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Functional characterisation of the S512Y mutant vanilloid human TRPV1 receptor

*,[1]Kathy G. Sutton, [1]Elizabeth M. Garrett, [1]A. Richard Rutter, [1]Timothy P. Bonnert, [1,2]Wolfgang Jarolimek & [1,3]Guy R. Seabrook

[1]The Neuroscience Research Centre, Merck Sharp & Dohme, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR

1 Mammalian transient receptor potential (TRP) channels include the nonselective cation channel TRPV1, which is activated by a range of stimuli including low pH, vanilloids and heat. Previously, selective mutagenesis experiments identified an intracellular residue (S512Y) critical to discriminating between pH and vanilloid (capsaicin) gating of the rat TRPV1 receptor.

2 In this study, switching the equivalent residue in the human TRPV1 (which has some significant differences with the rat TRPV1) also rendered this channel relatively insensitive to activation by capsaicin and proved critical in determining the receptor's sensitivity to the putative endovanilloid *N*-arachidonoyl-dopamine (NADA), suggesting a similar mode of activation for these two agonists.

3 Potency of pH gating was reduced; however, voltage-dependent outward rectification properties of the pH-dependent current and gating by heat and pH sensitisation of the S512Y heat response remained unaffected.

4 Surprisingly, residual capsaicin gating was detected and could be sensitised by pH even in the presence of a competitive antagonist. Taken together, these findings indicate that effective functional interaction of capsaicin with the S512Y channel still occurred, although the vanilloid-dependent gating *per se* was severely compromised.

5 This observation provides additional evidence for capsaicin interacting at multiple sites, distinct from the S512 residue located close to the intracellular face of the pore.
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Abbreviations: CHO, Chinese hamster ovary; Compound 1, *N*-(3-methylisoquinolin-5-yl)-*N*′-[4-(trifluoromethyl)benzyl]urea; DMSO, dimethyl sulphoxide; MOPS, 3-(*N*-morpholino)propanesulphonic acid; NADA, *N*-arachidonoyl-dopamine; TRPV1, VR1, transient receptor potential vanilloid receptor subtype 1

Introduction

Painful stimuli following tissue damage are detected primarily at the peripheral terminals of nociceptor sensory neurons. A proalgesic response creating a milieu of chemical, mechanical and thermal stimuli is translated into action potential firing projected to the CNS, ultimately eliciting a perception of pain or discomfort. Capsaicin sensitivity has long served as the functional signature of a subset of nociceptive sensory neurons and the transient receptor potential vanilloid receptor subtype 1 (TRPV1) is a nociceptor-specific ligand-gated cation channel that is capable of integrating a cocktail of noxious stimuli, including capsaicin, protons and heat (Caterina *et al*., 1997; Tominaga *et al*., 1998; Caterina & Julius, 2001). Identified by expression cloning, TRPV1 is homologous to members of the transient receptor potential family of ion channels first identified in the *Drosophila* phototransduction pathway (Montell & Rubin, 1989; Caterina *et al*., 1997), and TRPV1 knockout mice have been shown to have deficits in pain detection. The TRPV1 channel is suggested to form a multimeric complex (Kedei *et al*., 2001; Kuzhikandathil *et al*., 2001; Rosenbaum *et al*., 2002), while mutagenesis studies identifying specific residues involved in ligand binding, ion permeability, gating and desensitisation have revealed the complexity of a channel activated in a polymodal manner by a multitude of allosterically interacting agonists. Gating by capsaicin and other related endovanilloids is by no means straightforward. A number of distinct regions of the channel are thought to mediate capsaicin interaction, including the well-documented intracellular binding site. Moreover, ligand gating has also been shown to involve cooperative allosteric interaction between the bound vanilloid and activation of the channel by heat and protons. For example, external acidification has two primary effects on TRPV1 function, increasing the potency of vanilloid and thermal stimuli, in part, by lowering the threshold of channel activation, in addition to mediating proton-dependent channel activation *per se* (Tominaga *et al*., 1998; Jordt *et al*., 2000).

In an effort to clarify these findings further, we have carried out a detailed charactersation of the gating and sensitisation properties of the mutant human S512Y TRPV1 channel. Previous studies have demonstrated that mutation of this

*Author for correspondence; E-mail: kathy_sutton@merck.com
[2]Current address: GlaxoSmithKline, S.p.A., Medicines Research Centre, Via Fleming 4, 37135 Verona, Italy.
[3]Current address: Merck Research Laboratories, West Point, PA 19486-0004, U.S.A.

single residue can selectively disrupt the capsaicin-dependent gating of the rat TRPV1 receptor (Jordt & Julius, 2002). The polar S512 residue located at the transition between an intracellular loop and trans-membrane domain 3 is conserved in rat, mouse, rabbit, guinea pig and chick TRPV1 and is though to represent a critical component of a rudimentary vanilloid-binding site (Jordt & Julius, 2002). Despite known species-specific differences between human and rat TRPV1 (McIntyre *et al*., 2001; Smart *et al*., 2001; Witte *et al*., 2002), as predicted from the original rat study (Jordt & Julius, 2002), vanilloid-dependent activation of this mutant human channel was significantly compromised (with heat and proton responsiveness remaining relatively intact). The effective functional separation of capsaicin gating from activation by pH and heat in this clone has enabled a detailed examination of potential allosteric crosstalk between the different activation pathways. Moreover, because a small degree of capsaicin-dependent gating was detectable, it was also possible to characterise the ability of protons to sensitise the residual capsaicin response mediated through interaction(s) at sites distinct from S512. Some of these results have been published previously in abstract form (Sutton *et al*., 2003).

Methods

S512Y construct

Human TRPV1 cDNA (gift from D. Julius) was cloned as an *Nhe*I–*Sma*I fragment in pIRES2-EFGP (Clontech, Palo Alto, CA, U.S.A.). The serine to tyrosine mutation at amino acid 512 was introduced with the Quickchange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's instructions using the sense and antisense oligonucleotides 5′-ACCCTGTTTGTGGACAGCT ACGCTGAGATGCTTTTCTTTCTG and 5′-CAGAAAGA AAAGCATCTCAGCGTAGCTGTCCACAAACAGGGT. The construct was verified by sequencing (CEQ2000XL, Beckman Coulter, Fullerton, CA, U.S.A.).

Cell culture

Human TRPV1-Chinese hamster ovary (CHO) KI cells were stably transfected using pCI-neo vector containing human TRPV1. Following selection with Geneticin, clones were assessed for functionality using a fluorometric imaging plate reader and single cell cloned by limiting dilution. CHO cells were transiently transfected with human TRPV1 S512Y cDNA in a pIRES-eGFP vector (Clontech) using Lipofectamine 2000 (Invitrogen, Carlsband, CA, U.S.A.). Cells were grown in Iscove's Modified Dulbecco's medium with glutamine, 10% FBS at 30°C, 5% CO_2 and plated onto poly-D-lysine-coated glass coverslips.

