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Functional Mapping of the Transient Receptor Potential Vanilloid 1 Intracellular Binding Site

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ABSTRACT

Capsaicin (vanilloid) sensitivity has long served as the functional signature of a subset of nociceptive sensory neurons. Mutagenesis studies have revealed seemingly distinct regions involved in mediating ligand binding and channel activation at the capsaicin binding site. Residue 547 (transmembrane region 4) mediates significant species differences in resiniferatoxin (RTX) sensitivity, and the Ser^{512} residue is critical in discriminating between pH and capsaicin gating. In the present study, the pharmacological profiles of a variety of ligands were studied to investigate cross-talk between these two regions. Exchange of residue 547 between species mediated a difference in capsaicin and RTX-dependent gating. Likewise, the potency of iodoresiniferatoxin (I-RTX) and a novel transient receptor potential vanilloid 1 antagonist were also altered. Experiments using the S512Y mutant channel have confirmed the importance of residue 512 for functional interaction of capsaicin and our novel antagonist. In this study, we were surprised to find that the mutation S512Y converted the activity of the antagonist I-RTX into an intrinsic agonist, albeit with a lower potency than its parent compound, RTX. Recent studies have proposed a novel model for the receptor, based on the X-ray crystal structure of the voltage-dependent potassium channel, in which both the 512 and 547 amino acid residues are in close proximity. Our data support the model whereby intracellular ligand interaction occurs within an S3–S4 "sensor" domain, enabling binding of ligands to be transduced to functional gating of the channel. The binding pocket also seems to be exquisitely sensitive to residue-specific interaction with ligands, because subtle changes in either ligand or channel structure can have profound effects on channel activity.

The transient receptor potential vanilloid 1 (TRPV1) receptor is a nonselective cation channel with high permeability for calcium first identified through functional expression cloning by virtue of its sensitivity to activation by the vanilloid capsaicin (Caterina et al., 1997). TRPV1 acts as a key integrator of noxious stimuli such as heat and protons and is sensitive to a number of endogenous ligands such as certain inflammatory lipoxygenase products and the endocannibinoid anandamide. In addition, TRPV1 is also sensitized by intracellular substances such as protein kinase C (Cesare and McNaughton, 1996; Bhave et al., 2003), nerve growth factor, and bradykinin (Chuang et al., 2001). The mechanism by which the channel integrates all of these inputs is by no means trivial, and a number of different intra- and extracellular regions are implicated in mediating ligand binding, channel gating, and desensitization.

Capsaicin desensitization of TRPV1 activity has been used as a treatment for pain, and the therapeutic potential of TRPV1 makes it important to determine the molecular components of the channel that make up the vanilloid binding site. Human and rat amino acid sequences coding for the TRPV1 receptor share 86% identity; however, small species differences in coding have been shown to have major functional consequences. The antagonist capsazepine completely blocks the human receptor but is only a partial antagonist for rat TRPV1 responses to low pH (Seabrook et al., 2002; Phillips et al., 2004). Previous studies have also shown differences between the rat and human isoforms with regard to sensitivity to agonist binding, with the rat receptor binding resiniferatoxin (RTX) at a higher affinity than the human

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ABBREVIATIONS: TRPV1, transient receptor potential vanilloid 1; TM, transmembrane domain; RTX, resiniferatoxin; I-RTX, iodoresiniferatoxin; HEK, human embryonic kidney; compound A, *N*-(3-methylisoquinolin-5-yl)-*N*′-[4-(trifluoromethyl)benzyl]urea; WT, wild type; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; MES, 4-morpholineethanesulfonic acid.

(Chou et al., 2004). To maximize the therapeutic potential of TRPV1, it is important to determine which residues are the source of these species differences to improve the predictive quality of animal data through to humans in the clinic.

We and others have identified a number of critical residues within the S3 and S4 (sensor) regions that have been shown to be responsible for major species-specific differences in vanilloid activity (Chou et al., 2004; Gavva et al., 2004; Phillips et al., 2004). A single mutation embedded in the S4 transmembrane domain of hTRPV1, L547M, produced a 30-fold increase in sensitivity to [^{3}H]RTX, whereas the reverse mutation in the rat isoform caused a decrease in sensitivity of equal amplitude (Chou et al., 2004). Capsaicin is believed to bind to and activate the TRPV1 receptor at a specific intracellular site (Jung et al., 1999). Functional studies with the capsaicin-insensitive avian and rabbit orthologs of TRPV1 (Jordt and Julius, 2002; Gavva et al., 2004) have determined that capsaicin gating is mediated by a domain on the receptor that includes residues at positions 511 and 512, found at the transition between transmembrane domain 3 (TM3) and an intracellular loop. Other evidence supports the notion that apparently distinct regions of the TRPV1 channel are also associated with the vanilloid activation pathway. For example, Welch et al. (2000) identified three amino acid residues near the pore of the receptor that are involved in capsaicin gating but not in heat or proton activation of the channel. These findings have led to a reassessment of the traditional six-transmembrane structure proposed for this channel in light of the new X-ray crystal structure recently determined for the voltage-dependent potassium channel (K_vAP; Jiang et al., 2003). Together, the structure-activity data available for TRPV1 supports the notion that gating of this channel may involve movement of a paddle structure. In this model, the TM3/4 region of the channel is predicted to form a gating paddle, with residues such as 547 forming part of the binding site accessible from the intracellular interface, as opposed to being buried deep within the transmembrane domain in the traditional homology model associated with TRPV1 (Jordt and Julius, 2002; Chou et al., 2004; Gavva et al., 2004; Phillips et al., 2004).

