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Molecular cloning, functional characterization of the porcine transient receptor potential V1 (pTRPV1) and pharmacological comparison with endogenous pTRPV1

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ABSTRACT

In the present study, we cloned a porcine orthologue of transient receptor potential V1 (pTRPV1) and heterologously expressed it in human embryonic kidney (HEK) 293 cells to characterize its pharmacological properties. At the amino acid level, pTRPV1 was highly homologous (83–90%) to other orthologues of TRPV1. The expression of receptors was examined with current and $[Ca^{2+}]_i$ responses to capsaicin using whole-cell patch-clamp and fura-2 ratio imaging techniques, respectively, and by immunostaining with an anti-TRPV1 antibody. The receptors were characterized by changes in $[Ca^{2+}]_i$ in response to various vanilloid agonists, low pH and heat and by the effects of TRPV1 antagonists on them. The various TRPV1 agonists activated pTRPV1 in a dose-dependent manner in the order of potency of resiniferatoxin (RTX) > olvanil > capsaicin > phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV), phorbol 12,13-dinonanoate 20-homovanillate (PDNHV). Isovelleral and scutigeral had no effect. Endogenous vanilloids (anandamide > 15 (s)-HPETE ≫ NADA), low pH and noxious heat (>42 °C) activated pTRPV1. Comparison of amino acid sequences with various mammalian TRPV1 homologues suggested some novel putative vanilloid recognition sites. TRPV1 antagonists, iodoRTX, ruthenium red and capsazepine suppressed capsaicin-induced responses. Similar to human TRPV1, but not rodent TRPV1, capsazepine was effective in blocking pH- and heat-induced responses. Similar pharmacological profiles were observed in cultured porcine dorsal root ganglion neurons. We discuss putative amino acid residues related to pharmacological differences among mammalian TRPV1 homologues.

1. Introduction

The nociceptive neuron is activated by a variety of noxious mechanical, thermal and chemical stimuli. Capsaicin, the pungent main ingredient of hot chili peppers, selectively activates the peripheral termini of relatively small sensory neurons known as nociceptors and evokes the sensation of burning pain [1]. The activation of nociceptors by capsaicin and other vanilloids is thought to be mediated by the activation of vanilloid receptors composed of ion channels [2].

Recently, a functional capsaicin receptor termed vanilloid receptor 1 (VR1) has been cloned and shown to be a non-selective cation channel with high Ca^{2+} permeability. It is expressed predominantly in primary sensory neurons and is subject to polymodal activation [3,4]. According to the new nomenclature, VR1 is termed TRPV1 [5], because it belongs to a

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superfamily of ion channels known as transient receptor potential (TRP) channels. Mice lacking the TRPV1 gene are insensitive to capsaicin and do not develop inflammation-induced thermal hyperalgesia [6,7]. Therefore, TRPV1 appears to play a key role in the pain sensation under inflammatory conditions.

The nature of the endogenous ligands and mechanism of activation of TRPV1 in vitro remain outstanding questions. Structure–function studies of the channel are in their infancy. To date, however, in mammalian species, TRPV1 has been cloned from the human [8–10], guinea-pig [11], rabbit [12], mouse [13] and dog [14]. Cloning of a number of orthologues from different species is expected to greatly facilitate understanding of the molecular pharmacology of TRPV1 and to provide insights into which amino acids are responsible for interspecies variation in response to vanilloids and other stimuli [2]. Seven chemical classes of agonists for vanilloid receptors have been reported [15]. The five traditional classes include capsaicinoids (e.g. capsaicin, olvanil), phorboid vanilloids (e.g. phorbol 12-phenyl-acetate 13-acetate 20-homovanillate [PPAHV]), resiniferanoids (e.g. resiniferatoxin [RTX]), unsaturated dialdehydes (e.g. isovelleral) and triprenyl phenols (e.g. scutigeral). The sixth and seventh classes, identified recently, are represented by anandamide [16] and eicosanoids, products of lipoxygenases [17], both of which are structurally related to capsaicin. The sensitivity of these agonists to vanilloid receptors has not been fully examined in cloned TRPV1. There are some notable species differences in the compound sensitivities of these channels. For instance, capsaicin has an agonistic action in most mammalian orthologues except for rabbit TRPV1 [12]. Indeed, rabbit dorsal root ganglion (DRG) neurons are resistant to the acute toxicity of capsaicin [18] and have no resiniferatoxin-binding site [19]. Furthermore, human [10] and guinea-pig TRPV1 [11] have little sensitivity to PPAHV, while rat [10,20], mouse [13] and dog TRPV1 [14] are significantly sensitive to PPAHV. RTX is more potent than olvanil in guinea-pig TRPV1 [11], but it is the opposite in other species [13–15,20]. Capsazepine, a TRPV1 antagonist, inhibits the response to acidic pH in human [10] and guinea-pig TRPV1 [11], but not in rat [10] and mouse TRPV1 [13]. For studying pain research in vivo, a number of reports have been published using rodent models. However, because of the inability of capsazepine to inhibit all modes of rat and mouse TRPV1 activation, it is suggested that use of a rodent model for studying TRPV1 antagonists may not accurately reflect the role of TRPV1 in human pathophysiology [13]. Due to the presence of significant evidence for species differences in the molecular pharmacology, it is of interest to characterize porcine TRPV1 (pTRPV1), which has not been identified. Concerning pTRPV1, the presence of radiolabelled RTX-binding sites in capsaicin-sensitive neurons in the pig spinal cord and dorsal root ganglia has been reported [21]. However, because there are only a limited number of reports related to biological responses to capsaicin in the pig [22–24], capsaicin/vanilloid pharmacology has been hardly clarified in pTRPV1.

In the present study, we cloned a functional pTRPV1 orthologue for the first time and transfected it into human embryonic kidney (HEK) 293 cells to characterize its pharmacological profile. The expression of functional receptors was examined by current recording using the whole-cell patch-clamp technique and fura-2 ratiometrical measurement of $[Ca^{2+}]_i$ in response to capsaicin. Immunocytochemical staining was carried out with an anti-TRPV1 antibody. In the present study, $[Ca^{2+}]_i$ was monitored using the fura-2 ratio imaging system in cells transfected with pTRPV1, which were selected by GFP-fluorescence co-expressed as a fusion protein. To characterize compound sensitivity, we examined effects of various TRPV1 agonists, including endogenous agonists, on $[Ca^{2+}]_i$ in pTRPV1-expressing HEK293 cells. Furthermore, the effects of pH and noxious heat were investigated to evaluate whether pTRPV1 functions as a polymodal receptor. Finally, we examined pharmacological profiles of native receptors expressed in cultured porcine DRG neurons. From functional analysis of pTRPV1 and comparison with other TRPV1 orthologues, we herein present putative amino acid residues related to differences of compound sensitivity among mammalian TRPV1s, including porcine TRPV1.

2. Materials and methods

2.1. Isolation of porcine DRG and culture of DRG neurons

All protocols for experiments on animals were approved by the Committee on Animal Experimentation, Graduate School of Veterinary Medicine, Hokkaido University. Crossbred male pigs (*Sus scrofa domestica*, 2–4 weeks after birth, 5–10 kg) were deeply anesthetized by sodium pentobarbital (50 mg/kg, i.p.) after tranquilization with ketamine (15 mg/kg, i.m.) and then killed by bloodletting from the cervical artery. All efforts were made to minimize animal suffering. Thoracic dorsal root ganglia were quickly removed, dissected and freed from connective tissue under optical control using fine forceps and scissors in ice-cold phosphate-buffered saline (PBS: in mM, 134.8 NaCl, 8.1 Na_2HPO_4, 1.5 KH_2PO_4, 2.7 KCl) supplemented with 100 U/ml penicillin G (Meiji-Seika, Japan) and 100 μg/ml streptomycin (Banyu, Japan). For preparation of cultured DRG neurons, isolated ganglia were cut into small pieces and enzymatically dissociated with collagenase- (1 mg/ml, type II, Worthington, USA) and DNase- (1 mg/ml, Roche, USA) containing PBS for 30 min at 37 °C. Then, ganglia were immersed in PBS with trypsin (10 mg/ml, Sigma, USA) and DNase (1 mg/ml) for a further 30 min at 37 °C. After enzyme digestion, the enzyme-containing solution was aspirated and ganglia were washed with culture medium; M199 (Sigma) supplemented with fetal bovine serum (10%, Sigma, USA), penicillin G (100 U/ml) and streptomycin (100 μg/ml). DRG neurons were obtained by gentle trituration with a fine-polished Pasteur pipette. Then, the cell suspension was centrifuged (800 rpm, 5 min, 4 °C) and the pellet containing cells was resuspended with the culture medium. Aliquots were placed onto glass cover slips coated with poly-D-lysine (Sigma, USA) and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The culture medium was renewed every day. In the present experiment, cells cultured within 1–2 days were used.