Electrophysiology

Coverslips were placed in a recording chamber and perfused at room temperature (22°C) at a rate of 1 ml min^{-1}. Recording of whole-cell currents under voltage-clamp were made with a Axopatch 200B amplifier (Axon Instuments, Foster City, CA, U.S.A.). All recordings were made at −60 mV. Capacitance transients were canceled and series resistance compensation was >70%. Fire-polished patch pipettes (Harvard Apparatus, Kent, U.K., 120TF-10) had a tip diameter ~1 μm, resistances were approximately 2–3 MΩ. The intracellular pipette solution contained (in mM): CsF 110; TEA-Cl 30; Cs-BAPTA 20; $MgCl_2$ 1; Mg-ATP 2; HEPES 10, pH 7.2 adjusted with TEA.OH. The extracellular solution contained (in mM): NaCl 165; KCl 2; $MgCl_2$ 1; $CaCl_2$ 1.67; D-glucose 17; HEPES 10, pH 7.3 (NaOH). HEPES was used to buffer solutions within the range of pH 7.3–6.3 and 3-(*N*-morpholino)propanesulphonic acid (MOPS) (10 mM) was included as a substitute for HEPES to buffer more acidic solutions (pH 5.8–3.3). Drugs were applied to the cell by a fast perfusion system (RSC-200 Biologic, Claix, France) using a large internal diameter (500 μm) triple-barrel pipette assembly. Except where stated, agonists were applied for 5 s followed by a 30 s wash period. Inhibition of the agonist response was determined following a 30 s applications of the antagonist with no intervening period of wash. Current–voltage relationships were constructed from voltage ramps (−80 mV to +80 mV in 500 ms). Temperature changes were generated by applying heated solution from a single barrel (500 μm), using a rapid solution heater (MSDRL Research Engineering). The external solution applied to the recorded cell was preheated over 10 s period using a defined temperature ramp from 22 to 50°C. The temperature of the solution was monitored in the bath by a thermoresistor placed within 50 μm distance from the recorded cell. Recordings were filtered at 2 kHz and digitized at 500–1000 Hz using pClamp hardware (Axon Instruments). All heat data were additionally filtered at 40 Hz and reduced with a decimation factor = 10. The 10-degree temperature coefficient (Q10) values were determined using the following formula

$$Q10 = \exp(\Delta T E_a / R T_1 T_2)$$

where E_a is the activation energy calculated from the slope of the slope of the Arrhenius plot and R is the gas constant (see Vyklicky *et al*., 1999). Capsaicin (Sigma-Aldrich, Dorset, U.K.) was dissolved in 100% ethanol to yield a stock concentration of 10 mM. Further dilutions were made with 100% ethanol. *N*-arachidonoyl-dopamine (NADA) and *N*-(3-methylisoquinolin-5-yl)-*N*′-[4-(trifluoromethyl)benzyl]urea (Compound 1) were dissolved in 100% dimethyl sulphoxide (DMSO). Final bath concentrations of DMSO and ethanol were <0.1%. All data are reported as mean ± s.e.m. and except where stated, statistical analysis undertaken using Student's unpaired *t*-test.

Results

S512Y disrupts gating by capsaicin

Previous studies have demonstrated that the S512Y mutation can selectively disrupt the capsaicin-dependent gating of the rat TRPV1 receptor (Jordt & Julius, 2002). Sequence alignments were performed using Clustalw with default parameters (Thompson *et al*., 1994) and revealed that the serine residue critical for effective gating of the rat receptor is conserved across all known mammalian members of the TRPV1 family (human, rat, mouse, guinea pig, rabbit and chicken, Figure 1a). Consistent with this finding, data presented in the current study confirmed the relative separation of pH- and

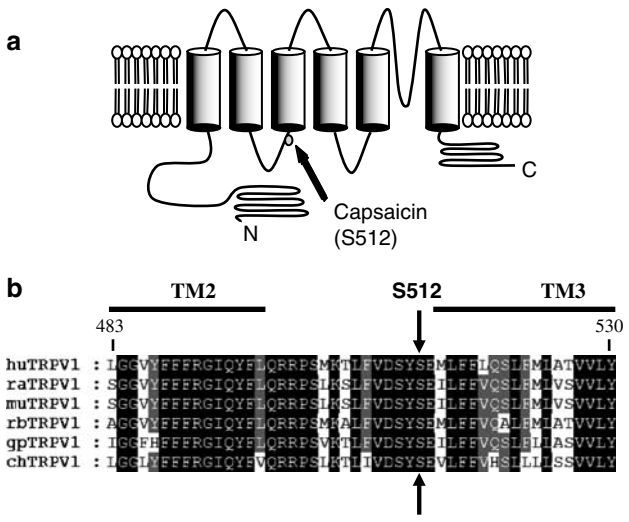

Figure 1 Sequence alignment. (a) Cartoon representing the topological structure of the TRPV1 receptor. Barrels represent the putative transmembrane regions, and the circle indicates the proposed location of the serine residue at position 512. (b) Multiple sequence alignment of human, np_542435.1; rat, np_114188.1; mouse, ENSMUSP00000006106; guinea pig, AAS13460.1; rabbit, AAR34458.1 and chicken, AY072909.1 TRPV1 receptors, of the sequence corresponding to amino acids 483–530 of human TRPV1. Amino acids conserved in all species are shaded black, >80% conservation are shaded grey. The horizontal bars represent the putative location of transmembranes 2 and 3.

vanilloid-dependent gating in the human S512Y TRPV1 homologue (hTRPV1; Figure 1).

Whole-cell currents recorded from CHO cells transiently transfected with the hTRPV1 S512Y mutant were readily activated by a proton stimulus (pH 5.5), while remaining insensitive to activation by capsaicin (500 nM, Figure 2a). Maximum steady-state currents recorded after a 5 s application of either agonist produced mean inward current amplitudes of -2.5 ± 0.7 nA ($n=7$) and -2.3 ± 1.0 nA ($n=7$) for wild-type receptors activated by capsaicin and pH, respectively. In contrast, S512Y capsaicin-dependent current amplitudes were virtually undetectable (-0.022 ± 0.017 nA, $n=10$), even though application of pH 5.5 produced a robust response (-3.5 ± 1.0 nA, $n=10$). The voltage-dependent properties of the S512Y pH-gated current remained unchanged. Membrane responses to voltage ramps (pH 5.5, Vramp = –80 to +80 mV, 320 mV s^{-1}) indicate that the mutation caused no shift in the voltage-dependence of the current (Figure 2b). Both mutant and wild-type proton-gated channels exhibited similar degrees of outwards rectification with no shift in the reversal potential (ratio +80 to −80 mV = 3.6 ± 0.9 and 3.3 ± 0.8, with mean Vrev = -1.6 ± 1.3 and 1.8 ± 1.2 mV, for mutant vs wild-type channel, respectively, $n=9$, 10).