In this study, we used mutant TRPV1 channels to look at the impact and potential cross-talk between mutations that affect the interaction of a variety of ligands with residues 512 and 547 (Fig. 1). Functional coincidence of these residues on the interaction of related TRPV1 ligands suggests the close physical association of these two residues within a common binding pocket (Chou et al., 2004). Functional mapping of the intracellular ligand binding site should give us a better insight into the gating mechanism of the receptor and a greater understanding of the structure-activity relationships that govern species differences between human and rat isoforms.

Materials and Methods

Cell Culture. Human embryonic kidney cells (HEKs, tsA-201-AEQ17) were stably transfected with rat TRPV1 receptors, and human TRPV1-Chinese hamster ovary KI cells were stably transfected using pCl-neo vector as described previously (Seabrook et al., 2002). Nucleotide substitutions in the cDNA sequences were generated by insertional mutagenesis using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). HEK cells were transiently transfected with mutant TRPV1 cDNA constructs in a pIRES-eGFP (Clontech, Mountain View, CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Chinese hamster ovary cells were transiently transfected with human TRPV1 S512Y cDNA also in a pIRES-eGFP vector (Clontech) using Lipofectamine 2000 (Invitrogen). Cells were grown in Iscove's modified Dulbecco's medium with glutamine and 10% fetal bovine serum, plated onto poly(D-lysine)-coated glass coverslips, and kept at 30°C for electrophysiological studies.

Electrophysiology. Coverslips were placed in a recording chamber and perfused at room temperature (22°C) at a rate of 1 ml/min. Recordings of whole-cell currents under voltage-clamp were made with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). All recordings were made at a holding voltage of −60 mV. Capacitance transients were cancelled, and series-resistance compensation was >70%. Fire-polished patch pipettes (Clarke instruments 120TF-10; Clarke Electromedical Instruments, Pangbourne, UK) had a tip diameter of ~1 μm with resistances of between 2 and 3 MΩ. The intracellular pipette solution contained 110 mM CsF, 30 mM TEA-chloride, 20 mM cesium-BAPTA, 1 mM $MgCl_2$, 2 mM magnesium-ATP, and 10 mM HEPES, pH 7.2 (TEA-OH). The extracellular solution contained 165 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.67 mM $CaCl_2$, 17 mM D-glucose, and 10 mM HEPES, pH adjusted to 7.3 with NaOH. MES (10 mM) was included as a substitute for HEPES to buffer more acidic solutions of pH 5.5. Drugs were applied to the cell by a fast-perfusion system (RSC-200; Biologic Inc., Claix, France) using a large internal diameter (500 μM) triple-barreled pipette assembly. Except where stated, agonists were applied for 5 s followed by a 30-s washout period. Inhibition of the agonist response was determined after a 30-s application of the antagonist with no intervening period of wash. To control for cross-contamination by highly potent ligands such as RTX and I-RTX, perfusion reservoirs and tubing were rinsed with 70% ethanol between experiments, only one cell per coverslip was used, and coverslips were regularly alternated between species and mutant constructs. Recordings were filtered at 2 kHz and digitized at 500 to 1000 Hz using pClamp

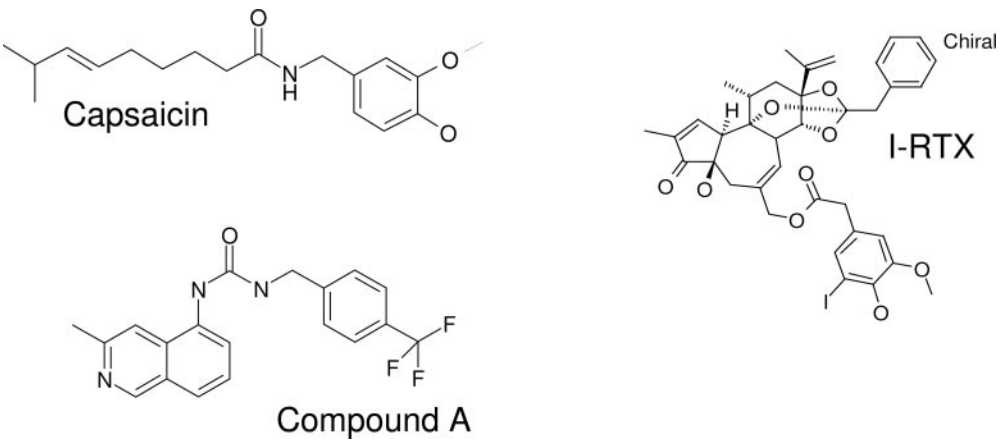


Fig. 1. Chemical structure of TRPV1 ligands.

hardware (Molecular Devices). Capsaicin (Sigma-Aldrich, Poole, Dorset, UK) was dissolved in 100% ethanol to yield a stock concentration of 10 mM. Further dilutions were made with 100% ethanol. Compound A [*N*-(3-methylisoquinolin-5-yl)-*N*′-[4-(trifluoromethyl)-benzyl]urea], RTX (Sigma-Aldrich), and I-RTX were dissolved in 100% dimethyl sulfoxide. Final bath concentrations of dimethyl sulfoxide and ethanol were <0.3%. All data are reported as mean ± S.E.M., and except where stated, statistical analysis was undertaken using Student's unpaired *t* test.

