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Cloning and functional characterization of dog transient receptor potential vanilloid receptor-1 (TRPV1)

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Abstract

Transient receptor potential vanilloid receptor-1 (TRPV1) is a sensory neuron-specific cation channel capable of integrating various noxious chemical and physical stimuli. The dog orthologue of TRPV1 was cloned using cDNA from nodose ganglia and heterologously expressed in HEK293^{OFF} cells. At the amino acid level, dTRPV1 displays 85–89% sequence identity to other TRPV1 orthologues. Molecular pharmacological characterization of HEK293^{OFF} cells expressing TRPV1 was assessed using a fluorescence imaging plate reader (FLIPR)-based calcium imaging assay. Dog TRPV1 was activated by various known TRPV1 agonists in a concentration-dependent manner: Ag23 = resiniferatoxin > olvanil ~ arvanil > capsaicin > phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) > *N*-oleoyldopamine (OLDA). In addition, select TRPV1 antagonists (capsazepine, I-resiniferatoxin and *N*-(-4-tertiarybutylphenyl)-4-(3-cholorpyridin-2-yl)tetrahydropyrazine-1(2*H*)-carbox-amide (BCTC)) were able to block the response of dTRPV1 to capsaicin. Furthermore, the dog TRPV1 lacked a conserved protein kinase A (PKA) phosphorylation site (117) found in other cloned orthologues, which may have physiological consequences on dog TRPV1 function. Taken together, these data constitute the first study of the cloning, expression and pharmacological characterization of dog TRPV1.

Keywords: TRPV1 (transient receptor potential vanilloid receptor-1); Cloning; Dog; Ganglia; FLIPR (fluorescence imaging plate reader); Capsaicin

1. Introduction

Capsaicin evokes a burning pain sensation via activation of specific vanilloid receptors on sensory nerve endings (Caterina et al., 1997). The cloned capsaicin receptor, transient receptor potential vanilloid receptor-1 (TRPV1 (formerly VR1)), is a sensory neuron-specific cation channel that serves as a polymodal transducer of both thermal and inflammatory stimuli (Caterina et al., 1997; Caterina and Julius, 2001). To date, mammalian TRPV1 has been cloned and characterized from human (Hayes et al., 2000), rat (Caterina et al., 1997), guinea pig (Savidge et al., 2002), rabbit (Gavva et al., 2004) and mouse (Correll et al., 2004). Members of the TRPV1 family respond to a variety of selective activators including chemical compounds such as capsaicin, resiniferatoxin, olvanil and *N*-oleoyldopamine (Chu et al., 2003), as well as physiological conditions such as changes in extracellular pH (Tominaga et al., 1998), temperatures above 42 °C (Szallasi and Blumberg, 1999) and direct phosphorylation via protein kinase C (PKC) (Numazaki et al., 2002). The various TRPV1 homologues share conserved consensus regions, including a span of six hydrophobic transmembrane domains as well as three intracellular ankyrin repeats contained within the intracellular N-terminal portion of the protein. Despite conservation of sequence and function among mammalian orthologues, TRPV1 proteins do not always exhibit similar pharmacological profiles. For example, both human and guinea pig TRPV1s differ from rat TRPV1 in that they show virtually no response to the potent rat agonist 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) at concentrations greater than 10 μM (Phillips et al., 2004; McIntyre et al., 2001). Species variations also exist with respect to the pungent vanilloid receptor agonist, capsaicin. Gavva et al.

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(2004) demonstrated species variation with respect to capsaicin by recently cloning and characterizing rabbit TRPV1 (oTRPV1), which is largely capsaicin-insensitive below 10 μM. It was found that the substitution of a single amino acid residue at position 550 from an isoleucine to a threonine was sufficient to confer vanilloid sensitivity to the rabbit orthologue. Given the significant evidence for species differences in the pharmacology of the human, rat, guinea pig and rabbit TRPV1, it is of interest and of value to characterize dog TRPV1, particularly since the canine model has been used to investigate the relationship between TRPV1 and neurogenic inflammatory pain. Recent studies have shown that injecting dogs with a single dose of resiniferatoxin, a potent TRPV1 agonist, resulted in prolonged relief of inflammatory pain by irreparably damaging TRPV1-expressing neurons (Karai et al., 2004). These findings suggest the potential benefit of TRPV1-based research delineating similarities and differences in the relationship between dog TRPV1 and the other TRPV1 orthologues. Presumably, such data would determine the suitability of dog models for the evaluation of TRPV1 function in vivo and potential relationship to human TRPV1 functionality.

In this study, a functional dTRPV1 orthologue was cloned, for the first time, and stably transfected into HEK293^{OFF} cells to characterize its molecular pharmacology using a variety of known TRPV1 activators and inhibitors. Analysis of the primary amino acid sequence revealed a high degree of homology between dog TRPV1 and the other TRPV1 family members. Additionally, upon sequence alignment with the human, rat, mouse and guinea pig orthologues, it was determined that the dTRPV1 sequence lacked a conserved protein kinase A (PKA) phosphorylation site at amino acid position 117. Phosphorylation of TRPV1 by PKA has been shown to regulate desensitization of the receptor to certain TRPV1 agonists (Gavva et al., 2004). Measurement of intracellular calcium influx via the fluorescence imaging plate reader (FLIPR) assay demonstrated that dog TRPV1 was responsive to known TRPV1 agonists and antagonists with a pharmacological profile that was unique when compared to previously identified and characterized TRPVs. Furthermore, results of the mutation of dTRPV1 at the conserved PKA phosphorylation site (dTRPV1-K117S) suggest that this mutation may have implications on TRPV1 function in vivo. Nonetheless, the findings presented here suggest that the dog may be a suitable large animal model for studying the role of TRPV1 function in vivo.

2. Materials and methods

2.1. Animal and tissue preparation

A male beagle dog (15 kg, discontinued from another experiment) was euthanized by a bolus intravenous infusion of sodium pentobarbital sodium (Sleepaway™, Fort Dodge, NJ, USA at 100 mg/kg). The nodose ganglia were dissected and flash-frozen in liquid nitrogen prior to total RNA isolation. All animals used for these studies were treated in accordance to the National Institutes of Health Guide to the Care and Use of Laboratory Animals and the Animal Welfare Act in Association for the Assessment and Accreditation of Laboratory Animal Care Program.