S512Y disrupts gating by NADA

TRPV1 receptors are gated by a variety of 'vanilloid-like' endogenous ligands that are also predicted to act at the same intracellular site as capsaicin (Huang *et al*., 2002; Chu *et al*., 2003; Toth *et al*., 2003). Nanomolar concentrations of a putative endovanilloid, NADA, have been shown to exhibit 'agonist-like' properties at the TRPV1 receptor (Huang *et al*., 2002). Likewise in the present study, NADA was found to activate wild-type hTRPV1 receptors with a similar high degree of potency ($EC_{50}=150$ nM; Figure 3). Cumulative applications of increasing concentrations of NADA were observed to produce a slowly activating inward current (Vh = −60 mV), which exhibited profound outward rectification, a characteristic voltage-dependent feature of all TRPV1-mediated currents. At the highest concentration tested, NADA (10 μM) produced a maximal activation of the wild-type hTRPV1 equivalent to $51.4\pm8.4\%$ ($n=3$) of the pH 5.5 response (Figure 3b). As for capsaicin, NADA-activated currents exhibit strong outward rectification (Vramp = −80 to +80 mV, 320 mV s^{-1}, Vrev = 2.1 ± 0.3 mV, ratio +80 to −80 mV = 3.4 ± 1.5, $n=3$; Figure 3c). In contrast, even a 10-fold increased concentration of NADA (30 μM) failed to activate any detectable current in cells transfected with the S512Y compared to the wild-type response (% current normalised to pH 5.5 response = $0.05\pm0.07\%$; $n=3$; Figure 3d). Disruption of the capsaicin-binding site, therefore, proved critical in determining the receptor's sensitivity to NADA, suggesting a similar mode of activation for these two agonists.

S512Y reduced efficacy of proton-dependent activation but not gating by heat

The mutant S512Y gave robust responses to pH in comparison to wild-type currents. Surprisingly, S512Y also caused a marked reduction in channel sensitivity to protons, with a significant decrease in the half-maximal response from pH 6.0 ± 0.1 to pH 5.7 ± 0.1 for wild-type vs S512Y, respectively (Hill slope −2.7 and −2.2, respectively; $n=5$, $P<0.01$; Figure 4).

In contrast, gating of the channel by heat was unaffected by this mutated residue. Both the wild-type TRPV1 and the S512Y mutant channel produced substantial and repeatable heat-activated currents in response to a 10 s heat ramp from room temperature (~25°C) to 50°C. Moreover, there was no significant difference in the temperature-dependent gating of the mutant channel, as both wild type and S512Y exhibited identical thresholds for activation (36.6 ± 0.9°C and 35.7 ± 0.3°C, $n=4$, 5, respectively; $P=0.3$; Figure 5).

Proton-dependent sensitisation of the heat-activated current remained unchanged

Previous studies have shown that acidifying conditions sensitise the response of TRPV1 to heat by lowering the threshold for channel activation and increasing the size of the activated current (Tominaga *et al*., 1998; Vlachova *et al*., 2001). Application of a mildly acidified external saline (pH 6.8) did not in itself activate a pH-dependent current in these cells, yet still induced a substantial sensitisation of the TRPV1-mediated heat response. At pH 6.8, the shift in activation threshold for either channel was below the threshold for detection, nevertheless, pH-dependent sensitisation was readily quantified as a significant change in current amplitude (Vh = −60 mV, 45°C) for both channels (Figure 6a–d). Application of pH 6.8 produced a reversible and repeatable enhancement of the heat-evoked current amplitude for both channel types (2.49 ± 0.44 and 2.70 ± 0.45 fold increase, $n=5$, 4 for wild type and S512Y, respectively; $P=0.8$, Figure 6d).

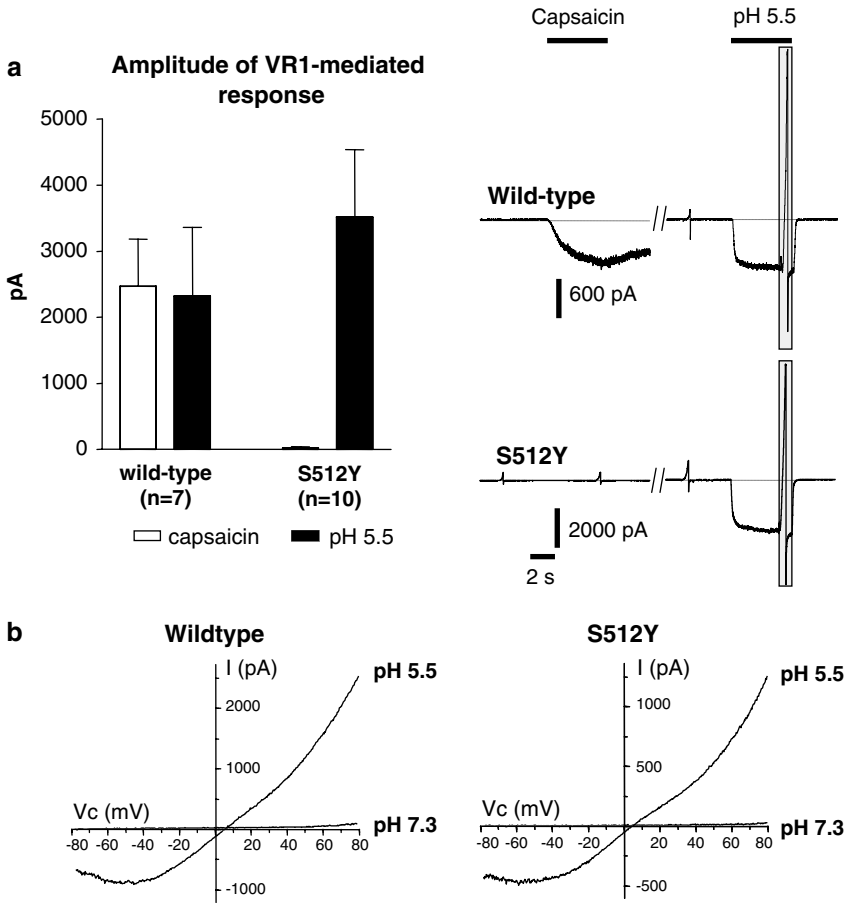

Figure 2 S512Y selectively eliminates capsaicin sensitivity. (a) Bar graph of mean (±s.e.m.) current amplitudes illustrates how introduction of the S512Y mutation into human TRPV1 virtually eliminates capsaicin sensitivity. Inset current trace of capsaicin- and pH-dependent currents recorded from a cell expressing wild type and S512Y TRPV1 (Vh = −60 mV). Grey boxes signify current responses to voltage ramp, plotted in (b). (b) Proton-dependent membrane responses to voltage ramps (pH 5.5, Vramp = –80 to +80 mV, 320 mV s^{-1}) recorded from wild-type and S512Y cells.