Binding Studies. HEK 293 cells were transiently transfected in parallel with TRPV1 cDNAs and cultured for 48 h. Human TRPV1 cDNAs were expressed in pIRES_eGFP and rat TRPV1 cDNAs in pcDNA3.1. Transfected cells were harvested and collected by centrifugation at 1000*g* for 10 min. Cell pellets were homogenized in ice-cold 25 mM HEPES buffer, pH 7.2, supplemented with protease inhibitors (1 tablet/50 ml; Roche, Indianapolis, IN) and centrifuged at 3000*g* for 10 min to remove nuclei and cellular debris. The partially purified membranes were then pelleted by centrifugation at 48,000*g* for 10 min and resuspended in binding buffer (20 mM Tris-HCl, pH 7.5) to a final protein concentration of 200 μg/ml. Cell membranes were incubated with [^{3}H]compound A in a final volume of 0.5 ml for 1 h at room temperature. Nonspecific binding was determined using 300 μM capsaicin. Binding was terminated by rapid filtration through Whatman GF/B glass fiber filters presoaked in 0.5% polyethylenimine. Filters were washed three times with 5 ml of wash buffer (10 mM Tris-HCl, pH 7.5), and bound radioactivity was measured by liquid scintillation detection.

Results

Residue 547 Mediates Functionality of Both Resiniferatoxin and Iodo-Resiniferatoxin. Initial studies confirmed clear species-specific differences in RTX potency as described previously (Chou et al., 2004). This was achieved by comparing RTX-induced currents in both wild-type and mutant TRPV1 receptors using whole-cell, patch-clamp electrophysiology. Activation of TRPV1 by RTX was achieved using sequential 3-min applications of the agonist at increasing concentrations without any intervening washout period. Using this protocol, RTX activated rat wild-type (WT) TRPV1 with an EC_{50} value of 75 ± 14 pM (n = 4), which was 4-fold more potent than at the human WT TRPV1 receptor (EC_{50} = 298 ± 52 pM; n = 6, $p < 0.05$). When the residue at position 547 is switched between the two receptor homologs, the M547L rat mutant and the L547M human mutant receptor are produced. Replacement with the rat-specific methionine in the human construct (L547M) produced a significant gain of function, with the EC_{50} value increasing to 61 ± 20 pM (n = 4; $p < 0.01$), a figure comparable with that of the wild-type rat receptor (Fig. 2a). Conversely, when the reverse mutation was made in the rat construct (M547L) a loss of function was observed, although in this instance, the change was not significant (EC_{50} = 197 ± 63 pM; n = 4). From these