2.2. RNA extraction and cDNA preparation

Total RNA was prepared from nodose ganglia using the Ambion Totally RNA kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Briefly, nodose ganglia from the dog were sonicated in the appropriate volume of denaturing solution. Phenol/chloroform/isoamylalcohol was then added to the tissue homogenate and the RNA was extracted from the aqueous phase after the sample was spun at 14,000×g. The RNA was re-extracted in one starting volume of acid phenol/chloroform and then precipitated with 70% ethanol. The RNA quality was assessed by gel electrophoresis and by absorbance ratios of 260 nm/280 nm. Aliquots of 5 μg of total RNA from the nodose sample were treated with 5 U of DNase I (Ambion) at 37 °C for 15 min. The first strand cDNA synthesis was carried out as follows: 5 μg of treated RNA was reversed transcribed into first strand cDNA using 1000 U of MMLV reverse transcriptase in the presence of 10 mM dNTP, 20 μM random hexamer primers and 200 U of recombinant RNase Inhibitor (all BD Bioscience Clontech). The reaction (total volume 100 μl) was incubated at 42 °C for 1 h and terminated by a 5 min incubation at 94 °C. Exactly 5 μl of each cDNA sample was taken to carry out polymerase chain reaction (PCR) reactions using an Ex Taq Kit (Pan Vera, Madison, WI, USA). The dog cDNA was cloned in three pieces: the 5′ end of the gene was cloned utilizing primer 1: AATATTGCAAATTGGGCCACAGAGGAT and primer 2: CCCATTCGGTGAACTTCCTGGA. The internal sequence was generated using primer 3: GACAGCTACTACAAGGGCCAGACAG and primer 4: GTGCGCTTCACGCCCTCACAGTTGC. The 3′ end was cloned using primer 5: CACCATCCTGGACACGGAGAAGAG and universal primer mix from SMART Race Kit (Clontech). Multiple isolated clones were analyzed for determination of sequence consensus and a full-length sequence was assembled. The cDNA from two separate dogs was used to establish a consensus sequence.

2.3. Site-directed mutagenesis for K117S substitution on dTRPV1

Site-directed mutagenesis was carried out using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA,

USA). The sense mutagenic primer for substitution K117S was 5′-ctcaagctctatgatcgcaggagtatatttgaggctgtcgctcag-3′. PCR was carried out in a 50 μl mixture using 10 ng of wild-type template plasmid DNA (dTRPV1 TRE2 Hyg) 12.5 ng/ml of each primer, 10 nmol of dNTPs, 2.5 U of cloned Pfu DNA polymerase and 1× reaction buffer (all Stratagene). The thermal cycler was programmed as follows: initial denaturation at 95 °C for 30 s, 12 cycles at 95 °C for 30 s, 55 °C for 1 min and 68 °C for 16 min. To digest the parental strand of DNA 1 μl (10 U) of Dpn1 endonuclease (Stratagene) was added to the sample (50 μl) and incubated at 37 °C for 1 h. Four microliters of the final sample was then used to transform competent *E. coli* cells (NovaBlue GigaSingles Competent Cells from Novagen, Madison, WI, USA) according to the manufacturer's instructions. Mutagenesis was confirmed by nucleotide sequencing (performed by GENEWIZ Inc., North Brunswick, NJ).

2.4. Mammalian cell culture and electroporation

cDNA for dTRPV1 was subcloned into the pTRE2hyg vector (BD Biosciences Clontech Palo Alto, CA, USA), between the *Pvu*II and *Not*I sites, for regulated mammalian expression. Plasmid DNA was purified from 100 ml of bacterial cultures using a Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions. The DNA pellet was dissolved in sterile water to approximately 1 μg/μl.

Transformed primary HEK293Tet^{OFF} cells, purchased from BD Bioscience Clontech, were maintained in MEM medium (supplemented with 10% Tet System Approved FBS/penicillin/streptomycin/L-glutamine/geneticin G418, all from Invitrogen) at 37 °C and 5% CO_2 in a humidified atmosphere. For transfection, cells were harvested at approximately 90% confluency by treatment with trypsin/EDTA and, subsequently, resuspended in DMEM medium to a density of 40×10^6 cells/ml. The cell suspension (0.250 ml) was mixed with 10 μg of purified plasmid DNA in a Bio-Rad Gene Pulser Cuvette (0.4 cm). Electroporation was carried out at room temperature using a Bio-Rad Gene Pulser at 220 V and 500 μFD, resulting in a mean pulse time of 22 ms. After transfection, cells were immediately diluted in 30 ml of DMEM culture medium and seeded onto 100 mm poly-D-lysine coated dishes (BD Biosciences) containing 10 ml of complete DMEM medium supplemented with 50 μg/ml doxycycline (to suppress gene activity). Stably transfected cells were obtained after 3 weeks of selection with 125 μg/ml of hygromycin B (BD Biosciences Clontech).

2.5. Drugs

Capsaicin, capsazepine, olvanil, arvanil, resiniferatoxin and phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) were purchased from Sigma (St. Louis, MO, USA). *N*-Oleoyldopamine (*N*-oleoyldopamine) and iodo-resiniferatoxin (I-resiniferatoxin) were purchased from Tocris (Ellisville, MO, USA), and 2-(3,4-dimethylbenzyl)-3{[(hydroxyl-3-methoxy-benzyl)amino]carbothioyl}-propylpivalate (Ag23) was purchased from Alexis (San Diego, CA, USA). *N*-(-4-Tertiarybutylphenyl)-4-(3-cholorpyridin-2-yl)tetrahydropyrazine-1(2*H*)-carbox-amide (BCTC) a TRPV1 antagonist (Valenzano et al., 2003) was synthesized according to standard methods and was tested in all experiments as its free base (molecular weight 372.89). All drugs were dissolved in dimethylsulfoxide (DMSO) and stored at −20.0 °C. The final concentration of DMSO was less than 0.1% (v/v) in all assays.

2.6. Measurement of Ca^{2+} via fluorometric imaging plate reader (FLIPR)

Dog TRPV1-HEK293^{OFF} cells were seeded at a density of 40,000 cells per well into black-walled, clear-base 96-well poly-D-lysine-coated plates (BD Biosciences) in 200 μl of DMEM media without doxycycline to allow for gene expression. The plates were incubated for 2 days at 37 °C and 5% CO_2 to allow for maximum expression of dTRPV1. On the day of the experiment, the cells were incubated for 1 h at 37 °C in Hank's Balanced Salt Solution (HBSS) containing 10 mM HEPES pH 7.4, 1% BSA and 2.5 mM probenecid, with the addition of the calcium indicator 4 μM Fluo-4 AM (Molecular Probes, Eugene, OR, USA) solubilized in 22 μl DMSO and combined with an equal volume of a 20% pluronic acid solution (Molecular Probes). The cells were washed three times in 200 μl of the above-mentioned Hank's buffer and allowed to incubate for an additional 30 min at 37 °C in 100 μl of prewarmed buffer prior to the assay. The plates were then placed into a FLIPR (Molecular Devices, Sunnyvale, CA, USA) with a heated stage (37 °C) to monitor cell fluorescence before and after the addition of various agonists and antagonists. After addition of compound, change in fluorescence was monitored for a period of 5 min and maximal increase in fluorescent signal was noted. Antagonists were added to cells in a volume of 50 μl via the FLIPR and allowed to incubate for 6 min prior to the addition of agonist. To assess activation, responses were measured as the difference between the maximum and the minimum change in fluorescence activity upon agonist addition. Data are presented as the percentage of the maximal response for each agonist. Antagonist assay design responses were normalized for comparison based on maximal signal generated (capsaicin, 30 nM) upon addition of the cells expressing dTRPV1. Curve fitting and calculation of both the EC_{50} and IC_{50} values were determined using GraphPad Prism v3.02 (GraphPad Software, Inc., CA, USA).