Consequently, S512Y does not affect the amplitude of pH-dependent sensitisation of the TRPV1 heat response.

An Arrhenius plot was also constructed to determine the Q10 of channel activation. The Q10 corresponds to the inverse slope of log current (I) plotted against $1/T$, where T is absolute temperature. Q10 values for the membrane conductance of relatively 'temperature-insensitive' channels are approximately 1.5–2.0. There was no significant difference in the Q10 values obtained for both wild-type and S512Y channel (14.1 ± 2.3 and 15.8 ± 0.8, $n = 5$, 4, respectively, Figure 6e). These data indicate that, unlike gating by capsaicin or pH, heat-dependent activation of the TRPV1 channel is completely independent from the effects of the S512Y mutant. pH-dependent sensitisation also produced a slight (but nonsignificant) decrease in the Q10 value that was similar for both channels (12.3 ± 2.4, 12.8 ± 1.6; $n = 5$, 4, for the wild-type and S512Y channel, respectively, $P = 0.14$, 0.6). The nonsignificant decrease in Q10 value seen for both channels could indicate a pH-dependent sensitisation or uncoupling of the channel from the temperature dependence associated with its gating properties under control conditions ($P = 0.3$ and 0.1 for wild-type and S512Y channel, respectively, Student's paired t-test). These data are in agreement with previous findings undertaken in DRG neurons (see Vlachova *et al*., 2001) where it is suggested that in acidified conditions, gating of the channel is not so steeply temperature-dependent as sensitisation by low pH facilitates gating and the channel has a less specific response to heat *per se*. From this data, we conclude that mutation of S512Y does not affect the ability of protons to sensitise this temperature-dependent response even though there is a significant change in the pH-dependent gating of the channel.

pH-dependent sensitisation of S512Y gating by capsaicin

We also examined if we could detect pH-dependent sensitisation of the capsaicin response, even though capsaicin-dependent gating is profoundly reduced. Amplitude of the capsaicin-gated current was normalized to the inward pH 5.8-gated current and found to activate over the concentration range 3-100 μM with an estimated EC_{50} value of just 10 μM. At maximal concentration tested (300 μM), capsaicin only produced 32% of the equivalent pH EC_{50} (pH 5.8) response, confirming that S512Y produces a substantial reduction in gating efficacy for this ligand (see Figure 7a and b).

Surprisingly, even though gating by capsaicin was severely compromised, proton-dependent sensitisation of the capsaicin response remained. Coapplication of capsaicin at 500 nM, a concentration 50-fold lower than that which produced any detectable inward current, was found to enhance the pH 5.8

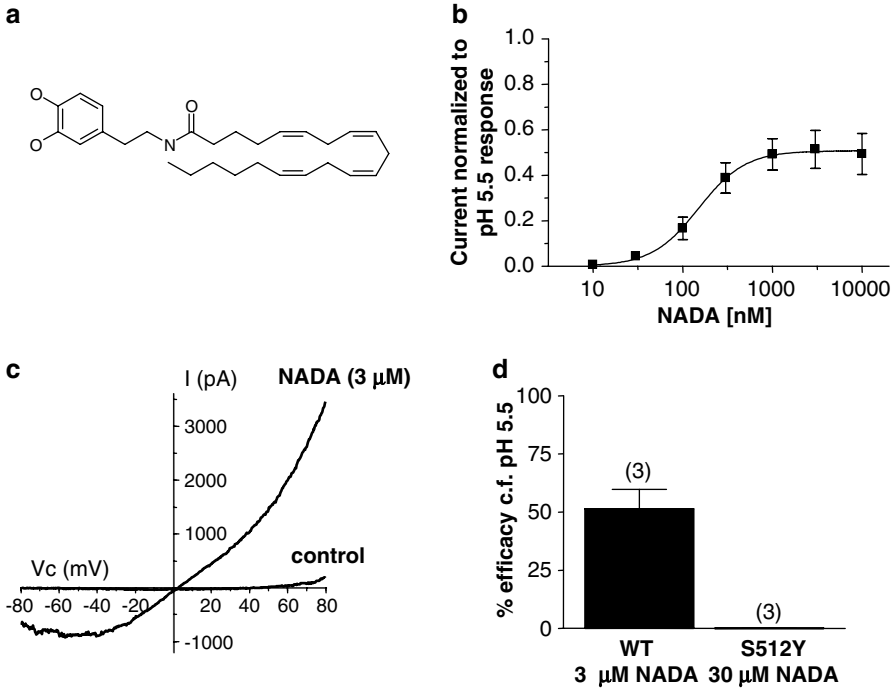

Figure 3 *N*-arachidonoyl-dopamine (NADA). (a) Structure of the putative endovanilloid TRPV1 agonist, *N*-arachidonoyl-dopamine (NADA). (b) Concentration–response curve of NADA activation of wild-type human TRPV1 (10 s cumulative applications, at 30 s intervals, mean ± s.e.m., $n = 3$). Currents were normalised to the pH 5.5 response and the EC_{50} was determined from a fit of the data (solid line) (c). Voltage-dependence of wild-type currents activated by NADA. (d) Bar graph comparing mean (± s.e.m.) NADA current amplitude normalized to the pH 5.5 response for wild-type and S512Y cells.

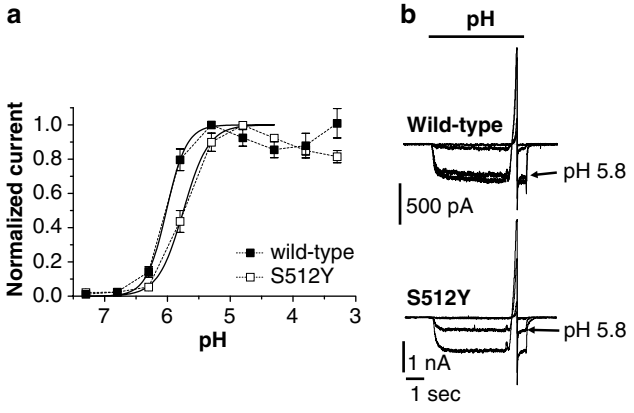

Figure 4 S512Y decreases efficacy of the pH-dependent activation of TRPV1. (a) Concentration–response curve for pH-dependent activation of mutant S512Y vs control human TRPV1. Currents were normalised to the maximum response (pH 5.3 for wild type, pH 4.8 for S512Y) and the EC_{50} was determined from a fit of the data (solid line) over the range from pH 7.3 to the maximum in each case. (b) Current traces in response to pH 6.8, 6.3, 5.8 and 5.3, illustrating the difference in efficacy for proton activation for the mutant vs wild-type channel.