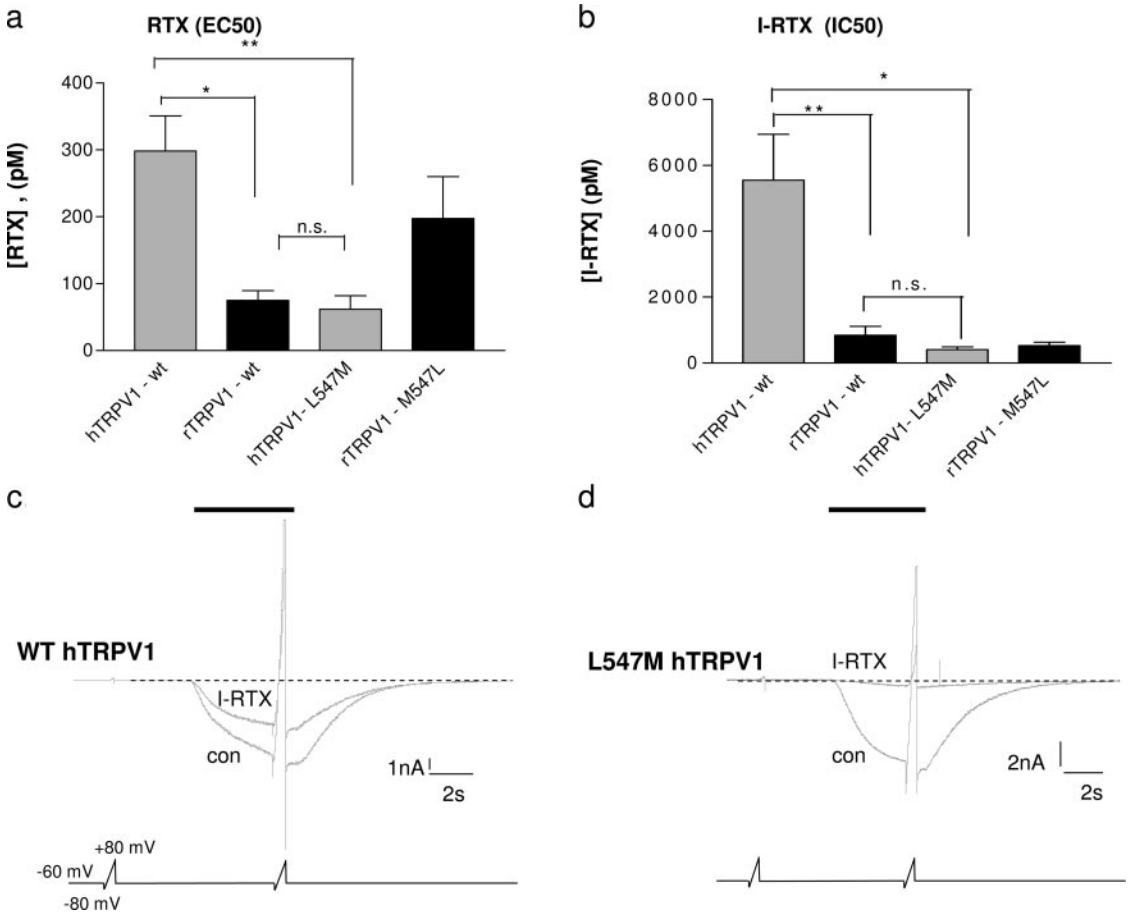


Fig. 2. Bar graph of mean EC_{50} values of RTX and IC_{50} values of I-RTX (± S.E.M.). a, the introduction of the leucine residue in place of the methionine at residue 547 in human TRPV1 significantly increases the potency of RTX (n = 4–6). b, the introduction of the leucine residue at position 547 in human TRPV1 increases the potency of I-RTX compared with wild type when challenging the cells with an application of 500 nM capsaicin (n = 3–6). c, a typical current trace showing 3 nM I-RTX inhibits the 500 nM capsaicin response by less than 50% at the wild-type human TRPV1 receptor. The black bar indicates capsaicin application. The voltage protocol is illustrated below the trace indicating when voltage ramps from −80 to +80 mV (0.5 mV/s^{-1}) were applied to the cell. d, a typical current trace showing 3 nM I-RTX inhibits the 500 nM capsaicin response by more than 90% at the L547M human TRPV1 receptor.

initial results, it can be seen that the species differences that we originally observed via binding assays and oocyte recordings (Chou et al., 2004) show functional correlation in a mammalian expression system.

The halogenated version of RTX, I-RTX, has been identified previously as a high-affinity antagonist of the TRPV1 receptor, also with differing potencies at both the human and rat receptors (Seabrook et al., 2002). Hence, we also looked at the effect of the 547 residue on the ability of I-RTX to antagonize the response of TRPV1 to 500 nM capsaicin. As for RTX, the antagonist I-RTX was found to be significantly more potent at the rat receptor versus human receptor, inhibiting the capsaicin response with IC_{50} values of 0.85 ± 0.26 ($n = 9$) and 5.5 ± 1.4 nM ($n = 8$), respectively (Fig. 2b; $p < 0.01$). After substitution of the rat-specific methionine into the human construct, I-RTX gained functional potency (L547M, $IC_{50} = 0.40 \pm 0.09$ nM, $n = 4$, $p < 0.05$), although in this instance, the converse change showed little effect (M547L, $IC_{50} = 0.53 \pm 0.10$ nM, $n = 4$). Therefore, introduction of the methionine residue enables the human receptor to interact more effectively with both agonist and antagonist alike in a manner that is not matched by leucine.

Residue 547 Also Mediates the Activity of Other Agonists and Antagonists. We then investigated the species differences seen in capsaicin sensitivity. Activation of TRPV1 by capsaicin was achieved using sequential 5-s applications of the agonist at increasing concentrations with a 30-s intervening washout period. In this instance, capsaicin was found to be significantly more potent at the human WT receptor than at the rat WT receptor, displaying a reverse species specificity to RTX (166 ± 21 and 387 ± 69 nM, $n = 10$ and 7, respectively; $p < 0.05$). When the 547 residue was switched between the rat and the human constructs, the sensitivity to the agonist was again altered. In this instance, this mutation caused a significant decrease in potency in the human mutant compared with the human wild type, with the EC_{50} value decreasing to 377 ± 89 nM ($n = 4$; $p < 0.01$; Fig. 3a), a value comparable with that of the rat wild type. As for both RTX and I-RTX, when the reverse mutation was made in the rat TRPV1 construct, there was no significant difference (421 ± 143 nM, $n = 4$; Fig. 3b).

We have previously reported that the S512Y mutant causes a small but significant decrease in the ability of protons to gate the TRPV1 receptor (Sutton et al., 2005). In this study, it was also interesting to note that, as for the S512Y mutation, switching the 547 residue from the rat receptor caused a similar decrease in the potency of protons at the human L547M mutant receptor (mean EC_{50} value decreasing from pH 6.00 ± 0.05 to pH 5.45 ± 0.10, $n = 6$ and 3, $p < 0.001$ for WT versus mutant receptor, respectively; data not shown). Conversely, no increase was seen when the reverse switch was made in the rat receptor (M547L), paralleling the lack of effect for this mutation on capsaicin gating.

A novel TRPV1 antagonist, compound A, which shows cross-species differences in activity, was also tested at the various TRPV1 constructs. Binding affinities were determined in a radioligand binding assay using tritiated compound A. Specific binding to the WT human receptor was best fit by a single high-affinity binding site yielding a K_d value of 6.2 ± 0.9 nM. Mutation of the leucine 547 residue to the rat-specific methionine caused a significant decrease in binding affinity ($K_d = 150 \pm 40$ nM). Because binding to the mutant construct occurred with such low affinity, true K_d values were difficult to determine accurately. K_d values were therefore estimated by extrapolating the sigmoidal curve to the B_{max} value derived for the WT construct. The reverse effect on binding was observed for rat constructs, such that WT TRPV1, containing methionine at residue 547, bound compound A with comparable affinity ($K_d = 180 \pm 30$ nM) to the mutant human receptor. Mutation of residue 547 to the human equivalent, leucine, caused an increase in affinity ($K_d = 29 \pm 5$ nM). Because K_d values were again difficult to determine accurately for low-affinity binding to the rat WT, construct they were estimated by extrapolating the sigmoidal curve fit to the B_{max} value derived for the rat M547L mutant.