2.2. RNA extraction and cDNA preparation

To isolate total RNA from porcine DRG, isolated ganglia were quickly extracted with Trizol reagent (Isogen, Nippongene,

Japan). Briefly, a piece of porcine DRG was homogenized in 0.5 ml Trizol reagent and subjected to chloroform extraction and isopropanol precipitation. Then, total RNA was treated with DNase I (Promega, USA) at 37 °C for 30 min. The RNA quality was assessed by absorbance ratios of 260 nm/280 nm. First-strand cDNA was synthesized from oligo (dT)-primed total RNA with Superscript II reverse transcriptase (Gibco, USA). The reaction mixture was then subjected to PCR amplification with the use of Taq DNA polymerase (Platina Taq polymerase, Invitrogen, USA) using several primer sets of the rat TRPV1 open reading frame (GeneBank accession number, AF327067). Samples were heated to 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 2 min and then 68 °C for 7 min. The PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining followed by UV transillumination. The objective DNA bands in agarose gels were picked and purified using a gel extraction kit (Qiaex II, Qiagen, USA) and subcloned into a vector, pCR®4-TOPO (TOPO TA cloning kit, Invitrogen, USA). Clones were sequenced from standard vector primers and, where necessary, internal gene-specific primers, using an automated sequencer (CEQ 8000, Beckman Coulter, USA). In the present study, we could obtain partial sequences of a putative TRPV1 clone from porcine cDNA using the following rat TRPV1 primers (forward: 5′-CAATGGGCAGAATGACACCA-3′, reverse: 5′-CCGGTGACTCGGAAATAGTC-3′), which correspond to amino acid sequences in regions 162–167 (NGQNDT) and 466–472 (DVFRVIG), respectively (Fig. 1). To identify the full-length of the putative porcine TRPV1 cDNA, we used RNA ligase-mediated rapid amplification of 5′- and 3′-cDNA ends (RLM-RACE) (GeneRacer™ kit, Invitrogen) using some appropriate primer pairs. Multiple isolated clones were analyzed for determination of sequence consensus and the full-length sequence was assessed. The cDNA from three pigs was used to establish a consensus sequence. Finally, to obtain full-length cDNA for functional expression study, two primer sets were prepared based on our sequence data. For directional TOPO cloning of blunt-ended PCR products into an entry vector using the Gateway™ system (Invitrogen), four bases (CACC) were added to the 5′-end of the forward primer and two reverse primers with and without the stop codon were prepared as follows: forward-1, 5′-CACCATGGAGAAATGGGAGAGCTTA-GAC-3′, reverse-1, 5′-AGGATCCTTGACGATCTCAGCATCT-3′ (without the stop codon) and reverse-2, 5′- CTAAGGATCCTT-GACGATCTCAGCA-3′ (with the stop codon). Synthesized cDNA was subjected to PCR amplification with the use of Pfx DNA polymerase (Platina Pfx polymerase, Invitrogen) using two sets of primers (forward-1 and reverse-1, forward-1 and reverse-2). Samples were heated to 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 3 min and then 68 °C for 7 min. Purified PCR products were subcloned into pENTR™/D-TOPO vector (pENTR directional TOPO cloning kits, Invitrogen). Thereafter, pENTR™/D-TOPO vectors inserted with the objective sequences were recombined with the *destination* vectors as expression vectors using *attL* and *attR* reaction with Gateway™ LR Clonase™ enzyme mix (Invitrogen). We used the following two expression vectors, pcDNA-DEST47 Gateway™ vector and pcDNA-DEST53 Gateway™ vector (Fig. 2C). Both the former and the latter contain the 'cycle 3 mutant' of the green fluorescent protein (GFP) gene at the C- and N-terminal in vector sequences, respectively, and are expressed as fusion proteins in the gene of interest. We recombined pENTR™/D-TOPO inserted with the PCR products obtained using the primer set with forward-1 and reverse-1 and with forward-1 and reverse-2 for pcDNA-DEST47 Gateway™ vector and pcDNA-DEST53 Gateway™ vector, respectively. The pTRPV1-expressing cells were detected by GFP-fluorescence. Human embryonic kidney (HEK) 293 cells (a kind gift from Dr. Y. Mori, Kyoto University, Japan) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin and streptomycin. Cells were transiently transfected with the *destination* vectors encoding an appropriate pTRPV1 clone by using a transfection reagent (Lipofectamin 2000, Gibco, USA).

2.3. Measurement of intracellular Ca^{2+} concentration

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in individual cells was measured with a fluorescent Ca^{2+} indicator, fura-2 [25], by dual excitation using a fluorescent imaging system controlling illumination and acquisition as described previously [26]. To load fura-2, cells were incubated for 1.5 h at room temperature with 10 μM fura-2 acetoxymethyl ester (fura-2 AM) in normal external solution (in mM): 134 NaCl, 6 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 glucose, 10 HEPES (pH 7.4 with NaOH). A cover slip with fura-2-loaded cells was placed in an experimental chamber mounted on the stage of an inverted microscope (Olympus IX71, Japan) equipped with an image acquisition and analysis system (Aqua Cosmos, Hamamatsu Photonics, Japan). Cells with GFP-fluorescence were selected for measurement of $[Ca^{2+}]_i$ and the patch-clamp experiment. Cells were illuminated every 5 s with lights at 340 and 380 nm and the respective fluorescence signals of 500 nm were detected. The fluorescence emitted was projected to a CCD camera (ORCA-ER, Hamamatsu Photonics, Japan) and the ratio of fluorescent signals (F340/F380) was stored on the hard disk of a PC (Pro-600L, EPSON, Japan). Calibration of fura-2 was performed with a Ca^{2+} calibration buffer solution (Molecular Probes, USA) containing 5 μM fura-2. Cells were continuously superfused with the external solution at a flow rate of 1 ml/min through a Y-tube pipette placed close to the cells [27]. All experiments were carried out at room temperature (20–24 °C), except for the experiment determining temperature effects.

2.4. Whole-cell current recording

Membrane currents were recorded using the conventional whole-cell configuration of the patch-clamp technique [28]. Whole-cell recordings were made with 3–4 MΩ electrodes mounted on the head stage of a patch-clamp amplifier (Axopatch200B, Axon, USA). Current data were filtered at 1 kHz and sampled at 2–5 kHz by an A/D converter (PowerLab System, AD Instruments, Australia) in conjunction with a personal computer (Macintosh G3, Apple, USA) and stored on the hard disk of the PC. The standard pipette solution contained (in mM): 120 CsCl, 20 tetraethylammonium Cl, 1.2 $MgCl_2$, 2 $ATPNa_2$, 0.2 $GTPNa_3$, 10 HEPES and 10 EGTA (pH 7.2 with CsOH). For simultaneous measurement of $[Ca^{2+}]_i$ with the current–response to capsaicin, EGTA was omitted and