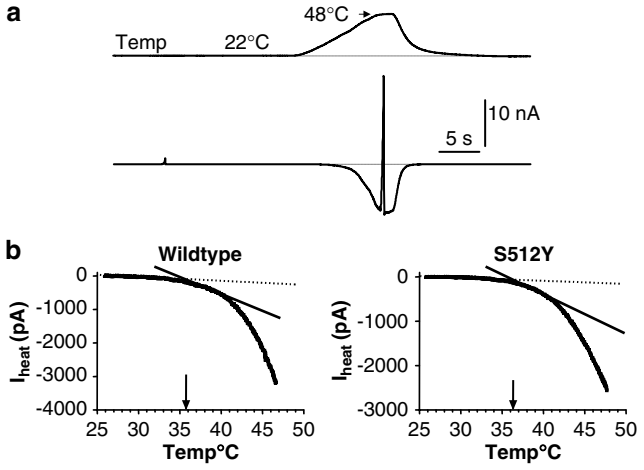

Figure 5 S512Y does not affect the threshold of heat-mediated activation. (a) Current trace showing the response of the S512Y mutant channel activated by a 10 s heat ramp from room temperature (~22°C) to 48°C. (b) Graph of heat current amplitude vs temperature. The straight black lines indicate manual fits of the data in which threshold for activation is determined as deviation from a linear response to an increase in temperature (intersection with the dotted line).

response by 31.7 ± 5.8% ($n = 13$, Figure 7c). Despite the fact that capsaicin alone did not produce a measurable inward current, gating by capsaicin over this concentration range (100–500 nM) could be detected by examining the larger outwardly rectifying current. Sensitisation of the pH response was found to significantly correlate with the capsaicin-dependent increase in the outward current at these low concentrations (Figure 7d). The pH-sensitised capsaicin response, therefore, correlates directly with capsaicin gating *per se* and indicates that, even though gating is barely detectable at these concentrations, the channel interaction with capsaicin still remains available for allosteric modulation by pH as for the wild-type channel. Protons are known to

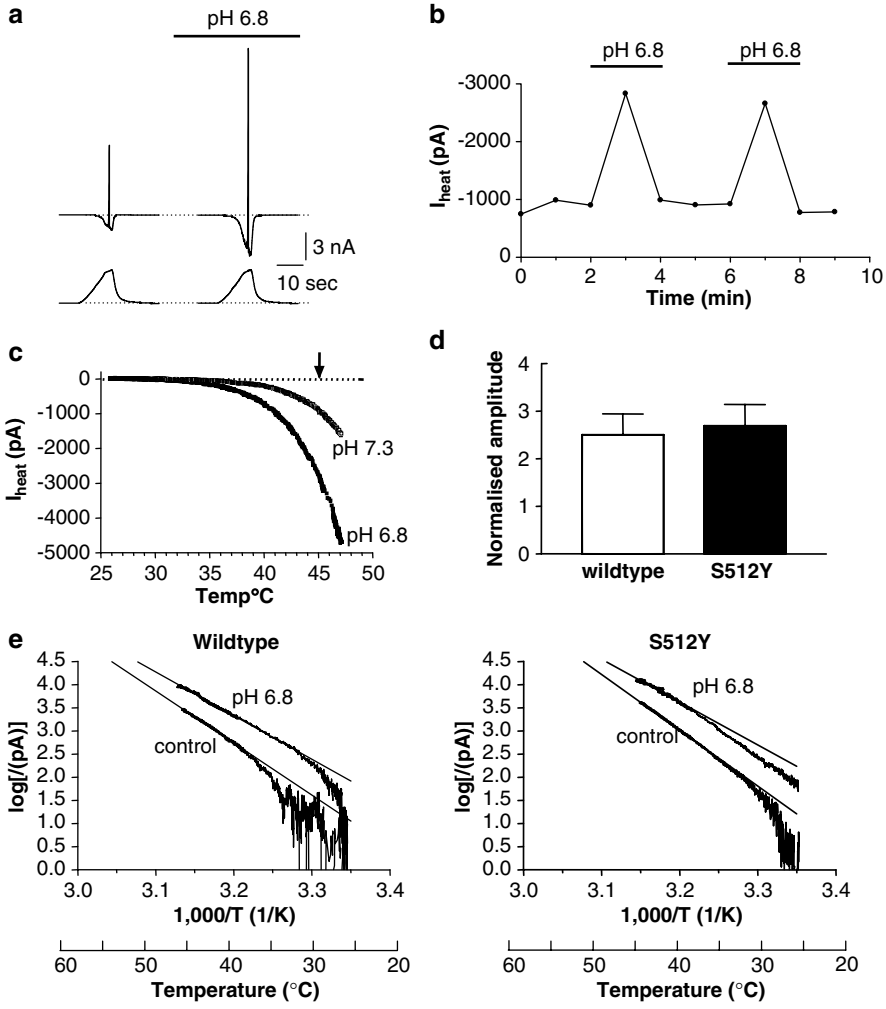

Figure 6 S512Y does not affect – amplitude of pH-dependent sensitisation of the TRPV1 heat response. (a) Current trace showing the response of the S512Y mutant channel activated by a 10 s heat ramp from room temperature (~22°C) to 48°C under control conditions and in the presence of pH 6.8. (b) Time course of current amplitudes of S512Y heat response showing reversible and repeatable enhancement by pH 6.8. (c) Graph of S512Y heat current amplitude vs temperature under control conditions and in the presence of pH 6.8. Current amplitude was measured at 45°C (arrow) (d). Bar chart of mean (±s.e.m.) sensitised heat current amplitude relative to control. S512Y does not affect – pH-dependent shift in temperature coefficient (Q_{10}) of TRPV1 activation. (e) Arrhenius plot. Straight lines indicate fit of data (linear range from 38 to 45°C) under control conditions, pH 7.3, and in the presence of pH 6.8. There is no significant difference in the absolute or pH-sensitized Q10 value obtained from the S512Y compared to the wild-type TRPV1 cells.

enhance gating by capsaicin of wild-type TRPV1 channels through a concentration-dependent decrease in capsaicin EC_{50} with no change in the maximum current amplitude (Tominaga *et al*., 1998; McLatchie & Bevan, 2001). From the literature, the shift in capsaicin efficacy seen with pH 7.4–5.5 is ~70–80-fold for native TRPV1 receptors recorded from rat DRGs. From this we deduce that pH-dependent sensitisation of the S512Y capsaicin response appears to be unaffected by the mutation, as the channel gains a current increase equivalent to a control application of 30 μM capsaicin (30%) for the pH 5.8 response observed in the presence of 500 nM capsaicin. Assuming no pH-dependent change in the maximum current amplitude, through extrapolation this represents at least ~60-fold shift in potency. Thus, the mutant channel retains the ability to increase capsaicin-binding affinity under acidified conditions and/or mediates interaction with capsaicin at a site that is distinct from that affected by S152Y (although its efficacy is still limited).