3. Results

3.1. Cloning of dog TRPV1

The dog TRPV1 receptor was cloned from nodose ganglia cDNA. PCR was performed using primer pairs described in Section 2 and a full cDNA nucleotide sequence of 2523 bp was obtained (GenBank accession no. AY568758) and inserted into the pTRE2Hygromycin vector and sequenced. The open reading frame of dog TRPV1 encoded an 840-amino acid polypeptide. Like other TRPV1 members, the dog orthologue contained the six hydrophobic transmembrane domains, the three N-terminal ankyrin repeat regions and putative conserved protein kinase C PKC (S503, S801) sites of phosphorylation (Fig. 1). Upon alignment with the other TRPV1 orthologues, the overall primary sequence

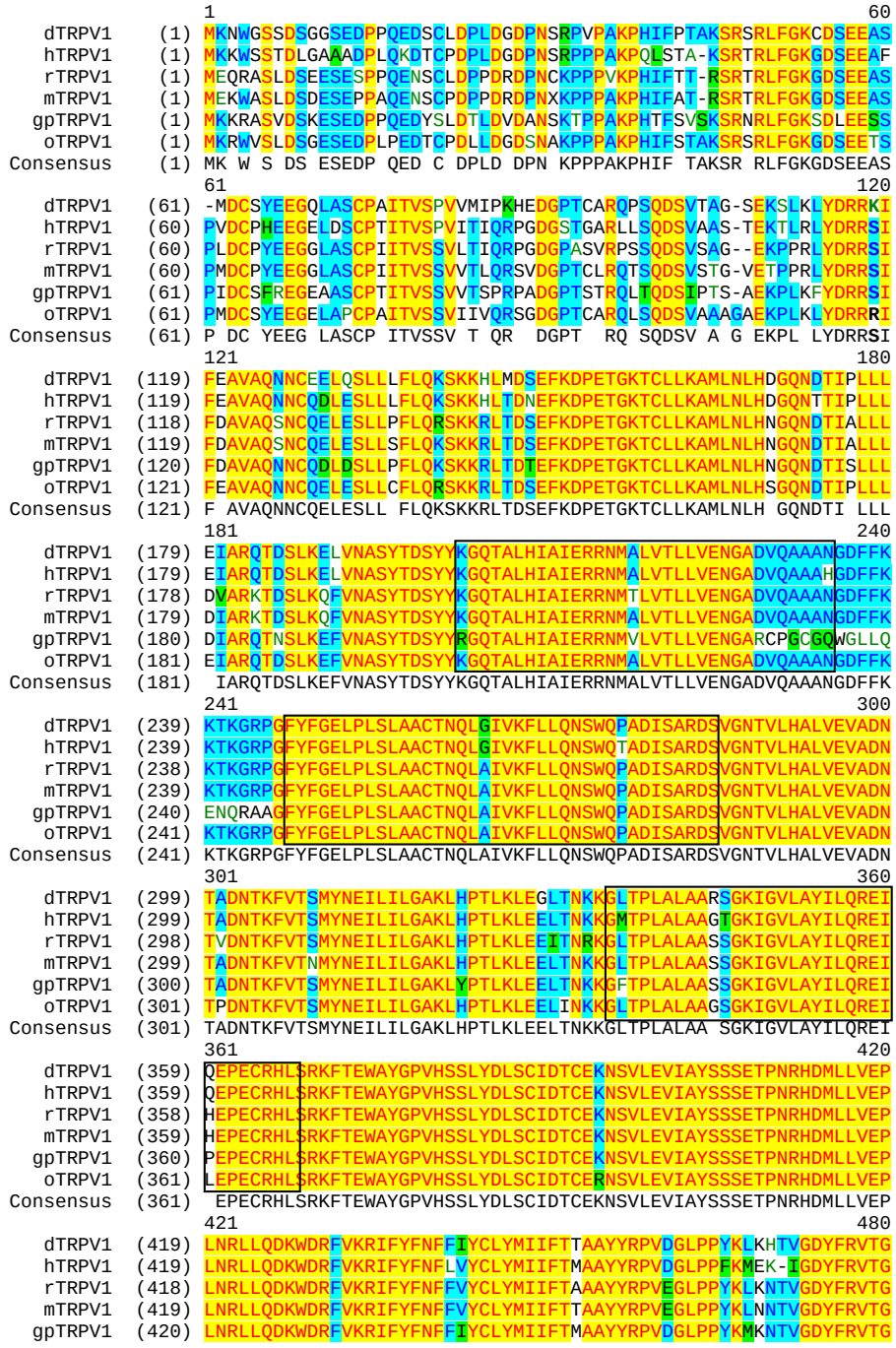


Fig. 1. Sequence alignment of TRPV1 family members. The alignment of dog, mouse, human, rat, guinea pig, rabbit and chicken amino acid sequences was generated using Vector NTI software (Informax) and the accession numbers listed in Fig. 2. Conserved N-terminal ankyrin repeat regions are shown in boxes. The putative transmembrane domains are underlined and the proposed important PKA (S117) and PKC (S503, S801) phosphorylation sites are shown in bold.