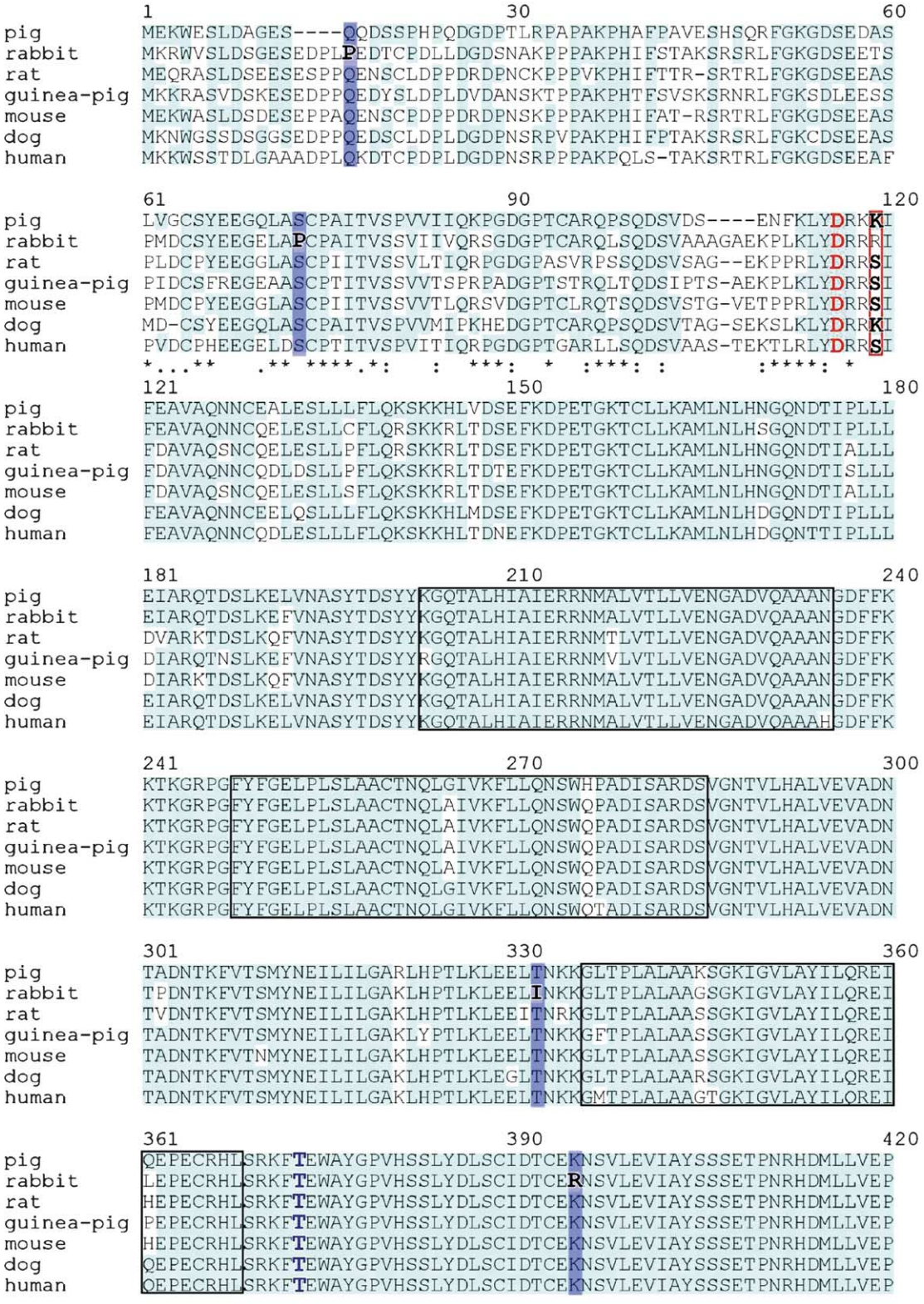

Fig. 1 – Alignment of pig, rabbit, rat, guinea-pig, mouse, dog and human TRPV1 amino acid sequences. The predicted protein sequences of the pig (this paper), rabbit (GeneBank accession number, AY487342), rat (AF029310), guinea-pig (AY513245), mouse (AY445519), dog (AY568758) and human (AJ277028). TRPV1 aligned using Clustal W. Conserved ankyrin repeat regions are shown in boxes. The putative transmembrane domains are underlined and potential N-linked glycosylation sites are shown with yellow boxes. The pore loop is indicated by a dashed line. Proposed PKA (S769, S815, blue) and PKC (S497, S796, black) phosphorylation sites and conserved residues involved in pH sensitivity of TRPV1 (E594,

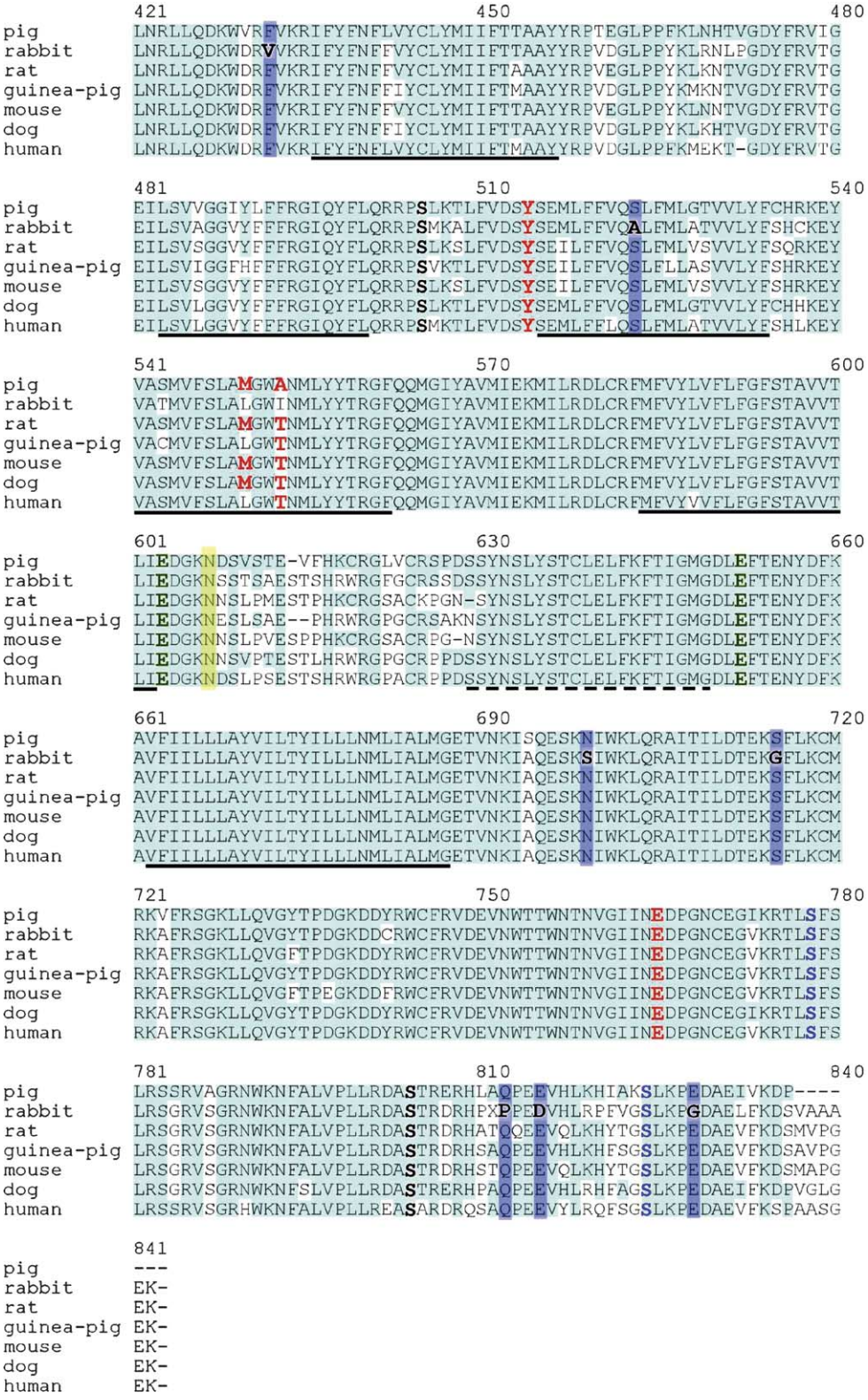

Fig. 1. (*Continued*).

E643, green) are shown in boldface. Lysine (K111) in the N-terminal region is emphasized with a red box, since this amino acid is suggested to be one of the putative PKA phosphorylation sites. Putative capsaicin- and RTX-sensitive sites are shown in boldface (red). Amino acids with the differences between rabbit and other TRPV1s are boxed in dark blue. Light blue boxes show amino acid residues that are the same as in pig TRPV1. Numbering of amino acids is in regard to the rabbit TRPV1 sequence and assignment of important residues is done by sequence alignment with other orthologues.

fura-2 (0.1 mM) was added to the pipette solution. A Y-tube system similar to that used for $[Ca^{2+}]_i$ measurement was used for compound application and external perfusion. For simultaneous measurement of $[Ca^{2+}]_i$ and membrane current, we used an inverted microscope (Diaphot 300, Nikon, Japan) equipped with a fluorometer (CAM-200, Jasco, Japan) as described previously [29], which produced alternate beams of excitation light at 340 and 380 nm by a wheel spinning at 100 Hz. The emission light passing through a pinhole diaphragm slightly larger than the cell was collected by a photomultiplier through a 500 nm filter. Fluorescent signals and their ratios and membrane currents were stored on the cassette tape of a PCM data recorder (NF Electronic, Japan) and the hard disk of the PC through an A/D converter (PowerLab, AD Instruments, Australia).

2.5. Immunocytochemistry

To obtain evidence for TRPV1 protein expression, transfected cells were subjected to immunostaining with an anti-TRPV1 antibody. In brief, after $[Ca^{2+}]_i$ responses to capsaicin were observed, cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature and subsequently rinsed with 10 mM phosphate-buffered saline with 0.3% Triton X-100 (Triton-PBS). Non-specific binding sites were blocked with 10% normal goat serum in Triton-PBS for 1 h. The cells were next incubated with a guinea-pig antiserum for TRPV1 (diluted 1:5000, Chemicon, USA) for 1 h at room temperature. After several rinses with Triton-PBS, the antibody was visualized by incubation with Alexa-labelled goat anti-guinea-pig IgG (10 μg/ml, Molecular Probes, USA) in Triton-PBS for 1 h. Finally, cells were extensively rinsed with Triton-PBS, then with distilled water. Preparations were examined with a laser confocal microscope (FV500, Olympus, Japan). A negative control was prepared by omission of the primary antibody, resulting in no staining.