Compound 1

We therefore utilised a novel antagonist of the TRPV1 channel, Compound 1, to determine the role of residue S512 in mediating the proton-dependent increase in affinity for capsaicin. Tritiated Compound 1 also acts at the intracellular vanilloid-binding site, as its binding is fully displaced by capsaicin. The S512Y mutation significantly attenuated inhibition of proton and capsaicin-mediated activity by Compound 1. Even though wild-type pH 5.5 responses were completely inhibited with an $IC_{50} = 3.2$ nM (data not shown), application of 10 μM Compound 1 produced just 41±3.8% ($n = 10$) inhibition of the S512Y pH 5.8 response (Figure 8a and b). Inhibition of capsaicin gating of the channel was also compromised. Despite the fact that capsaicin (500 nM) did not produce a measurable inward current, effects of Compound 1 on gating by capsaicin at this concentration could be detected when looking at the larger outward current. Following

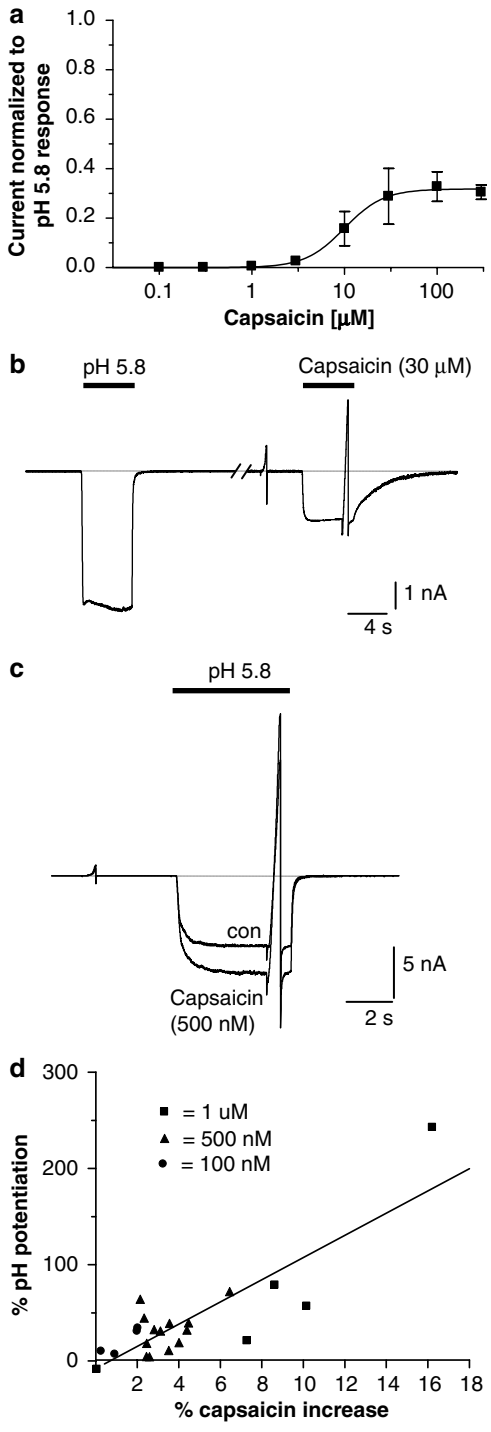

Figure 7 Characterisation of residual S512Y capsaicin activity. (a) Concentration response of capsaicin-dependent activation of S512Y (mean ± s.e.m., $n = 3$–4). Currents were normalised to the pH 5.8 response and the EC_{50} was determined from a fit of the data (solid line). (b) Current traces from the same cell comparing the maximum capsaicin response (30 μM) of the S512Y mutant channel to the current activated by pH 5.8. (c) Current trace illustrating capsaicin-dependent enhancement of the pH 5.8 current. (d) Linear regression analysis showing significant correlation between % capsaicin gating (calculated as the outward capsaicin current normalized to the inward pH 5.8 response), vs % enhancement of the inward pH-activated current. Slope = 11.5 ± 1.6, $r^2 = 0.71$, $P < 0.001$.

application of Compound 1 (10 μM), outward capsaicin-dependent currents normalised to the pH 5.8 response were only partially decreased by 67%, from 3.3 ± 0.4 to 1.1 ± 0.6%, respectively ($P < 0.05$, paired t-test $n = 6$; Figure 8b), significantly less than the 100% inhibition achieved with the wild-type channel. Radioligand binding of Compound 1 was also reduced to undetectable levels in S512Y (data not shown). This suggests that, as for capsaicin and NADA, Compound 1 interaction with the TRPV1 channel is also mediated by the residue S512, at the intracellular face of the channel. As the activity of both Compound 1 and capsaicin appear to be mediated *via* the same critical residue, this provided us with a tool to investigate the role of S512 in mediating pH-dependent enhancement of capsaicin efficacy.

Application of Compound 1 did not interfere with proton-dependent sensitisation of the capsaicin response, even though Compound 1 significantly inhibited gating by either capsaicin or pH 5.8 alone. S512Y currents potentiated by pH 5.8 in the presence of 500 nM capsaicin were enhanced by 36.2 ± 7.1 and 31.8 ± 4.8% under control conditions and following application of 10 μM Compound 1, respectively ($n = 6$, $P = 0.22$, paired t-test values; Figure 8c). Thus, the increase in capsaicin efficacy by acidification is sufficient to overcome any inhibition by Compound 1. Even though S512Y significantly compromised the ability of capsaicin to independently gate the channel, interaction with the channel in acidic conditions is sufficient enough to remain susceptible to sensitisation by pH and/or successfully out-compete inhibition by Compound 1. This data therefore supports the theory of capsaicin interaction with the channel mediated by multiple distinct sites, some of which may be more distant from the S512 residue located close to the intracellular face of the pore.