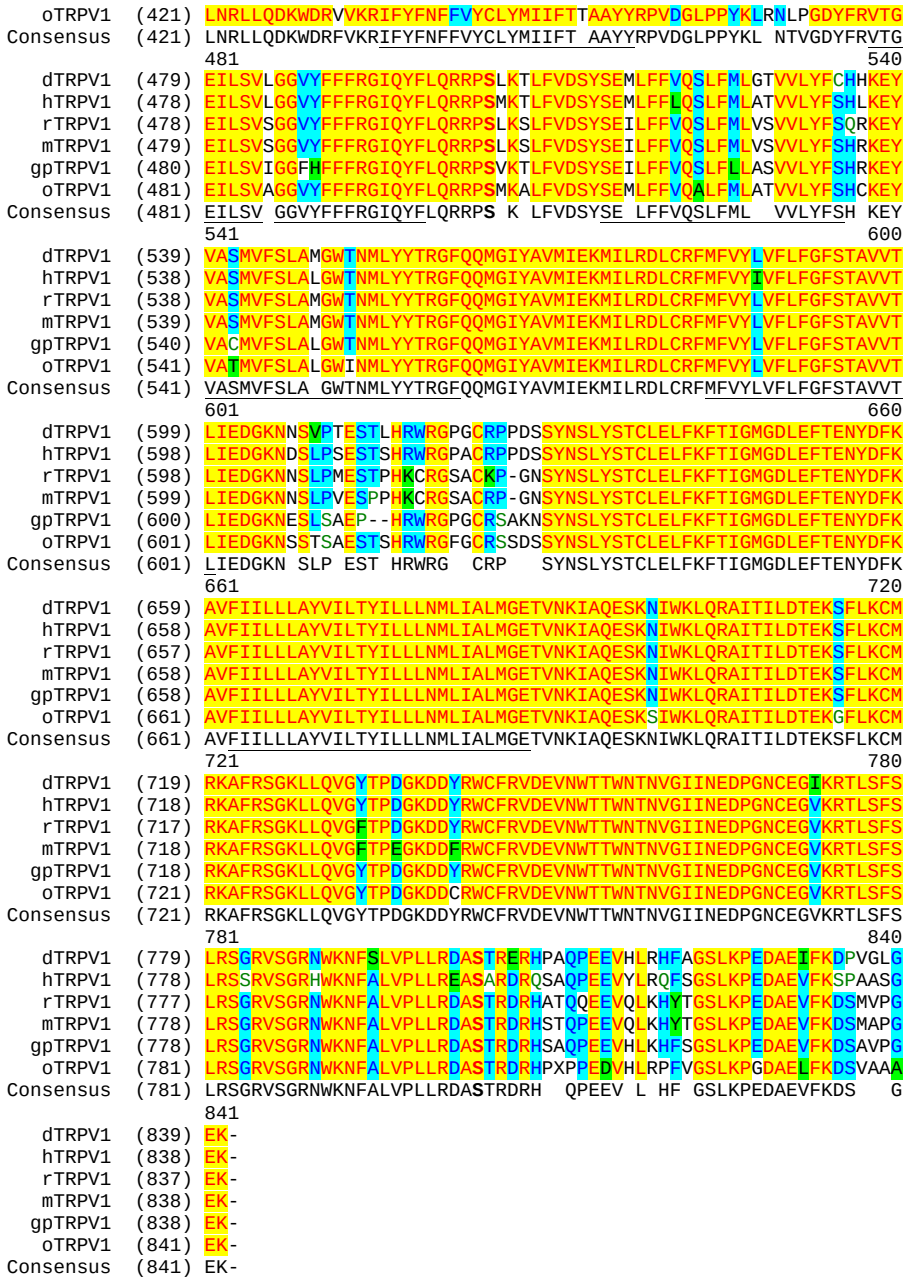
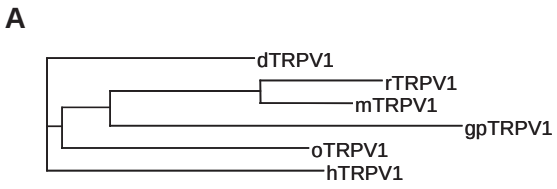


Fig. 1 (*continued*).

identities were as follows: 89.1% with hTRPV1, 85.2% with gpTRPV1, 87.5% with rTRPV1, 88.3% with mTRPV1 and 89.3% with oTRPV1 (Fig. 2A and B). Sequence analysis of the dTRPV1 protein revealed the presence of a threonine at position 550. It has been determined that the substitution of a single residue at position 550 in rabbit to the corresponding threonine residue found in rat and human TRPV1 was enough to confer sensitivity of rabbit TRPV1 to capsaicin, arvanil, olvanil and *N*-oleoyldopamine (Gavva et al., 2004). The fact that dTRPV1 contains a threonine at position 550 suggests the possibility that this orthologue might be sensitive to the aforementioned vanilloid receptor compounds. Upon closer inspection of the dog sequence, it was noted that the primary amino acid sequence contained a lysine at position 117, a putative protein kinase A (PKA) phosphorylation site, as opposed to a serine found in the other family members. Site-directed mutagenesis was performed on the dog construct to substitute the lysine for a serine residue to form a new construct: dTRPV1-K117S. Both wild-type dTRPV1 and dTRPV1-K117S were stably transfected into the HEK293^{OFF} cell line in order to fully pharmacologically characterize this novel TRPV1 protein and elucidate any functionality to the proposed conserved PKA phosphorylation site.



B

Sequence Identities of Dog TRPV1 and Other Family Members

	rTRPV1	hTRPV1	gpTRPV1	oTRPV1	mTRPV1
dTRPV1	**87.50%** **91.40%**	**89.10%** **93.30%**	**85.20%** **89.30%**	**89.30%** **92.50%**	**83.30%** **91.80%**
rTRPV1		85.70% 90.40%	85.50% 90.30%	86.70% 90.90%	95.10% 96.90%
hTRPV1			84.40% 88.70%	87.60% 91.40%	86.40% 90.70%
gpTRPV1				84.80% 89.40%	85.90% 89.90%
oTRPV1					87.90% 91.70%

Fig. 2. Dog TRPV1 is a member of the TRPV1 superfamily. (A) A phylogenetic evolutionary tree of the TRPV1 protein family. The following GenBank accession numbers were used to generate this tree: dog (AY568758), rat (AF029310), guinea pig (AJ492922), human (NM_080704), mouse (AY445519) and rabbit (AY487342). The tree was produced using the Neighbor Joining (NJ) method using Vector NTI software (Informax, Bethesda, MD, USA). (B) The identity and homology between TRPV1 of different species (dog, dTRPV1; rat, rTRPV1; human, hTRPV1; mouse, mTRPV1; guinea pig, gpTRPV1; and rabbit, oTRPV1). For each species, identity is shown on top, and similarity on the bottom.

3.2. Mammalian expression and pharmacological characterization of wild-type dog TRPV1

The coding sequence of dTRPV1 was cloned into the pTRE2Hygromycin vector and stably transfected into HEK293^{OFF} cells for functional characterization of $[Ca^{2+}]_i$ responses using the FLIPR assay. All TRPV1 agonists led to concentration-dependent increases in $[Ca^{2+}]_i$ fluorescence in the dTRPV1 cell line, but not in non-transfected HEK293^{OFF} cells (data not shown). The EC_{50} values of the six agonists tested in dTRPV1 are listed in Table 1. The rank order of potency from the most potent to the least potent agonist for the dTRPV1 expressing cells was: Ag23 (2.18 ± 0.29 nM)=resiniferatoxin (2.27 ± 0.18 nM)>olvanil (8.43 ± 1.74 nM)~arvanil (16.50 ± 6.08 nM)>capsaicin (40.75 ± 8.34 nM)>PPAHV (170.5 ± 46.7 nM)>*N*-oleoyldopamine

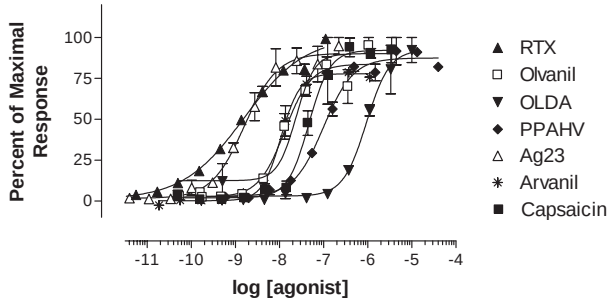


Fig. 3. Comparison of the ability of TRPV1 agonists to stimulate $[Ca^{2+}]_i$ uptake in dTRPV1 expressing HEK293^{OFF} cells. Cells were dye loaded with Fluo-4 AM and $[Ca^{2+}]_i$ was monitored before and after the addition of capsaicin, resiniferatoxin, olvanil, *N*-oleoyldopamine, PPAHV, arvanil and Ag23. Data are expressed as percent of maximal response to the highest agonist value obtained for that experimental run. Error bars represent S.E.M. EC_{50} values derived from these data are shown in Table 1.

