only in the cohort of EGEEA.