2.6. Chemicals

The following compounds were used: adenosine 5′-triphosphate (ATP), arachidonylethanolamide (anandamide), arvanil, capsaicin, capsazepine, isovelleral, 8*N*-[2-(34-dihydroxyphenyl)ethyl]-5Z8Z11Z14Z-eicosaetraenamide (NADA), *N*-oleoyldopamine (OLDA), olvanil, phorbol 12-phenylacetate 13-acetate 20-homovanillate, phorbol 12,13-dinonanoate 20-homovanillate (PDNHV), resiniferatoxin, ruthenium red and scutigeral from Sigma (MO, USA). Iodoresiniferatoxin (iodoRTX) was from Tocris (MO, USA) and 15-hydroperoxyeicosa-5Z,8Z,11Z,13E-tetraenoic acid (15 (S)-HPETE) from Biomol (PA, USA). Fura-2 and fura-2 acetoxymethyl ester (fura-2/AM) were from Molecular Probes (OR, USA). In the present study, all vanilloid agonists and antagonists used except for capsaicin were dissolved in dimethyl sulfoxide (DMSO). Capsaicin was dissolved in absolute ethanol.

2.7. Data analysis

The data are presented as the mean ± S.E.M. (n = number of observations). Comparisons were made by the paired Student's t-test and differences with a P-value of less than 0.05 were considered significant. EC_{50} and IC_{50} values were determined using Origin software (OriginLab, USA).

3. Results

3.1. Amino acid sequence of porcine TRPV1

In the present study, we cloned the porcine TRPV1 receptor from synthesized cDNA isolated from DRG, inserted it into pCR®4-TOPO vector and sequenced it. The full cDNA nucleotide sequence was 2484 bp and the predicted protein coding domain of the pTRPV1 was 828 amino acids (Fig. 1). Comparative analysis of cDNA homology among different mammalian TRPV1 orthologues showed that the pTRPV1 cDNA possessed significant homology to the human (88%), dog (88%), rabbit (85%), mouse (85%), guinea-pig (84%) and rat (84%) based on Clustal W alignment (EMBL-EBI, USA). When alignment of the open reading frame of pTRPV1 was compared with those of other mammalian TRPV1 orthologues, the sequence homologies were 86% with the human, 84% with the rat, 87% with the mouse, 83% with the guinea-pig, 86% with the rabbit and 90% with the dog. The phylogenic tree showed that pTRPV1 was near dog and human TRPV1 (Fig. 2A). Sequence analysis revealed that the pTRPV1 contained three ankyrin repeat domains at the N-terminal region and six hydrophobic transmembrane domains and conserved putative phosphorylation sites by protein kinase C (PKC; S497, S796) and protein kinase A (PKA; T364). At one putative PKA phosphorylation site of the N-terminal region, pTRPV1, like dog TRPV1 [14], possessed a lysine (K111), but this amino acid is a serine (S) in the other mammalian orthologues. There were residues related to pH sensitivity (E594, E643) in pTRPV1. Surprisingly, sequence analysis of pTRPV1 protein revealed the presence of an alanine (A545) instead of threonine (T) in various species, except for rabbit TRPV1 [12], the amino acid of which is suggested to be responsible for sensitivity to capsaicin. Other putative vanilloid-sensitive portions (Y506, M542) were conserved in pTRPV1. Unlike other mammalian homologues, pTRPV1 was characterized by the lack of six amino acids at the C-terminal region (Fig. 1).

3.2. Expression of pTRPV1 in HEK 293 cells

The protein coding sequence of pTRPV1 was inserted into two expression vectors, which led to constitutive expression of GFP as a fusion protein in the N- or C-terminal of pTRPV1, since GFP is useful to detect functional expression by its color [30]. However, it is possible that such large peptides may interfere with the objective protein's function. Therefore, we constructed pTRPV1 with two different expression vectors as C-terminal GFP- and N-terminal GFP-fusion proteins and transiently transfected them into HEK293 cells (Fig. 2C). Approximately, 80% of cells were GFP-positive in the present experiment. pTRPV1 expression was confirmed at the protein level with immunocytochemical study using an anti-guinea-pig TRPV1 antibody. Cells with GFP-fluorescence were TRPV1-immunoreactive positive. Capsaicin induced a $[Ca^{2+}]_i$ increase in pTRPV1-expressing cells but not in non-expressing ones (Fig. 2B). $[Ca^{2+}]_i$ responses to capsaicin were observed in cells

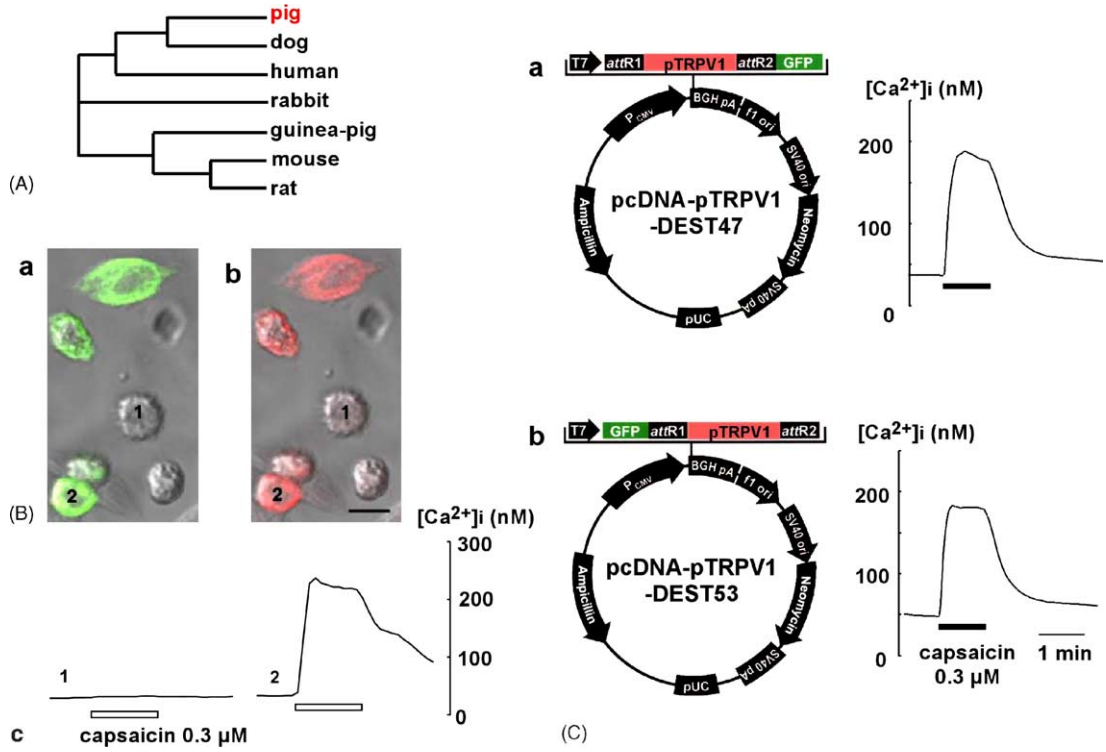

Fig. 2 – A phylogenic tree of TRPV1 and identification of expression of pTRPV1 in HEK293 cells. (A) A phylogenic tree based on Clustal W alignment of mammalian TRPV1 orthologues. (B) GFP-fluorescent image (a) and immunostaining of pTRPV1 using an anti-guinea-pig TRPV1 antibody (b). After measurement of $[Ca^{2+}]_i$ responses to capsaicin, cells were fixed and subjected to immuncytochemistry. Responses to capsaicin in cells numbered 1 and 2 (c) correspond to (a and b). (D) The elements of the expression vector used in the present experiments. pTRPV1 was translated as GFP-fusion protein using pcDNA-DEST47 (a) and pcDNA-DEST53 Gateway™ vectors (b). Note that capsaicin elicited $[Ca^{2+}]_i$ responses in cells transfected with both expression vectors.

that expressed pTRPV1 in both the N- and C-terminal of GPF-fusion proteins (Fig. 2C).