(1002.17 ± 177.12 nM). The response curves generated by TRPV1 agonists are shown in Fig. 3. The EC_{50} values for PPAHV in dTRPV1 were considerably lower than the reported EC_{50} value for rat TRPV1 (between 3 and 10 μM, Phillips et al., 2004). These data show that, similar to rat TRPV1, dog TRPV1 exhibits sensitivity to PPAHV unlike either human or guinea pig TRPV1. The fact that dTRPV1 is responsive to PPAHV is consistent with previous studies, as it possesses a methionine at position 547, a site recently identified to confer PPAHV sensitivity in the rat (Phillips et al., 2004). Capsaicin sensitivity is a distinguishing characteristic of many TRPV1 family members. As previously described for the human, rat and guinea pig orthologues, dTRPV1 was activated by capsaicin with an EC_{50} value of 40 nM (Table 1 and Fig. 3). These data demonstrate that dTRPV1 is activated by a variety of different published TRPV1 agonists in a way that is consistent with other TRPV proteins. The Hill slope values for the ligands tested are displayed in Table 1. All TRPV1 agonists displayed positive cooperativity, with the exception of resiniferatoxin, which had a substantially lower Hill coefficient of 0.6 ± 0.09.

The ability of TRPV1 antagonists BCTC, capsazepine and I-resiniferatoxin to inhibit a capsaicin response was analyzed via the FLIPR assay. As shown in Fig. 4, all three antagonists were able to inhibit a capsaicin response in a concentration-dependent manner. IC_{50} values and order of potency ranking for this inhibition of dTRPV1 were as follows: I-resiniferatoxin (0.264 ± 0.03 nM)>BCTC (0.272 ± 0.05 nM)>capsazepine (484 ± 153.37 nM).

Table 1
The EC_{50} values of TRPV1 agonists in HEK293^{OFF} cells expressing dTRPV1 as ranked by potency

	Capsaicin	RTX	Olvanil	OLDA	PPAHV	Arvanil	Ag23
EC50	40.8 ± 8.34	2.3 ± 0.18	8.4 ± 1.74	1002.2 ± 177.12	170.5 ± 46.70	16.5 ± 6.08	2.2 ± 0.29
Slope	2.1 ± 0.52	0.63 ± 0.09	1.46 ± 0.35	1.66 ± 0.41	1.29 ± 0.19	2.03 ± 0.55	1.16 ± 0.23

Data are expressed as means ± S.E.M.

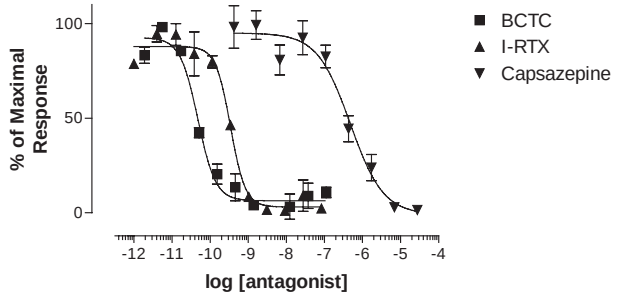


Fig. 4. Ability of BCTC, resiniferatoxin-I and capsazepine to antagonize capsaicin-induced responses in dTRPV1 expressing HEK293^{OFF} cells. Fluo-4 AM-loaded dTRPV1-HEK293^{OFF} cells were preincubated with antagonist for 6 min at 37 °C and then $[Ca^{2+}]_i$ was monitored before and after the addition of capsaicin (30 nM). Efficacies are expressed at percentage of maximal response and are given as ± S.E.M. IC_{50} values derived from these data are shown in Table 2.

3.3. Characterization of the capsaicin response in HEK293^{OFF} cells expressing dTRPV1-K117S using FLIPR assay

In order to compare the capsaicin-induced $[Ca^{2+}]_i$ kinetic responses of wild-type dTRPV1 with dTRPV1-K117S, the mutated construct was stably transfected into HEK293^{OFF} cells for functional analysis via FLIPR. Addition of capsaicin to dTRPV1-K117S-HEK293^{OFF} cells led to an increase in intracellular calcium levels that was concentration-dependent, with an EC_{50} of 11.47 ± 1.85 nM (Fig. 5). Analysis of $[Ca^{2+}]_i$ response curves revealed that the addition of capsaicin at various concentrations led to a distinct type of kinetic profile that differed from the one exhibited by dTRPV1 (Fig. 6A). While wild-type dTRPV1 response to capsaicin demonstrated a quick return to baseline, the dTRPV1-K117S mutation revealed a sustained response to capsaicin (Fig. 6B).

4. Discussion

In response to pain, sensory nerves release a variety of neuropeptide mediators from their peripheral endings (Brian, 1997). TRPV1 is a cation channel present in sensory nerve endings capable of sensing pain-producing stimuli and believed to play a central role in promoting neurogenic inflammation via the release of neuropeptides.

Table 2
Antagonist affinities vs. capsaicin in dTRPV1

Antagonist	dTRPV1
Capsazepine	484.5 ± 153.37
I-RTX	0.265 ± 0.03
BCTC	0.273 ± 0.05

Antagonist affinities at recombinant dog TRPV1 receptor expressed in HEK293^{OFF} cells.
The IC_{50} ± S.E.M. was determined and expressed in nM concentrations. Each data point represents triplicate determinations from at least three separate experiments.

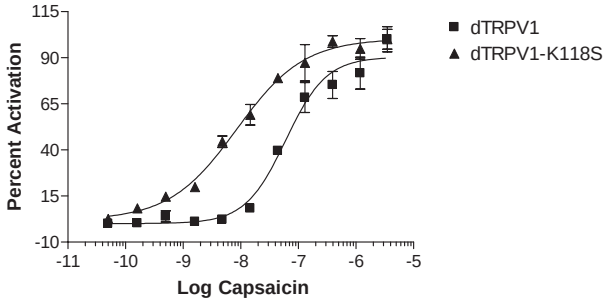


Fig. 5. TRPV1-mediated $[Ca^{2+}]$ responses for capsaicin in cells expressing wild-type or mutant TRPV1. Peak agonist-induced responses to 3.00E^{-6} capsaicin exhibited by each cell line are the result of duplicate experiments. Results are expressed in nM concentrations as the mean ± S.E.M. Representative data from at least four separate experiments is shown.