As for RTX, this change in binding affinity was also reflected by a change in functional potency, with a significant increase in IC_{50} value observed for the human mutant (L547M). Using whole-cell patch clamp, the potency of compound A against the mutant human and rat TRPV1 receptors was investigated for inhibition of both capsaicin and pH 5.5 responses. The compound exhibited a profile similar to that of capsaicin, because it was more potent at the WT human receptor compared with the rat. When challenged, the IC_{50} values were 2.49 ± 0.49 and 3.7 ± 1.4 nM ($n = 3$ and 3) at the human receptor, as opposed to 12.5 ± 2.1 and 40 ± 10.2 nM ($n = 4$ and 5) at the rat wild-type receptor versus capsaicin

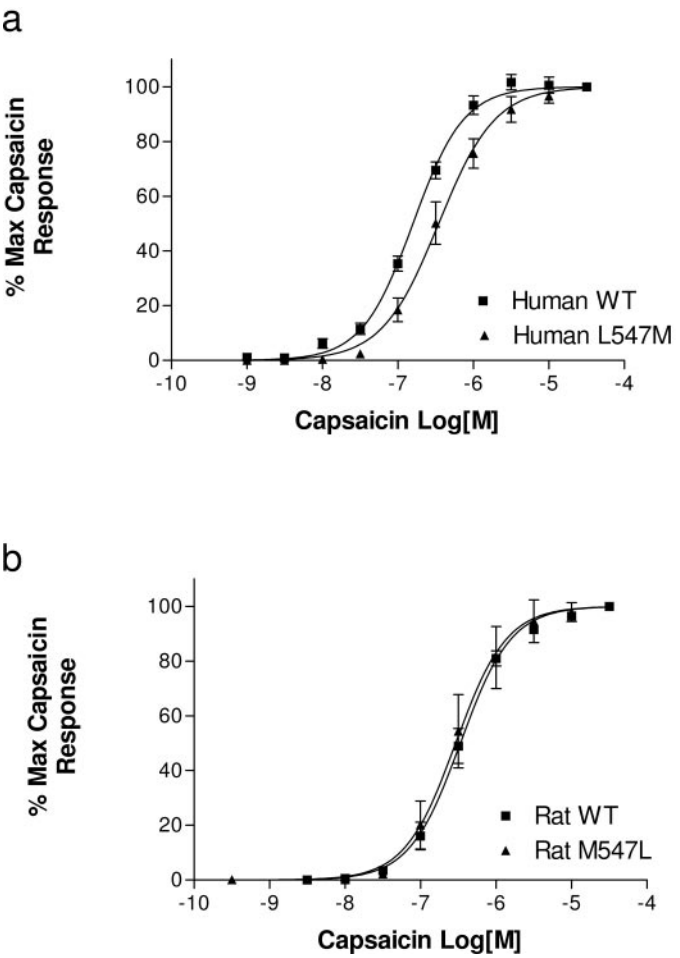
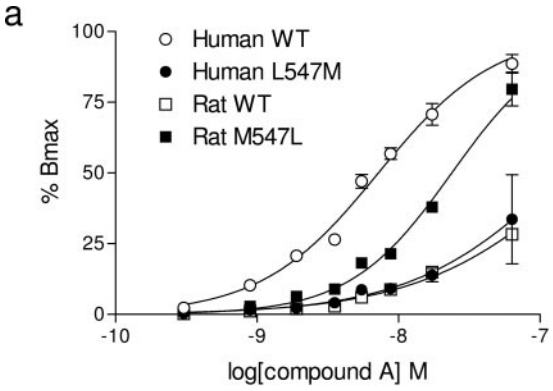


Fig. 3. a and b, concentration responses of capsaicin-dependent activation of WT and mutant TRPV1 receptors (mean ± S.E.M., $n = 4$–10). Currents were normalized to the maximum capsaicin response (30 μM), and the EC_{50} value was determined from a fit of the data (solid line).

and pH, respectively ($p < 0.05$). The compound showed a significant loss of potency when tested against both capsaicin and pH at the mutant human receptor (L547M, 12.6 ± 1.4 and 59 ± 13.3 nM, $n = 3$ and 4, respectively; $p < 0.05$). Conversely, an increase in potency was observed in the mutant rat receptor (M547L, 6.4 ± 0.8 and 14.9 ± 2.9 nM, $n = 4$ and 4, respectively; Fig. 4, b and c), although this only reached significance for capsaicin as an agonist ($p < 0.05$). Therefore, as for capsaicin, the methionine substitution disrupted compound A inhibition of the human TRPV1; however, in this instance, switching the leucine to the rat receptor also imparted a reciprocal significant gain of function.

Altering Residue 512 Converts I-RTX from an Antagonist to an Agonist. To further investigate the binding domain of TRPV1 we looked at the involvement of residue 512, which is also located within the binding pocket proposed by Chou et al. (2004). It has been reported previously that the S512Y mutation in both the rat and the human TRPV1 significantly compromises the receptor's ability to be gated by capsaicin but does not affect activation by pH (Jordt and Julius, 2002). Moreover, switching this residue causes a significant decrease in the ability of compound A to antagonize the proton response (Sutton et al., 2005). We investigated how this mutation would alter the properties of the antagonist I-RTX. Most surprisingly, however, in the present study, I-RTX was found to have intrinsic agonist properties when tested against the S512Y mutant. Cumulative applications of increasing concentrations of I-RTX caused a dose-dependent increase in receptor activation (EC_{50} = 259 nM), which could be detected as an increase in outward current (Fig. 5, a and b). There was no indication of any residual antagonist activity because I-RTX did not inhibit the pH-dependent activation of TRPV1. As for other agonists such as capsaicin, acidification with pH 5.8 was shown to enhance the agonist potency of I-RTX, producing a 20-fold decrease in the EC_{50} value (= 11.7 nM). This was calculated from the dose-dependent enhancement of the inward current response to pH 5.8 (Fig. 5, a and c). Thus, one unpredicted effect of the S512Y mutation was to convert I-RTX into an agonist with nanomolar potency, albeit with much lower efficacy than its counterpart (RTX) for the WT channel. Taken together, these data indicate that S512Y is a critical residue for mediating the functional influence and/or interaction of both agonists and antagonists with the channel.