3.3. *Current and $[Ca^{2+}]_i$ responses to capsaicin in HEK293 cells transfected with pTRPV1*

In the present experiments, all studies were conducted using HEK293 cells transiently transfected with pTRPV1. To examine whether pTRPV1-transfected HEK293 cells expressed functional ion channels as reported for rat and human TRPV1 [9,31], GFP-fluorescent-positive cells were whole-cell voltage-clamped. At a holding potential of −60 mV, application of capsaicin (0.3 μM) evoked an inward current, which was sustained during its presence (Fig. 3A). The average inward current induced by capsaicin with the standard internal solution was 202.5 ± 29.6 pA (n = 12). A voltage-ramp from −80 to +80 mV for 100 ms was applied before and during the application of capsaicin. In the current–voltage (I–V) relation, the capsaicin-induced current showed an outward rectification with a reversal potential of −8.5 ± 1.4 mV. The capsaicin-induced inward current was greatly reduced by removal of external Na^+, which was replaced by N-methyl D-glucamine$^+$.

It is known that TRPV1 channels possess high Ca^{2+} permeability [1] and capsaicin produces increases of $[Ca^{2+}]_i$ in heterologously expressed cells with TRPV1 orthologues [10,13,14,20]. To examine the Ca^{2+} permeability of cells expressing pTRPV1, we simultaneously measured current and $[Ca^{2+}]_i$ responses to capsaicin under voltage-clamp conditions. An internal solution containing fura-2 (0.1 mM) without EGTA was used. At −60 mV, application of capsaicin elicited an inward current concomitant with a rise of $[Ca^{2+}]_i$ with some seconds' delay. Under these conditions, an I–V relation similar to that shown in Fig. 2A was obtained during the response to capsaicin. Capsaicin-induced current and $[Ca^{2+}]_i$ responses were examined at various holding potentials (Fig. 3C). At +60 mV, as expected, capsaicin elicited little $[Ca^{2+}]_i$ increase due to the decrease of the driving force for Ca^{2+} influx. These results clearly indicated that pTRPV1-transfected cells expressed functional ion channels with Ca^{2+} permeability.

3.4. *Activation of pTRPV1 by various vanilloid agonists and endogenous vanilloids*

We used the fura-2 ratiometric Ca^{2+} imaging method in single cells to estimate the sensitivities of vanilloid agonists to recombinant pTRPV1. The effects of putative vanilloid agonists, such as capsaicinoids, phorboid vanilloids, resiniferanoids, unsaturated dialdehydes, triprenyl phenols and endogenous vanilloids were examined. As shown in Fig. 4A, capsaicin evoked a rapid increase of $[Ca^{2+}]_i$ in a concentration-dependent manner. Resiniferatoxin, one of the most potent TRPV1 agonists isolated from a plant-derived product, the

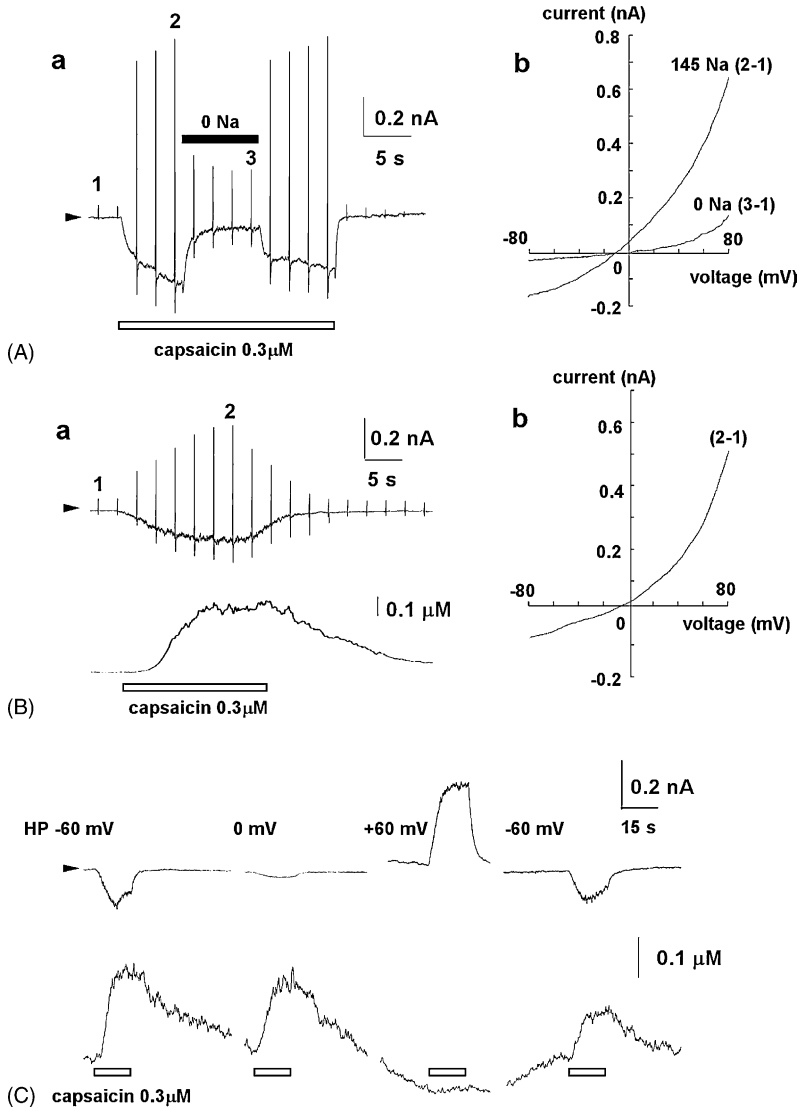

Fig. 3 – Capsaicin-evoked inward current and $[Ca^{2+}]_i$ increases in HEK293 cells transfected with pTRPV1. (A) Membrane current–responses to capsaicin in voltage-clamped HEK293 cells-expressing pTRPV1. At a holding potential of −60 mV, capsaicin (0.3 μM) was applied for 1 min with and without external Na^+. In Na^+-free solution (0 Na), Na^+ was replaced by N-methyl D-glucamine$^+$. Before and during capsaicin stimulation, ramp voltages from −80 to +80 mV for 100 ms were applied at the points shown as vertical lines in the current trace (a). Current–voltage (*I–V*) relations obtained with a ramp protocol are plotted (b) as the difference of currents before capsaicin application (trace 2 minus 1, trace 3 minus 1). Numbers correspond to those in the current trace. (B) Simultaneous measurement of current and $[Ca^{2+}]_i$ responses to capsaicin: (a) upper trace shows membrane current and lower $[Ca^{2+}]_i$ and (b) current–voltage (*I–V*) relations obtained with a voltage-ramp protocol. (C) Current and $[Ca^{2+}]_i$ responses to capsaicin at the various holding potentials indicated above (HP). Arrowheads beside the current traces are zero current level.

cactus *Euphorvia resinifera* [2], also evoked a concentration-dependent increase of $[Ca^{2+}]_i$ with very high potency. The pattern of the $[Ca^{2+}]_i$ response to RTX was quite different from that to capsaicin (Fig. 4B). In most cells, the capsaicin-induced $[Ca^{2+}]_i$ increase returned to the unstimulated level after its removal, but RTX produced a sustained increase of $[Ca^{2+}]_i$ that never returned to the original level after its washout. Increased $[Ca^{2+}]_i$ levels in response to other vanilloids were decreased after their washout, though there were some differences in their restoration speeds.

The concentration–response relations for various vanilloid agonists and endogenous vanilloids are shown in Fig. 4C and D. Like capsaicin, among these vanilloid agonists, arvanil, olvanil, PPAHV, PDNHV and RTX elicited concentration-dependent $[Ca^{2+}]_i$ increases in pTRPV1-expressing cells. OLDA had very slight agonistic action and no $[Ca^{2+}]_i$ increase occurred with isovelleral or scutigeral. The order of potency (EC_{50}, nM) was RTX (1.0 ± 0.4) > olvanil (2.9 ± 0.7) > arvanil (5.7 ± 0.9) > capsaicin (46.2 ± 9.8) > PPAFV (109.7 ± 24.3) > PDNHV (167.9 ± 71.4). Endogenous vanilloids also had agonis-