Antagonists that specifically target TRPV1 may prove to be valuable in the management and treatment of pain associated with neuropathic and inflammatory conditions. To date, TRPV1 from five different species have been cloned and functionally characterized. Although they share a high degree of sequence homology and structural

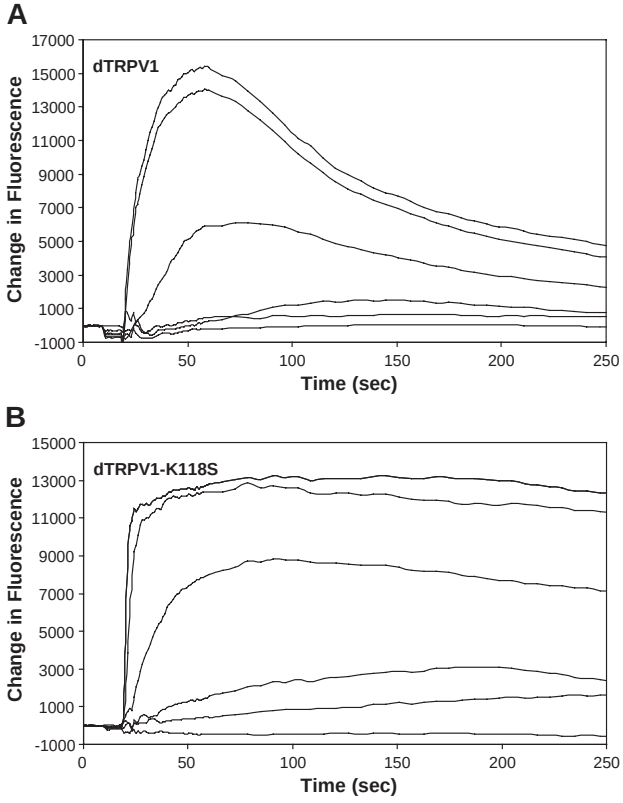


Fig. 6. dTRPV1 and dTRPV1-K117S exhibit distinct and different kinetic profiles in response to capsaicin. The effect of capsaicin (3.00E^{-6} to 6.0E^{-11}) on $[Ca^{2+}]_i$ responses in HEK293^{OFF} cells expressing either wild-type dTRPV1 or dTRPV1-K117S was assessed using the FLIPR assay (A, B). Capsaicin was able to induce a concentration-dependent Ca^{2+} response in cells expressing dTRPV1 or dTRPV1-K117S. Responses were monitored before and after the addition of capsaicin, and measured as peak increase in fluorescence minus basal, and expressed relative to the maximum response for each agonist.

similarity, they vary significantly in their selectivity and mode of activation with respect to various agonists and stimuli. This study describes the novel cloning and identification of dog TRPV1 cDNA from dog nodose ganglia and the functional characterization of this orthologue in stably transfected HEK293^{OFF} cells. In the present study, the full-length cDNA of dog TRPV1 was isolated by reverse-transcribing total RNA generated from dog nodose ganglia. Dog TRPV1 was sequenced in both directions, revealing a gene 2523 bp in length that encoded a polypeptide consisting of 840 residues. Peptide alignment of the dog orthologue with other species of the TRPV1 family revealed a high degree of sequence homology. The protein encoding the dog orthologue was closely related to other mammalian TRPVs including rabbit, human, rat, guinea pig and mouse sequences (89.3%, 89.1%, 87.5%, 85.2% and 83.3%, respectively). The dog protein contained six hydrophobic membrane-spanning domains, as well as three N-terminal ankyrin repeats, both of which are motifs characterizing this family of proteins.

TRPV family members can be subdivided into two groups based upon their species-specific pharmacological properties: those that are functionally responsive to capsaicin: human (Hayes et al., 2000), rat (Caterina et al., 1997) and guinea pig (Savidge et al., 2002), mouse (Correll et al., 2004) and those that are not: rabbit (Gavva et al., 2004). The molecular basis for capsaicin sensitivity can be localized to a single threonine residue at amino acid position 550 within the S3 to S4 region (Gavva et al., 2004). Sequence analysis of the dog TRPV1 revealed that it contained a threonine residue at position 550, suggesting that this orthologue of TRPV1 could be activated by capsaicin. The primary amino acid sequence of dTRPV1 also revealed the presence of a methionine at position 547, a site in rat that is responsible for ligand recognition of PPAHV (Szallasi and Blumberg, 1999).

To understand the pharmacological profile of this newly cloned TRPV1 orthologue, dog TRPV1 was subcloned into the pTRE2hygromycin vector and stably transfected into HEK293^{OFF} cells to generate a cell line for transcriptionally regulated expression. Using the FLIPR assay and Fluo-4 AM as an indicator of intracellular calcium, a series of TRPV1 agonists were tested. Capsaicin increased $[Ca^{2+}]_i$ levels in dTRPV1 expressing HEK293^{OFF} cells, with an EC_{50} of 40.7 ± 8.34 nM. Although this value for dTRPV1 is higher than that previously reported for rTRPV1, hTRPV1 and mTRPV1 (7.84 ± 0.07 nM, 7.90 ± 0.05 nM, respectively, Witte et al., 2002, 9.1 ± 1.2 nM, Correll et al., 2004), the order of potency of capsaicin with respect to other agonists for dTRPV1 was the same when compared to the other orthologues. The functional response to capsaicin was characterized by an initial rapid onset that peaked at about 50 s, followed by a slow decline back to base line over the duration of the 5 min assay (Fig. 6A). Such kinetics are consistent with those previously reported for the TRPV1 channel (Smart et al., 2001; Jerman et al., 2000). In addition to the ability of capsaicin to evoke $[Ca^{2+}]_i$ increases in the dog TRPV1-HEK293^{OFF} cells, PPAHV was also able to elicit a similar agonistic response. Previously, it was shown that rat TRPV1 but not human TRPV1 was sensitive to PPAHV (Phillips et al., 2004). Here we report that dTRPV1 is responsive to PPAHV. These findings support the results of Phillips et al. (2004), who determined that a methionine at amino acid position 547 was essential for PPAHV activation. Consistent with these preceding results, the dog sequence also contained a methionine at this critical position, leading to the aforementioned response.

An interesting pharmacological feature of the dTRPV1 receptor in this study is that the Hill slope value corresponding to resiniferatoxin is less than one. This differs from the Hill coefficients published for this agonist in both the rat and human TRPV1 orthologues (Jerman et al., 2000; Behave et al., 2002) and indicates the unique possibility of negative cooperativity of the dog receptor for this agonist.