	Kd, nM
Human WT	6.2 ± 0.9
Human L547M	150 ± 40
Rat WT	180 ± 30
Rat M547L	29 ± 5

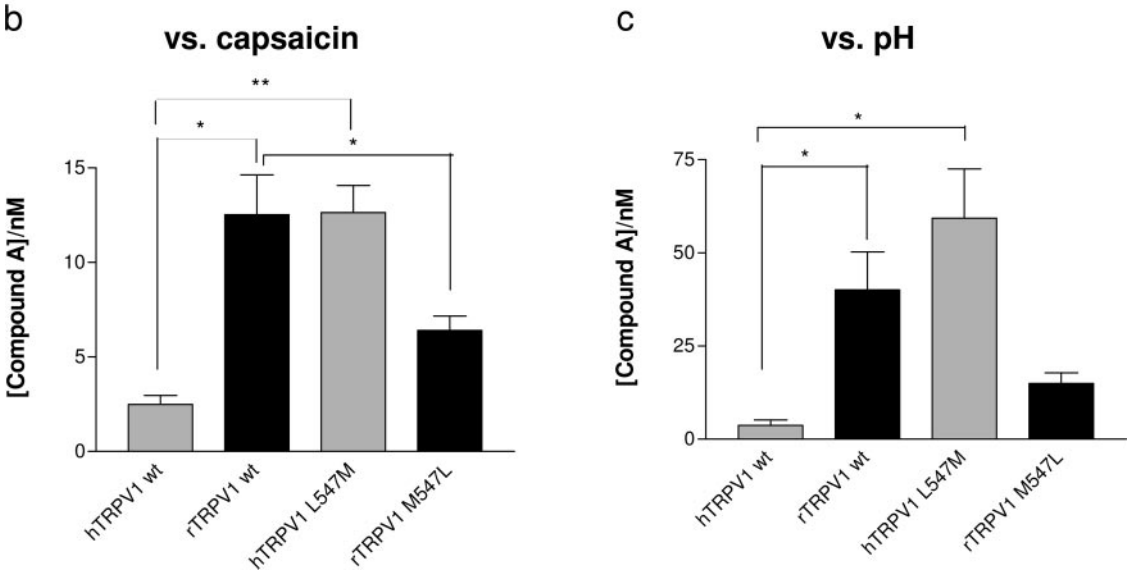


Fig. 4. a, radioligand binding studies carried out using tritiated compound A show the differences in binding affinity of the compound between rat and human TRPV1 and their respective mutants. Data were analyzed by nonlinear regression analysis and fit to sigmoidal dose-response curves to derive K_d and B_{max} values. K_d values for low-affinity binding to the human L547M mutant and rat WT constructs were determined by extrapolating curves to the B_{max} values that were derived from binding to human WT and rat M547L mutant constructs, respectively. Specific binding is presented as a percentage of the appropriate B_{max} value. The graph presented shows representative data from $n = 3$ independent determinations. b and c, bar graph showing the mean potency of compound A for various TRPV1 constructs ± S.E.M. values when tested against capsaicin or pH 5.5.

Discussion

We have characterized the influence of two regionally distinct residues, 512 and 547, on the functional interaction of a variety of agonists and antagonists of the TRPV1 receptor. The traditional six-transmembrane model of TRPV1 situates residue 547 deep within transmembrane domain 4. This location has no obvious structural association with Ser^{512}, although both residues are implicated in forming a critical part of the rudimentary vanilloid binding site. An alternative homology model for TRPV1 places both residues within a common binding pocket in positions that might be expected to influence the functional interaction of vanilloids such as capsaicin and RTX (Chou et al., 2004). Therefore, compounds interacting within this binding pocket that are affected by Ser^{512} might also be expected to be influenced by residue 547. In this study, the side-chain chemistry of both residues was found to have a significant impact on the activity of related agonists and antagonists, a finding which suggests the close physical association of these two residues within a common ligand interaction site.

We confirmed the species-specific interaction of RTX with residue 547 (Chou et al., 2004) using a mammalian expression system, and this translated to the related antagonist, I-RTX. The influence of Met^{547} proved to be pivotal in mediating species differences, regardless of whether the human channel was more or less affected by either agonist or antagonist. In both instances, Met^{547} enabled the human receptor to interact more effectively with both RTX and I-RTX in a manner that was not matched by Leu^{547}. The model proposed in Chou et al. (2004) situates Met^{547} alongside RTX, suggesting a direct steric influence on ligand binding. Capsaicin and capsazepine are competitive inhibitors of RTX binding (Szallasi et al., 1993). It is noteworthy that capsazepine exhibits the reverse species specificity compared with RTX, because it is more effective at the human channel and only a partial antagonist of the rat pH response (Seabrook et al., 2002). We