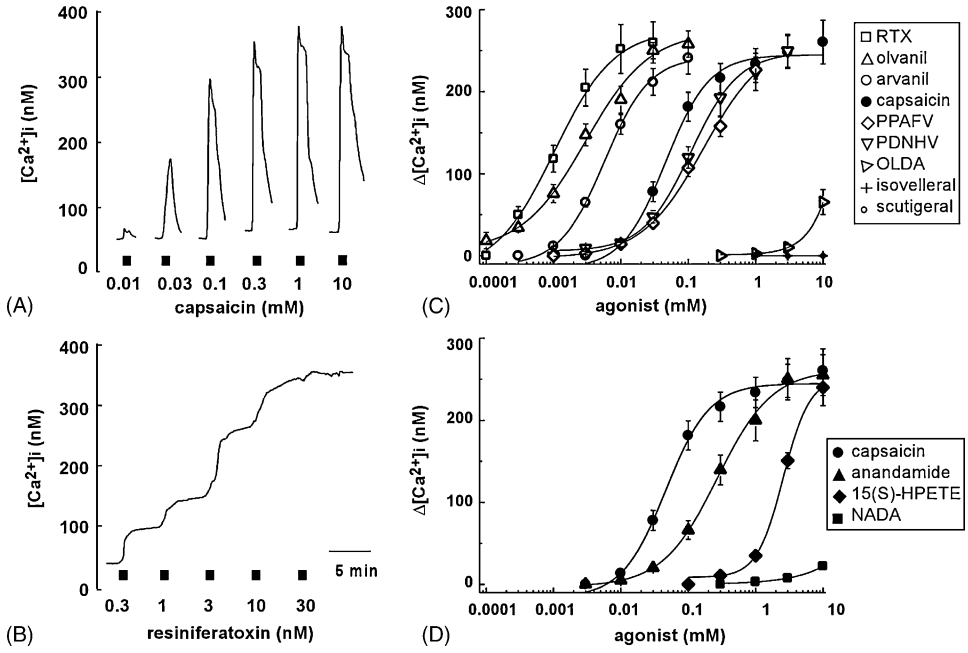

Fig. 4 – Comparison of the effects of vanilloid agonists and endogenous vanilloids on $[Ca^{2+}]_i$ in pTRPV1-expressing HEK293 cells. Representative traces of $[Ca^{2+}]_i$ responses to capsaicin (A) and resiniferatoxin (RTX, B) at various concentrations for 30 s. (C) Concentration–response relations for the peak $[Ca^{2+}]_i$ levels induced by RTX, olvanil, arvanil, capsaicin, PPAFV, PDNHV, OLDA, isovelleral and scutigeral. (D) Concentration–response relations for capsaicin, anandamide, 15(S)-HPETE and NADA. For comparison, the data of capsaicin were transcribed from (C). Data points from all experiments were pooled to yield the concentration–response relations. Symbols with vertical lines show mean ± S.E.M. (n = 33–45).

tic action, the potency of which was lower than that of capsaicin. EC_{50} values (μM) of anandamide and 15(S)-HPETE were 0.27 ± 0.07 and 2.56 ± 0.36, respectively. NADE caused only a slight effect, at least at 10 μM. HEK293 cells without GFP-fluorescence were unresponsive to the compounds tested.

3.5. *Activation of pTRPV1 by low pH and noxious heat*

Next, we determined the responses to low pH in HEK293 cells transfected with pTRPV1. In pTRPV1-expressing cells, extracellular solutions at pH 6.5 or lower evoked $[Ca^{2+}]_i$ increases (Fig. 5A). The $[Ca^{2+}]_i$ response to changing pH was rapid in onset and returned to the unstimulated level after switching from the external solution with low pH to pH 7.4. $[Ca^{2+}]_i$ responses were increased by decreasing the pH and the maximal response was obtained at around pH 5.5. A discontinuous pH–response relation was observed at pH 4.5 (Fig. 5A, inset), at which cells not expressing pTRPV1 also responded. It has been reported that HEK293 cells natively express a subtype of acid-sensing ion channels [32].

Next, responses to heat in pTRPV1-expressing cells were studied, because TRPV1 is activated by noxious heat [4]. Heat stimulation was carried out by application of an external solution warmed to the desired temperatures to the cells. Fig. 5B shows the temperature-dependent $[Ca^{2+}]_i$ rise in pTRPV1-expresssing HEK293 cells. At 42 °C, a small $[Ca^{2+}]_i$ response occurred and this was increased with increasing temperature. The peak response was obtained at 46 °C. The $[Ca^{2+}]_i$ response to heat was rapid in onset and was restored to the original level by changing the temperature of the external solution to 20–25 °C.

3.6. *Effects of vanilloid antagonists on responses to capsaicin, pH and heat*

The TRPV1 antagonists capsazepine [33,34], iodoRTX [35,36] and ruthenium red [2] were tested for their ability to inhibit pTRPV1. As shown in Fig. 6A (inset), sustained $[Ca^{2+}]_i$ increases in the presence of capsaicin (0.3 μM) were gradually inhibited by capsazepine applied cumulatively. All antagonists caused concentration-dependent inhibition of capsaicin-induced activation of pTRPV1 with IC_{50} (μM) values of 0.057 ± 0.015 for iodoRTX, 0.107 ± 0.069 for ruthenium red and 2.31 ± 0.16 for capsazepine (Fig. 6A). Unlike rodent TRPV1, $[Ca^{2+}]_i$ increases induced by pH 5.5 on pTRPV1-expressing cells were inhibited by capsazepine (Fig. 6B, inset). Fig. 6B shows the concentration–inhibition relation of capsazepine on pH 5.5-induced $[Ca^{2+}]_i$ increases with an IC_{50} of 1.04 ± 0.23 μM. $[Ca^{2+}]_i$ responses to heat (48 °C) were also significantly suppressed by 1 μM capsazepine (Fig. 6C).

3.7. *$[Ca^{2+}]_i$ responses to vanilloid agonists and antagonists in cultured porcine DRG neurons*

Finally, to compare pharmacological profiles of recombinant pTRPV1 with endogenous TRPV1-expressed in DRG neurons, the effects of vanilloid agonists and antagonists on cultured porcine DRG neurons were examined. In this study, cells were first stimulated with capsaicin (0.1 μM) to identify DRG

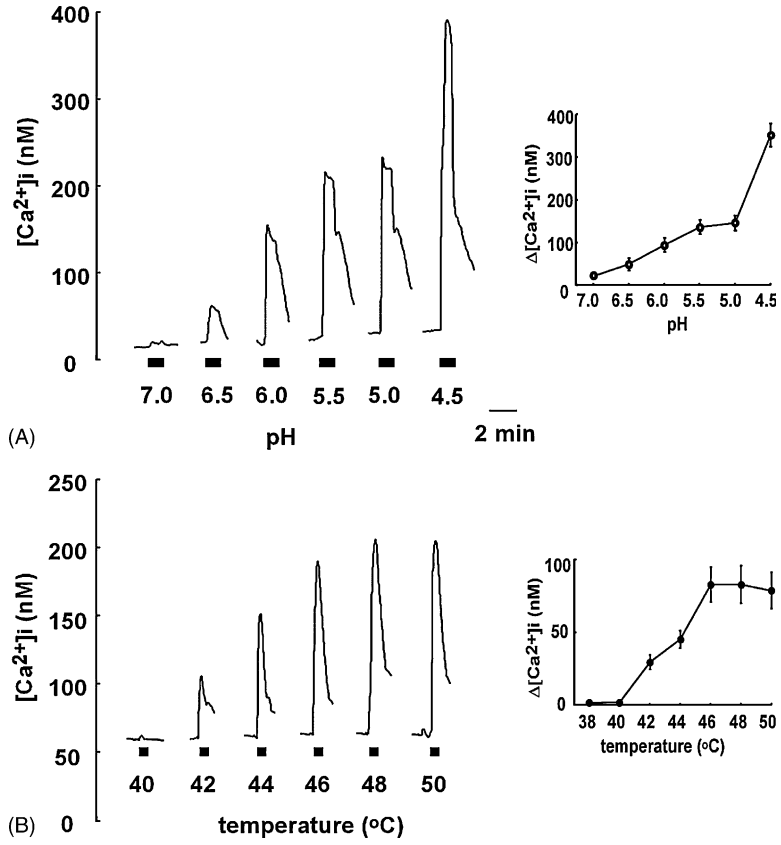

Fig. 5 – Protons- and heat-evoked $[Ca^{2+}]_i$ increases in HEK293 cells-expressing pTRPV1. Actual traces of $[Ca^{2+}]_i$ responses to various concentrations of protons (pH, A) and to various temperatures (B). Insets show pH (A, $n = 32$) and temperature (B, $n = 41$) relations for the peak $[Ca^{2+}]_i$ levels. Symbols with vertical lines show mean ± S.E.M.

neurons expressing TRPV1. Typical $[Ca^{2+}]_i$ responses to capsaicin and RTX in the same cells are shown in Fig. 7A-a. Similar to the recombinant pTRPV1, capsaicin produced a rapid $[Ca^{2+}]_i$ increase, which returned to the unstimulated level after its washout. However, in some cells, especially cells stimulated with high concentrations of capsaicin, the increased $[Ca^{2+}]_i$ levels hardly returned to the original levels. On the other hand, $[Ca^{2+}]_i$ responses to RTX never returned after its washout. The concentration–response relations for RTX and capsaicin are depicted in Fig. 7A-b and the EC_{50} values were estimated to be 2.4 ± 0.8 and 73.2 ± 21.1 nM, respectively. Protons (pH 5.5) and heat (48 °C) increased $[Ca^{2+}]_i$ in cultured DRG neurons (Fig. 7B and C) and both responses were significantly suppressed by capsazepine (1 μM).