Discussion

Critical role of S512Y in mediating interaction of ligands with TRPV1

The polar S512 residue located at the transition between an intracellular loop and TM3 is conserved in rat, mouse, rabbit, guinea pig and chick TRPV1 (but not TRPV2) and thought to represent a critical component of a rudimentary vanilloid-binding-site (Jordt & Julius, 2002). This original finding by Jordt and Julius is confirmed in this study where we have demonstrated that, despite pharmacological differences between rat and human TRPV1 (McIntyre *et al*., 2001; Smart *et al*., 2001; Witte *et al*., 2002), the impact of the S512Y mutation on gating of the channel by capsaicin is preserved between both species. Jordt & Julius (2002) have shown previously that the S512Y mutation converts the rat TRPV1 intracellular binding site to one with only very residual vanilloid sensitivity. Likewise, when tested in this study, sensitivity of the human S512Y mutant to both capsaicin and NADA was found to be significantly compromised. The decrease in both capsaicin efficacy and potency suggest that residue S512 serves as a key regulatory residue for the human TRPV1 receptor, mediating channel gating by both capsaicin and to a lesser extent, pH. Nevertheless, reduced binding of the related vanilloid, RTX (Jordt & Julius, 2002) would also suggest that residue S512 may also serve as an important component for mediating ligand interaction as well as gating.

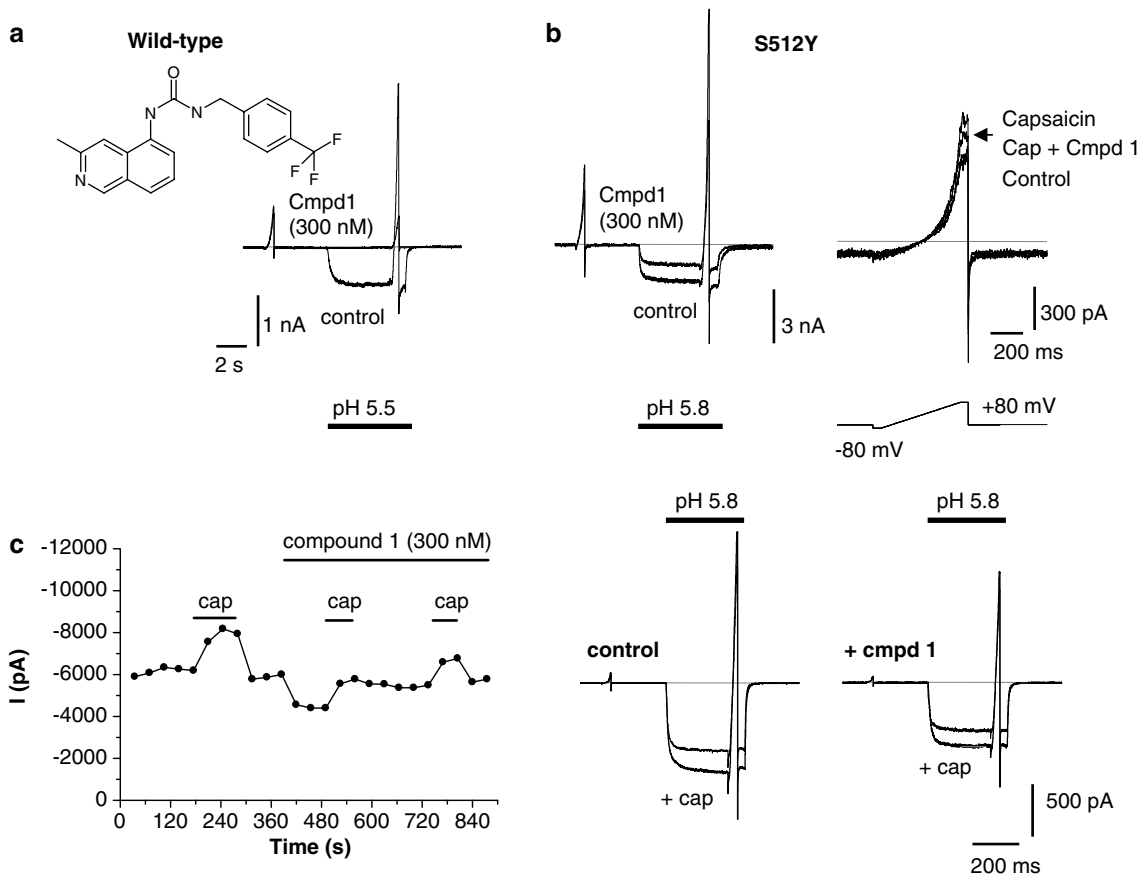

Figure 8 Compound 1 is ineffective at preventing pH-dependent sensitisation of the S512Y capsaicin response. (a) Structure of *N*-(3-methylisoquinolin-5-yl)-*N*′-[4-(trifluoromethyl)benzyl]urea (Compound 1) and current trace showing complete block of wild-type pH-mediated current. (b) S512Y current traces illustrating significant reduction in efficacy of Compound 1 for both the inward pH 5.8 current (Vh = −60 mV) and the outward capsaicin-mediated currents (activated using a voltage ramp from −80 to +80 mV, 320 mV s^{-1}). (c) Time course of current amplitudes of S512Y pH 5.8 response showing reversible and repeatable enhancement by capsaicin (500 nM) both prior to and following inhibition by Compound 1. Inset traces show capsaicin enhancement of the S512Y pH 5.8 response under control conditions and in the presence of Compound 1.

NADA has previously been shown to act as a putative ligand at the TRPV1 receptor with potency similar to capsaicin (EC_{50} ~50 nM). Efficacy of NADA in gating human TRPV1-mediated activity in this study was confirmed electrophysiologically, with an EC_{50} comparable to that determined previously using Ca imaging to monitor TRPV1 activity (150 nM; Huang *et al*., 2002). Activation by NADA was shown to be antagonised by both capsazepine and I-RTX, suggesting an interaction with the channel at the intracellular capsaicin-binding site (Huang *et al*., 2002). It is therefore clear that the S512 residue forms an important component in mediating the interaction of a number of vanilloid ligands at the interface of the channel, with disruption having profound consequences on the ability of these compounds to influence channel activity.