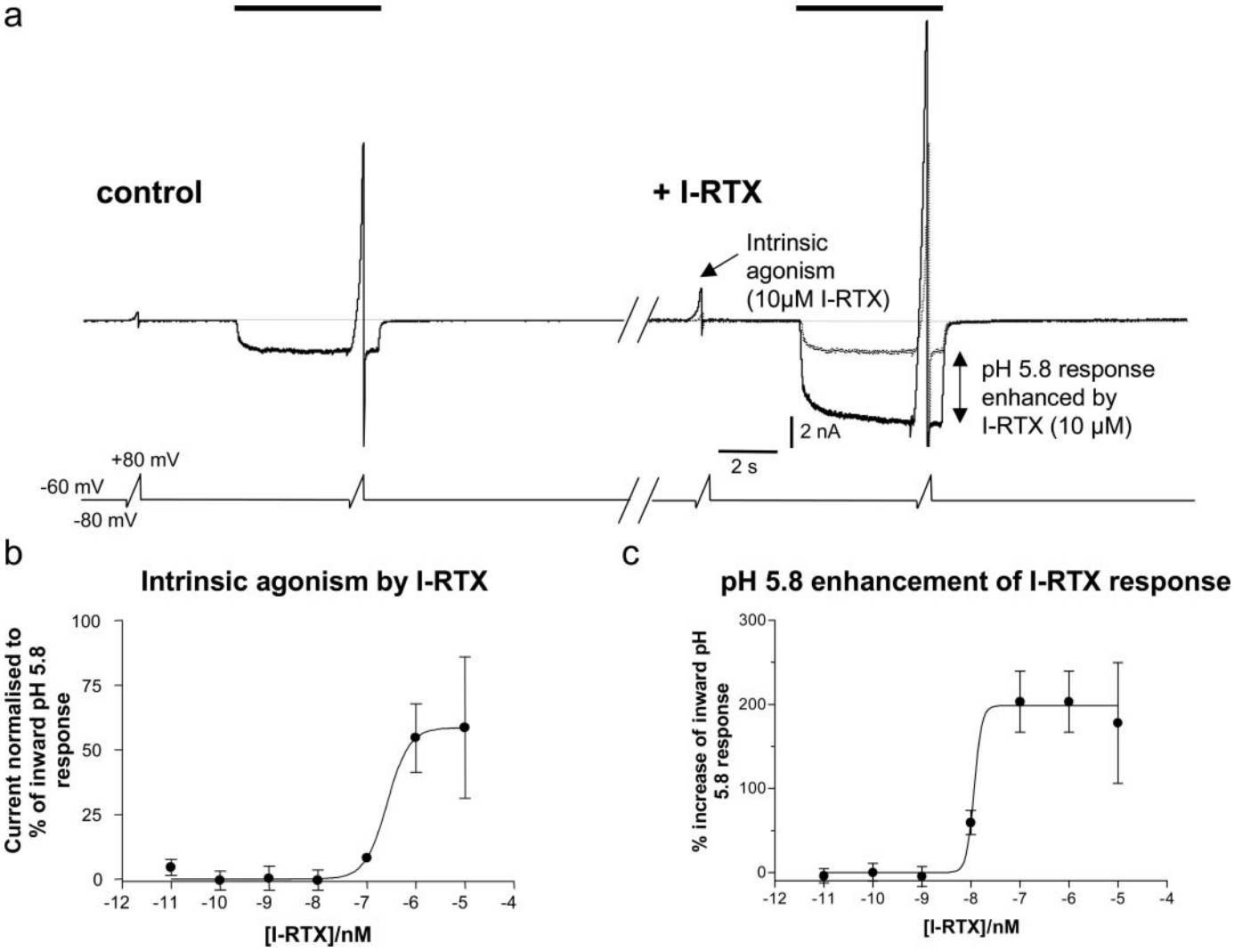


Fig. 5. a, current trace showing intrinsic activity of I-RTX detected as an increase in outward current in response to a voltage ramp (−80 to +80 mV, 0.3 mV/ms). Increasing concentrations of I-RTX caused a dose-dependent increase in the outward current. Acidification with pH 5.8 increased the potency of I-RTX as an agonist. The black bars indicate the application of pH 5.8 in the absence or presence of I-RTX. b, concentration response of I-RTX-dependent activation of S512Y (mean ± S.E.M., $n = 4–6$). Currents were normalized to the inward pH 5.8 response, and the EC_{50} value was determined from a fit of the data (solid line). c, I-RTX-dependent activation of S512Y in the presence of pH 5.8 (mean ± S.E.M., $n = 4–6$). Responses were calculated as the percentage increase in pH 5.8 response, and the EC_{50} value was determined from a fit of the data (solid line).

now show that capsaicin and the TRPV1 antagonist, compound A, are also more potent against human than rat TRPV1 and were similarly influenced by residue 547.

The reciprocal mutation in rat, M547L, only partially conferred a loss of function for I-RTX, although a decrease in binding affinity for both RTX and I-RTX has been reported (Chou et al., 2004; Gavva et al., 2004; Phillips et al., 2004) and may intimate a complex process for how binding translates into channel gating, an unknown for this channel type. Critical residues have been identified for the cysteine-loop receptor superfamily that forms part of an allosteric pathway linking neurotransmitter binding to gating (Lee and Sine, 2005; Lummis et al., 2005). Nevertheless, little is known with respect to the TRPV1 channel as to how a conformational change in the S3–S4 sensor domain relates to channel activation. In this respect, a certain degree of disconnect between ligand affinity, potency, and efficacy is not surprising, because alterations that affect conformational change of the sensor might not always be expected to translate to equivalent influence on channel opening. Moreover, there is evidence that additional residues elsewhere in the rat channel may also be involved in mediating species-specific activity (Kuzhikandathil et al., 2001; Jung et al., 2002; Phillips et al., 2004). Nevertheless, collectively, our data reveal that residue 547 is important not just for species-specific interactions but plays a more general role in mediating the interaction of ligands within the intracellular vanilloid binding pocket (Jordt and Julius, 2002; Chou et al., 2004; Gavva et al., 2004; Phillips et al., 2004).