4. Discussion

TRPV1 is an important potential target for the treatment of pain [3]. The cloning of TRPV1 from mammalian species, including humans, has provided substantial information concerning structure–function relations of this channel. Here, we reported the cloning of the cDNA sequence and pharmacological characterization of the porcine orthologue of TRPV1. The heterogeneous expression study showed that pTRPV1 was sensitive to various vanilloid agonists as well as endogenous vanilloids, low pH and heat. These results indicate that pTRPV1 plays a role as a polymodal receptor for pain sensation. The pharmacological characteristics of recombinant pTRPV1 were similar to those in cultured porcine DRG neurons.

The protein coding of pTRPV1 was highly conserved (>83%) when compared to the amino acid sequences of other mammalian TPPV1 sequences. pTRPV1 contains six hydrophobic and putative membrane spanning domains and three N-terminal ankyrin repeats, which are motifs characterizing the TRP family [5]. In addition, putative phosphorylation sites for protein kinase C and protein kinase A are present. Upon sequence alignment of pTRPV1 with other TRPV1 orthologues, the lack of six C-terminal amino acids became apparent. In the current study, however, these six amino acids did not seem to be important for TRPV1 function.

In the present experiment, we first detected the expression of channel protein in HEK 293 cells transfected with pTRPV1 by immunostaining and examined the sensitivity to capsaicin by measuring current and $[Ca^{2+}]_i$ responses. In pTRPV1-expressing cells, capsaicin evoked an inward current, which was greatly reduced by the removal of external Na^+ and the *I–V* relation to capsaicin-induced current showed the typical TRPV1 channel reported previously [31]. Capsaicin-induced $[Ca^{2+}]_i$ increases were due to the entry of Ca^{2+} because the magnitude of the $[Ca^{2+}]_i$ increase became small at +60 mV, a voltage near the equilibrium potential of Ca^{2+}. These results

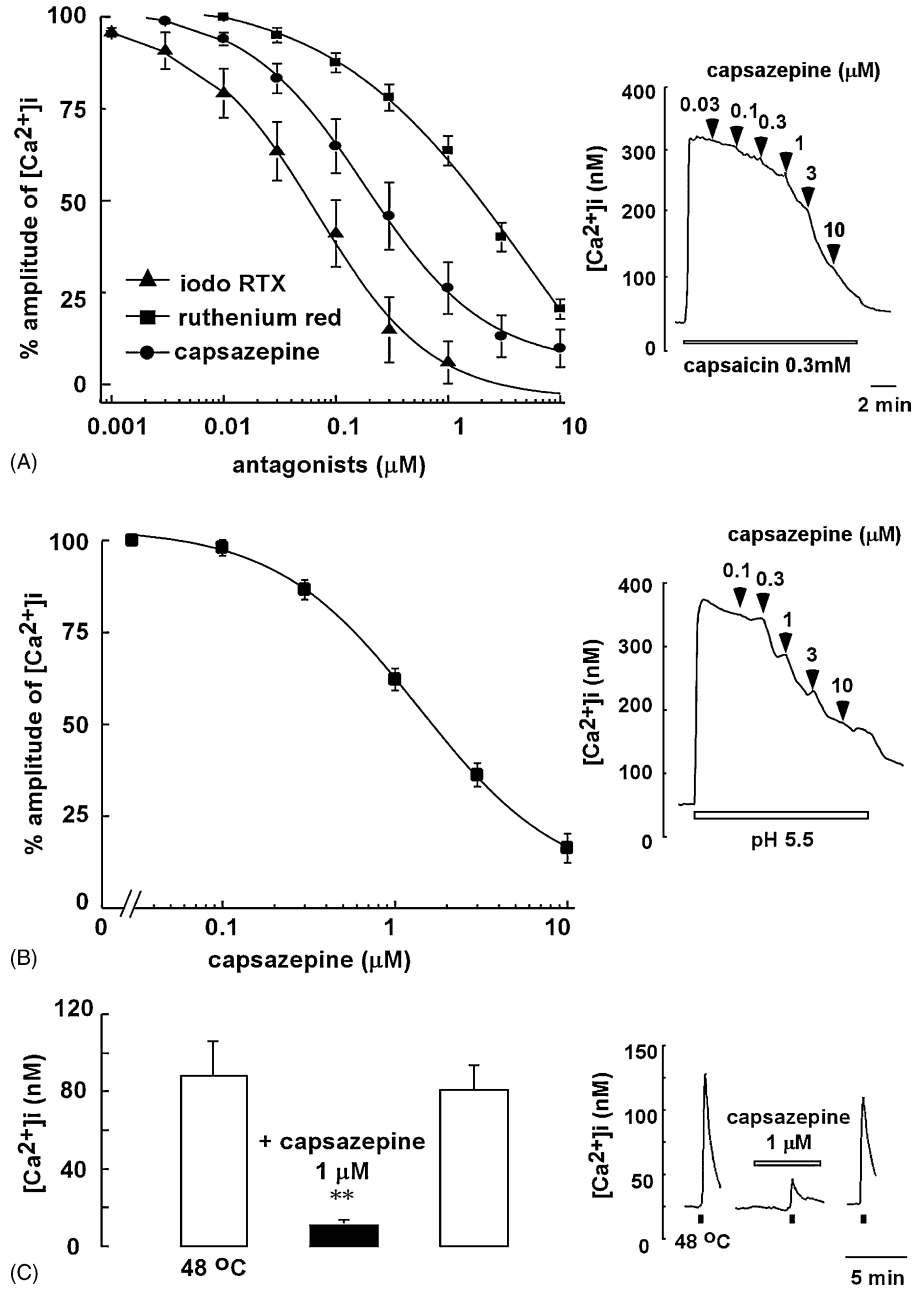

Fig. 6 – Inhibition of TRPV1-mediated increases in $[Ca^{2+}]_i$ by TRPV1 antagonists in HEK293 cells-expressing pTRPV1. (A) Concentration–inhibition curves for effects of iodoRTX, ruthenium red and capsazepine on capsaicin (0.3 μM)-induced $[Ca^{2+}]_i$ increase. Responses were estimated as a percentage of the amplitude of the capsaicin-induced $[Ca^{2+}]_i$ increase in the absence of these antagonists (capsazepine, $n = 30$; ruthenium red, $n = 25$; iodoRTX, $n = 18$). Inset shows a representative inhibitory effect of capsazepine with increasing concentrations on capsaicin-induced $[Ca^{2+}]_i$ increase. (B) Concentration–inhibition curves for capsazepine on pH 5.5-induced $[Ca^{2+}]_i$ increase ($n = 26$). Inset shows a representative inhibitory effect of capsazepine on pH-induced induced $[Ca^{2+}]_i$ increase. (C) Inhibitory effect of capsazepine (1 μM) on heat (48 °C)-evoked $[Ca^{2+}]_i$ responses ($n = 25$). Columns from left to right show increases of $[Ca^{2+}]_i$ in response to heat before and during the application of capsazepine and after its washout. Inset shows a typical inhibitory effect of capsazepine on the heat-induced $[Ca^{2+}]_i$ response. Symbols with vertical lines show mean ± S.E.M.

indicate that capsaicin-sensitive ion channels with Ca^{2+} permeability are functionally expressed in HEK293 cells transfected with pTRPV1.