Upon sequence alignment of dTRPV1 with the other TRPV1 orthologues, it became apparent that a conserved serine at position 117 was instead a lysine in the dTRPV1 amino aid sequence. A serine at this position has been shown to be a target for phosphorylation by PKA (Behave et al., 2002). PKA phosphorylation of TRPV1 increases the sensitivity of TRPV1 to capsaicin and inhibits desensitization of the cation channel via repetitive application of this agonist via a Ca^{2+}-dependent mechanism (Mohapatra and Nau, 2003). In order to determine if the lack of a serine at position 117 would in any way alter the pharmacologic and kinetic profile of dTRPV1, site-directed mutagenesis was performed on the dTRPV1 to substitute a serine for the lysine residue, creating the dTRPV1-K117S construct. This mutated sequence was stably transfected into the HEK293^{OFF} cells for functional comparison with wild-type dTRPV1 via FLIPR. The capsaicin-induced $[Ca^{2+}]_i$ response of dTRPV1-K117S-HEK293^{OFF} cells was approximately four-fold more sensitive than that of wild-type (11.47 ± 1.45 nM vs. 40.8 ± 8.34 nM). In a similar finding, using rat TRPV1, Mohapatra and Nau (2003) mutated the wild-type serine at position 116 to an alanine (S116A), which resulted in a decrease in capsaicin sensitivity and a rightward shift in the capsaicin concentration–response curve for mutant rat TRPV1. The EC_{50} value of dTRPV1-K117S for capsaicin is also similar to the EC_{50} values published previously for rTRPV1 and hTRPV1 (Witte et al., 2002), and for mTRPV1 (Correll et al., 2004) all of which contain a serine residue at position 117. Thus, the four-fold decrease that was observed in the EC_{50} of wild-type dog for capsaicin compared to the other TRPV1 orthologues can be interpreted as a phenotypical difference unique to this species, and not an example of differences in experimental paradigms between our laboratory and others. We have, in fact, cloned and functionally characterized the human, rat, mouse (Correll et al., 2004) and

guinea pig TRPV1s, using methodology reported in the literature; and our potency findings for these orthologues were similar to those reported by other laboratories and are consistent with the findings presented here.

In the HEK293^{OFF} cells expressing dTRPV1, treatment with capsaicin (1 μM) evoked a response that was typified by an initial rapid onset followed by a gradually declining secondary phase (Fig. 6A). The kinetics of HEK293^{OFF} dTRPV1-K117S cells in response to capsaicin had a similar initial primary phase compared to wild-type. In contrast, dTRPV1-K117S had a secondary phase that remained elevated, making only a small decrease toward baseline. The apparent increase in sensitivity of mutant TRPV1 to capsaicin is in agreement with the phenotype of a PKA phosphorylation point mutation at residue 116 previously described. The data presented here, which is in agreement with previous results, demonstrated that the lack of a serine at position 116 in rat has functional consequences on the properties of the TRPV1 receptor. From these data, it can be concluded that differences in the kinetic characteristics between the two cell lines as measured by the FLIPR functional assay can only be attributed to the mutagenesis of the wild-type lysine back to a serine residue within the conserved PKA phosphorylation region. The fact that a single amino acid mutation confers a change in the pharmacological sensitivity of dTRPV1 to capsaicin suggests that PKA phosphorylation not only regulates desensitization of TRPV1 but also sensitivity to vanilloids such as capsaicin. The ability of PKA-mediated phosphorylation to abrogate Ca^{2+}-dependent densitization of capsaicin-induced currents in sensory neurons might lead to an increase in receptor availability for painful stimuli and the sensitization of nociceptive information.

A significant finding of this study was the ability of capsazepine, BCTC and I-resiniferatoxin to inhibit capsaicin-induced activation in HEK293^{OFF} cells expressing the dTRPV1 receptor. Capsazepine is the best characterized of the TRPV1 antagonists and it is able to block a capsaicin response in all recombinant TRPV1 species (McIntyre et al., 2001; Brian, 1997). In dTRPV1, capsazepine was able to inhibit a 30 nM capsaicin-induced response in a concentration-dependent manner. BCTC has also been shown to be a potent antagonist of capsaicin-mediated channel activation in HEK293 cells expressing rTRPV1 (Pomonis et al., 2003). In this study, data from the FLIPR-based assay of HEK293^{OFF} cells expressing dTRPV1 revealed that BCTC was also able to inhibit channel activation in response to 30 nM capsaicin. The potency of BCTC in the dog was two orders of magnitude lower than what was reported in the heterologously expressed cloned rTRPV1 (Valenzano et al., 2003). To date, one of the most potent TRPV1 agonists is the plant-derived product resiniferatoxin, which was first isolated from the cactus *Euphorbia resinifera* (Szallasi and Blumberg, 1999). Interestingly, when resiniferatoxin becomes iodinated, it (I-resiniferatoxin) behaves as a high affinity antagonist against capsaicin-induced activation of both rat and human TRPV1 (Wahl et al., 2001; Rigoni et al., 2003). We found that the iodinated form of resiniferatoxin was a potent antagonist against dTRPV1 and we did not detect any agonist activity at the range of concentrations tested. The pathophysiology associated with inflammatory disease is characterized by the release of pro-inflammatory neuropeptides from peripheral terminals of sensory nerves. The TRPV1 receptor is responsible for detecting and integrating the stimuli that ultimately lead to neurotransmitter release from sensory neurons. It is believed that antagonists of TRPV1 may serve as effective agents in the management of pain associated with inflammation. Currently, rat models are used for the testing of inflammatory pain, but there is a great deal of species variation between rat and human TRPV1 receptor pharmacology. There is varying sensitivity across species with respect to TRPV1 agonists, and recent evidence has shown that there is also functional variability with respect to certain TRPV1 antagonists. Capsazepine, a compound that was first shown to antagonize capsaicin responses in cultured rat sensory neurons (Bevan et al., 1992), has been shown to exert diverse inhibitory effects in different TRPV1 receptors. For example, capsazepine inhibits both heat and pH responses in human and guinea pig TRPV1, yet is ineffective against either stimulus in native or cloned rat TRPV1 orthologue expressed in CHO cells (Savidge et al., 2002). This suggests that the use of rat models for TRPV1 antagonist studies may not accurately reflect the role of TRPV1 in human pathophysiology.

In conclusion, this is the first report of the cloning, functional expression and characterization of the dog TRPV1 orthologue. The data gathered from this study will help determine whether dTRPV1 is a suitable predictive model for the effects of TRPV1 antagonists in humans. The findings presented here suggest that recombinant dTRPV1 expressed in HEK293^{OFF} cells functions as a ligand-gated calcium channel with the requisite agonist and antagonist pharmacology, comparable to hTRPV1. The ability of dTRPV1 to display molecular pharmacology akin to other TRPV1 orthologues suggests that dog would be a good model for studying the role of TRPV1 in inflammatory diseases and nociception.

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References

Behave, G., Zhu, W., Wang, H., Brasier, D.J., Oxford, G.S., Gereau IV, R.W., 2002. cAMP-dependent protein kinase regulates desensitization

of the capsaicin receptor (VR1) by direct phosphorylation. Neuron 35, 721–731.