Given the potential for structural and functional disconnect between ligand binding and gating (Gavva et al., 2004; Phillips et al., 2004), it is encouraging that there is a close correlation between affinity and potency for the ligands used in this study. In this instance, channel functionality was assessed directly using the whole-cell patch-clamp technique to measure current through the membrane. Both the increase in potency seen for RTX and I-RTX and decrease for compound A in the human mutant (L547M) were matched by parallel changes in binding affinity (Chou et al., 2004). Moreover, the gain of function observed in this study is a good indicator that the switch in residue 547 is influencing a direct interaction between the channel and the ligand, as opposed to a nonspecific disruption in protein structure per se. A number of studies have implicated the involvement of additional residues within TM4, the putative S5–S6 pore region and the N and C termini on vanilloid activity (Welch et al., 2000; Jung et al., 2002; Vlachova et al., 2003; Gavva et al., 2004; Phillips et al., 2004). These findings leave open the option of an indirect influence of residue 547 on the vanilloid binding pocket via a conformational change—activity that could be supported by assuming a more traditional TM6 domain structure. Nevertheless, the complementary nature of residue 547 influence on both antagonists and agonists alike, coupled with the correlation between channel activity and ligand binding, points to a more direct influence of 547 on ligand interaction as opposed to acting through allosteric modulation.

If, as a number of groups have suggested, residues 547 and 512 are both located within the proposed S3–S4 sensor binding pocket, then compounds influenced by Ser^{512} might also be expected to be influenced by residue 547 and vice versa (Jordt and Julius, 2002; Chou et al., 2004; Gavva et al., 2004, 2005; Phillips et al., 2004). Capsaicin and RTX were originally used as diagnostic tools for defining the critical role of residues Tyr^{511} and Ser^{512} in forming part of the rudimentary vanilloid binding site (Jordt and Julius, 2002). RTX binding was reported to be undetectable in the S512Y clone (Jordt and Julius, 2002) and was also affected by residue 547 (Chou et al., 2004). Likewise, inhibition and binding by the novel antagonist compound A has been shown to be reduced by the S512Y substitution (Sutton et al., 2005). In the current study, compound A had a species-specific profile similar to that of capsaicin, mediated by residue 547 in the human receptor, with the binding profile for compound A tracking functional activity.

One particularly intriguing finding was that I-RTX lost the ability to antagonize S512Y TRPV1 yet maintained a rudimentary agonist activity at the mutant receptor. It has been reported that I-RTX exhibits partial transient agonist activity with strong outward rectification (Shimizu et al., 2005), a profile similar to the intrinsic agonist activity we detected in our study. Interaction with Ser^{512} is therefore critical for determining the inhibitory activity of I-RTX. In support of these findings, evidence exists that low-level agonist activity of capsaicin is also retained in the S512Y mutant (Sutton et al., 2005). Could the mutated Tyr^{512} residue be mediating the activity of vanilloids in a more general manner, by sterically influencing their position within a rudimentary binding pocket, as opposed to a more optimized direct chemical interaction facilitated by Ser^{512}? Interaction of the hydrophobic Tyr^{512} residue with the iodo-portion of the I-RTX could place this molecule in a position that is more favorable to gating of the channel (akin to RTX), albeit with much reduced efficacy—an interaction that might not be supported to such an extent by the WT hydrophilic serine. Reorientation of vanilloids within the mutated binding pocket may be sufficient to achieve a small degree of channel activation. That a single point mutation has such a significant affect on the potent antagonist I-RTX also suggests that the vanilloid binding pocket is exquisitely sensitive to the orientation and residue-specific interactions of its ligands.

There is also evidence of allosteric cross-talk between vanilloid and proton-dependent gating, because mutants which showed reduced interaction at the intracellular vanilloid binding site exhibit similarly reduced pH sensitivity. In this study, switching the 547 residue in human L547M not only caused a decrease in capsaicin potency but also resulted in a small but significant leftward shift in gating by pH, matching a finding reported previously for the human S512Y mutant (Sutton et al., 2005). A recent article by Gavva et al. (2005) looked into the influence of pH on the conformation of the vanilloid binding site and found no effect for the functional interaction of ligands that were tested. Nevertheless, the current data suggest that converse cross-talk does occur, in so far as structural changes associated with reduced ligand interaction at the vanilloid binding site can allosterically influence proton-dependent gating of the channel.

In conclusion, our data support the proposed model, whereby Met^{547} with Ser^{512} form part of an intracellular vanilloid binding pocket which functions as the S3–S4 sensor domain. Orientation of ligands within the binding pocket seems to be under tight control with respect to how ligand interaction is translated to channel activation. Because preclinical validation supporting the use of TRPV1 blockers as

potential antihyperalgesics continues to emerge, defining the key residues that are responsible for mediating antagonist versus agonist activity could prove crucial in the pursuit of TRPV1 modulators as therapeutic agents for the treatment of pain.

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