S512Y did not affect heat

Nevertheless, mediation of TRPV1 gating by capsaicin is not always intrinsic to overall channel activity *per se*. In this study, S512Y virtually abolished capsaicin-dependent activation with only a small (but significant) decrease in sensitivity to protons. Moreover, the heat-activation pathway remained intact, suggesting a degree of dichotomy in gating mechanisms for these different agonists. A number of other studies have demonstrated separation of changes that effect gating by heat from activation by protons and capsaicin (Jordt *et al*., 2000; Welch *et al*., 2000; Kuzhikandathil *et al*., 2001). In particular, Vlachova *et al*. (2003) demonstrated that a distal portion (Δ42) of the C-terminal region of the channel could selectively mediate thermal sensitivity with minimal impact on gating by capsaicin and protons (although more proximal sections of the tail were shown to impact upon gating by capsaicin, pH and voltage as well as heat). Recent data now suggest these findings in which capsaicin and proton sensitivity is reduced may be due to channel desensitisation as opposed to reduced ligand interaction (Liu *et al*., 2004). Nevertheless, in another study by Jung *et al*. (2002), the N- and C-terminal residues highlighted to participate in mediating capsaicin- and proton-dependent activation did not affect heat. In addition, a novel human TRPV1 splice variant has been found (named hVR1b) with a truncated N terminal, whereby activation by heat is preserved yet the channel is insensitive to capsaicin and acid (Lu *et al*., 2003). In the current study, thermal sensitivity also remained untouched in the S512Y mutant and as such represents a unique means of activating the channel, relatively independent from interaction by vanilloids or protons. The complex nature of polymodal activation of TRPV1 and the wide range of cooperativity between various ligands necessarily complicates interpretation of these studies. Consequently, the current findings of this study add weight to the theory of a certain degree of functional and regional segregation

of the various biochemical and physical activators of TRPV1, heat in particular.

Evidence for allosteric crosstalk between agonists

Mutagenesis studies also indicate that the properties mediated by capsaicin-dependent gating differ from those of other agonists (Welch *et al*., 2000). The suggestion is that TRPV1 undergoes conformational changes upon capsaicin binding, which it does not undergo in response to activation by protons, heat or voltage that is, stimulus-specific steps or pathways leading to TRPV1 activation. However, given the complex network of allosteric interactions involved in directing TRPV1 channel activity, it would perhaps be naïve to expect complete functional separation of capsaicin and proton gating. In the current study, changes in the ability of capsaicin to gate hTRPV1 were also reflected, in part, by a decrease for pH-dependent gating of the channel. Likewise, Jung *et al*. (2002) identified critical residues in the N- and C-cytosolic tails of TRPV1 that mediate capsaicin activation but also appeared to be linked functionally to activation by protons. TRPV1 is a polymodal receptor and analysis of single channel data suggests a complex allosteric gating mechanism in which capsaicin binding and channel activation are represented by at least five closed and three open states, which may in turn be influenced by pH and heat (Baumann & Martenson, 2000; McLatchie & Bevan, 2001; Premkumar *et al*., 2002; Hui *et al*., 2003; Ryu *et al*., 2003). As agonists are likely to act in 'allosteric combination' as activators of the channel, perhaps it is not surprising that in the present study, changes in capsaicin gating are also reflected to a degree by significant affects on S512Y activation by pH.

Modulation of S512Y by pH

Protons therefore are known to act not only as activators but also as modulators of TRPV1. They have been shown to set the affinity of the receptor for capsaicin by facilitating the transition between open and closed states of the channel. Modest increases in extracellular acidity enhance the potency of heat or capsaicin, in part by lowering the threshold for channel activation by either stimulus, increasing the magnitude of heat-activated currents and reducing cooperative binding of capsaicin (Caterina *et al*., 1997; Tominaga *et al*., 1998; Baumann & Martenson, 2000; McLatchie & Bevan, 2001; Ryu *et al*., 2003). In addition to existing evidence for residue-specific roles in mediating gating vs sensitisation by protons (Jordt *et al*., 2000; Kuzhikandathil *et al*., 2001), the findings of this study also supports the suggestion of a degree of functional separation, as sensitisation by pH is preserved in the S512Y mutant, even though gating efficacy *per se* is reduced.

Evidence for multiple regions mediating capsaicin interaction

Although capsaicin-dependent activation of S512Y was significantly compromised, evidence from the current study also suggests that S512Y TRPV1 still retains a rudimentary binding site for capsaicin. Capsaicin interaction was sufficient to mediate a detectable opening of the channel, albeit at relatively high concentrations. Moreover, susceptibility of capsaicin-dependent activation to sensitisation by protons persisted, supporting the theory of multiple sites for functional interaction of capsaicin with the channel. Retaining an ability to sensitise in the absence of apparent gating may go some way to explaining how a subset of dorsal root ganglion neuron may have little or no capsaicin-activated current, yet retain the ability for capsaicin to increase sensitivity to noxious heat (Vlachova *et al*., 2001).

Capsaicin-dependent activity is influenced by a variety of hydrophilic regions of the channel, which may include the N and C terminals (Jung *et al*., 2002; Vlachova *et al*., 2003; Liu *et al*., 2004) in addition to residues such as S512 and others forming part of the hydrophobic binding pocket located close to or within the intracellular pore (Welch *et al*., 2000; Jordt & Julius, 2002; Chou *et al*., 2004; Gavva *et al*., 2004; Phillips *et al*., 2004). Capsaicin activity is also associated with interactions between linkers of adjacent monomers (Kedei *et al*., 2001; Kuzhikandathil *et al*., 2001; Rosenbaum *et al*., 2002), external residues (Vyklicky *et al*., 2003) and allosteric conformational changes distinct from those specifically mediating capsaicin binding (Kuzhikandathil *et al*., 2001).

Through selective deletion of the C-terminal region of the TRPV1 receptor, Vlachova *et al*. (2003) have also demonstrated that the accompanying loss of channel sensitivity to capsaicin is associated with a loss of voltage-dependent sensitivity, suggesting a close association (functional and/or allosteric) between C-terminal-mediated capsaicin activity and the putative voltage-sensor of the channel (see also Liu *et al*., 2004). If the loss of activity in the truncated mutant reflects an altered structural conformation or loss of association between these two modules, it is perhaps not surprising that in the present study the voltage-dependent rectification properties were not affected by the S512Y mutation, which is regionally distinct from the C terminal.

In summary, binding and gating of TRPV1 by capsaicin appears to be highly complex in nature. There is therefore a growing body of evidence to suggest that activation of the vanilloid receptor requires cooperative interaction between several distinct regions within the channel complex. Single channel gating studies support this theory and suggest that capsaicin is able to interact with the TRPV1 receptor at multiple functional binding sites (Hui *et al*., 2003). Findings from this study support the view that the intracellular face of the TRPV1 channel and the S512 residue form a critical region responsible for mediating effective gating by capsaicin. Nevertheless, it also appears that other regions may also participate to a degree, enabling interaction of vanilloids with the channel that is sufficient for effective allosteric crosstalk between agonists to occur.

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