Bevan, S., Hothi, S., Hughes, G., James, I.F., Rang, H.P., Shah, K., Walpole, C.S.J., Yeats, J., 1992. Capsazepine: a competitive antagonist of the sensory neurone excitant capsaicin. Br. J. Pharmacol. 107, 544–552.

Brian, S.D., 1997. Sensory neuropeptides: their role in inflammation and wound healing. Immunopharmacology 37, 133–152.

Caterina, M.J., Julius, D., 2001. The vanilloid receptor: a molecular gateway to the pain pathway. Annu. Rev. Neurosci. 24, 487–517.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., Julius, D., 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389, 816–824.

Chu, C.J., Huang, S.M., De Petrocellis, L., Bisogno, T., Ewing, S.A., Miller, J.D., Zipkin, R.E., Daddario, N., Appendino, G., Di Marzo, V., Walker, J.M., 2003. *N*-Oleoyldopamine, a novel endogenous capsaicin-like lipid that produces hyperalgesia. J. Biol. Chem. 278, 13633–13639.

Correll, C.C., Phelps, P.T., Anthes, J.A., 2004. Cloning and characterization of mouse TRPV1. Neurosci. Lett. 370, 55–60.

Gavva, N.R., Klionsky, L., Qu, Y., Shi, L., Tamir, R., Edenson, S., Zhang, T.J., Viswanadhan, V.N., Toeth, A., Pearce, L.V., Vanderah, T.W., Porreca, F., Blumberg, P.M., Lile, J., Sun, Y., Wild, K., Louis, J.C., Treanor, J., 2004. Molecular determinants of vanilloid sensitivity in TRPV1. J. Biol. Chem. 279, 20283–20295.

Hayes, P., Meadows, H.J., Gunthorpe, M.J., Harries, M.H., Duckworth, D.M., Cairns, W., Harrison, D.C., Clarke, C.E., Ellington, K., Prinjha, R.K., Baarton, A.J., Medhurst, A.D., Smith, G.D., Topp, S., Murdock, P., Sanger, G.J., Terrett, J., Jenkins, O., Benham, C.D., Randall, A.D., Gloger, I.S., Davis, J.B., 2000. Cloning and functional expression of a human orthologue of rat vanilliod receptor-1. Pain 88, 205–215.

Jerman, J.C., Brough, S.J., Prinjha, R., Harries, M.H., Davis, J.B., Smart, D., 2000. Characterization using FLIPR of rat vanilloid receptor (rV1) pharmacology. Br. J. Pharmacol. 130, 916–922.

Karai, L., Brown, D.C., Mannes, A.J., Connelly, S.T., Brown, J., Gandal, M., Wellisch, O.M., Neubert, J.K., Olah, Z., 2004. Deletion of vanilloid receptor 1-expressing primary afferent neurons for pain control. J. Clin. Invest. 113, 1344–1352.

McIntyre, P., McLatchie, L.M., Chambers, A., Phillips, E., Clarke, M., Savidge, J., Toms, C., Peacock, M., Shan, K., Winter, J., Weerasakera, N., Webb, M., Rang, H.P., Bevan, S., James, I.F., 2001. Pharmacological differences between the human and rat vanilloid receptor 1 (VR1). Br. J. Pharmacol. 132, 1084–1094.

Mohapatra, D., Nau, C., 2003. Densensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent protein kinase pathway. J. Biol. Chem. 278, 50080–50090.

Numazaki, M., Toyooka, T., Tominaga, M., 2002. Direct phosphorylation of capsaicin receptor VR1 by protein kinase C epsilon and identification of two target serine residues. J. Biol. Chem. 277, 13375–13378.

Phillips, E., Reeve, A., Bevan, S., McIntyre, P., 2004. Identification of species specific determinants of the action of the antagonist capsazepine and the agonist PPAHV on TRPV1. J. Biol. Chem. 279, 17165–17172.

Pomonis, J.D., Harrison, J.E., Mark, L., Bristol, D., Valenzano, K.J., Walker, K., 2003. *N*-(-4-Tertiarybutylphenyl)-4-(3-cholorphyridin-2-yl)tertrahydropyrazine-1(2*H*)-carbox-amide (BCTC), a novel, orally effective vanilloid receptor 1 antagonist with analgesic properties: II. In vivo characterization in rat models of inflammatory and neuropathic pain. J. Pharmacol. Exp. Ther. 306, 387–393.

Rigoni, M., Trevisani, M., Gazzieri, D., Nadaletto, R., Tognetto, M., Cremionon, C., Davis, J.B., Campi, B., Amadesi, S., Geppetti, P., Harrison, S., 2003. Neurogenic responses mediated by vanilloid receptor-1 (TRPV1) are blocked by the high affinity antagonist, iodo-resiniferatoxin. Br. J. Pharmacol. 138, 977–985.

Savidge, J., Davis, C., Shah, K., Colley, S., Phillips, E., Ranasinghe, S., Winter, J., Kotsonis, P., Rang, H., McIntyre, P., 2002. Cloning and functional characterization of the guinea pig vanilloid receptor 1. Neuropharmacology 43, 450–456.

Smart, D., Jerman, J.C., Gunthorpe, M.J., Brough, S.J., Ranson, J., Cairns, W., Hayes, P.D., Randall, A.D., Davis, J.B., 2001. Characterization using FLIPR of human vanilloid VR1 receptor pharmacology. Eur. J. Pharmacol. 417, 51–58.

Szallasi, A., Blumberg, P.M., 1999. Vanilloid (capsaicin) receptors and mechanisms. Pharmacol. Rev. 51, 159–211.

Tominaga, M., Caterina, M.J., Malmberg, A.B., Rosen, T.A., Gilbert, H., Skinner, K., Raumann, B.E., Basbaum, A.I., Julius, D., 1998. The cloned capsaicin receptor intergrates multiple pain-producing stimuli. Neuron 21, 531–543.

Valenzano, K.J., Grant, E.R., Wu, G., Hachicha, M., Schmid, L., Tafesse, L., Sun, Q., Rotshteyn, Y., Francis, J., Limberis, J., Malik, S., Whittemore, E.R., Hodges, D., 2003. *N*-(4-Tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carbox-amide (BCTC), a novel orally effective vanilloid receptor 1 antagonist with analgesic properties: I. In vitro characterization and pharmacokinetic properties. J. Pharmacol. Exp. Ther. 306, 377–386.

Wahl, P., Foged, C., Tullin, S., Thomsen, C., 2001. Iodo-resiniferatoxin, a new potent vanilloid receptor antagonist. Mol. Pharmacol. 59, 9–15.

Witte, D.G., Cassar, S.C., Masters, J.N., Esbenshade, T., Hancock, A.A., Use of a fluorescent imaging plate reader-based calcium assay to assess pharmacological differences between the human and rat vanilloid receptor. J. Biomol. Screen. 72002. , 466–475.