Based on the species-specific pharmacological properties, members of TRPV1 family can be divided into two groups: one is functionally responsive to capsaicin (rat [20], human [10], guinea-pig [11], mouse [13], dog [14]), the other is not (rabbit [12]). Similar to the majority of mamalian TRPV1 orthologues, capsaicin was effective in activating porcine TRPV1. The molecular basis of capsaicin sensitivity is suggested to be

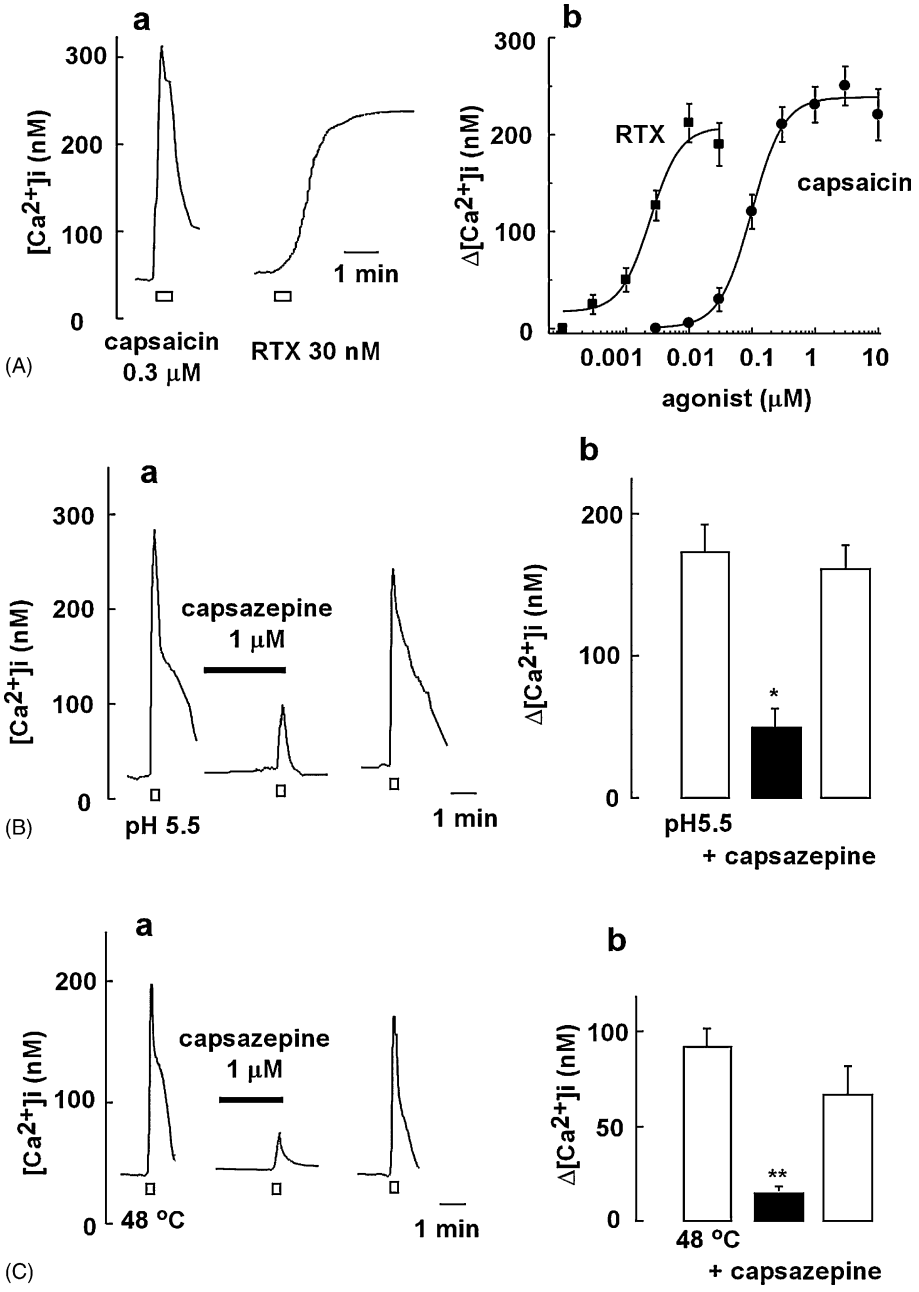

Fig. 7 – Effects of vanilloid agonists and antagonists on native pTRPV1 in cultured porcine DRG neurons. (A-a) Typical $[Ca^{2+}]_i$ response to capsaicin (0.3 μM) and subsequent response to RTX (30 nM) in the same cell. (A-b) Concentration–response relations for the peak $[Ca^{2+}]_i$ levels induced by RTX (n = 18) and capsaicin (n = 22). Proton-induced (B, pH 5.5) and heat-induced (C, 48 °C) $[Ca^{2+}]_i$ increases in cultured pig DRG neurons and the inhibitory effects of capsazepine on them. Proton or heat stimulation was applied three times and 2nd stimulation was done in the presence of capsazepine (1 μM), which was introduced 2 min before stimulation. Columns from left to right indicate before and during application of capsazepine and after its washout: (B-b) pH, n = 23; (C-b) heat n = 29. Mean ± S.E.M., $^{*}P < 0.05$, $^{**}P < 0.01$ when compared with before capsazepine application.

located to a single threonine residue (T) at amino acid position 550 within S3 to S4 region based on a mutagenesis study of rabbit TRPV1 [12]. This amino acid is conserved in all previous reported TRPV1 homologues except rabbit TRPV1 (Fig. 1). In the present experiment, surprisingly, the sequence analysis of pTRPV1 revealed the presence of an alanine (A) residue at this position instead of threonine. Therefore, the present cloning data indicate that threonine is not the only determinant of the vanilloid sensitivity of TRPV1, since pTRPV1 was substantially sensitive to capsaicin. It has been suggested that glutamate (E) at 761 in the cytoplasmic tail [37] and tyrosine (Y) at 511, are critical for vanilloid sensitivity [38]. However, these two amino acids were conserved in pTRPV1 and across species. In comparison with amino acid sequences of rabbit TRPV1,11

amino acids were different among other orthologues, including pTRPV1 (Fig. 1), suggesting that some of these amino acids are responsible for the vanilloid sensitivity of TRPV1. It has also been reported that the N-terminal intracellular domain is essential for the vanilloid sensitivity based on analysis of N-terminal splice variants [39]. Moreover, the C-terminal cytoplasmic tail is also reported to be functionally important in rat TRPV1 [40]. Therefore, there may be multiple sites for determination of vanilloid sensitivity in TRPV1. Further experiments, such as a mutagenesis study are necessary to confirm vanilloid recognition sites in TRPV1.

In the present study, on sequence alignment of pTRPV1 with other mammalian TRPV1 orthologues, it became apparent that the serine (S) at position 111, the putative PKA phosphorylation site [41], was replaced by a lysine (K) in pTRPV1 as in dog TRPV1 [14]. It has been suggested that PKA phosphorylation increases TRPV1 activity and inhibits desensitization [42]. This amino acid was suggested to be related to the sensitivity to capsaicin in a mutagenesis study on dog TRPV1 [14] and rat TRPV1 [42]. The similar EC_{50} value of pTRPV1 obtained in the present results to dog TRPV1 may be explained by the substitution of the amino acid at this site.

Many vanilloid agonists and endogenous vanilloids could activate pTRPV1 in a concentration-dependent manner and the order of potency of these agonists to pTRPV1 was RTX > olvanil > capsaicin > PPAHV > OLDA. Like other mammalian TRPV1s, RTX was more potent than olvanil in pTRPV1, except that olvanil was one order of potency stronger than RTX in guinea-pig TRPV1 [11]. pTRPV1 possesses a methionine (M) at position 542, a site recently identified to confer sensitivity to PPAHV and RTX [12,43], which was clearly shown in the present study. Isovelleral and scutigeral, vanilloid receptor agonists in native tissues [44], had no effect on pTRPV1 at any concentration, as reported for rat TRPV1 [15]. OLDA, an endogenous vanilloid agonist [45], showed only a slight agonistic action in pTRPV1. It is reported to have full agonistic action in mouse [13] and dog TRPV1 [14]. On the other hand, anandamide, a partial agonist to guinea-pig TRPV1 [11] acts as a full agonist in pTRPV1. In native pig DRG, the EC_{50} values of capsaicin and RTX were larger than for recombinant pTRPV1. Similar differences have been reported in native rat DRG cells and rat TRPV1-expressing HEK293 cells [46]. These differences may be explained by differences in post-translation modifications of recombinant TRPV1 and the native channels.

In pTRPV1-expressing HEK293 cells, a $[Ca^{2+}]_i$ rise occurred rapidly in response to capsaicin and vanished after its washout. On the other hand, the RTX-induced $[Ca^{2+}]_i$ increase displayed a different pattern from the capsaicin-induced one, with a markedly slow onset followed by a subsequently maintained phase. A similar slow and irreversible response to RTX was observed in cultured DRG cells. This seems to be an intrinsic property of this compound rather than an artificial effect on the heterogeneous expression system. A similar difference between RTX and capsaicin has been demonstrated in rat DRG [15,47] and recombinant rat TRPV1 expressed in *Xenopus* oocytes [31] and HEK293 cells [15,47].

Vanilloid antagonists, such as capsazepine, iodoRTX and ruthenium red effectively inhibited the activation of pTRPV1 in response to capsaicin, as for mouse and dog TRPVI [13,14]. It has been reported that there are clear species differences in inhibitory effects of capsazepine on pH- and heat-induced responses. Capsazepine is ineffective for pH-induced responses of mouse [13] and rat TRPV1 [10], but is effective for human [10] and guinea-pig TRPV1 [11]. Moreover, $[Ca^{2+}]_i$ response to heat is inhibited by capsazepine for human, but not for rat, TRPV1 [10]. In the present experiment, pTRPV1 activations by pH and heat were suppressed by capsazepine. This was also the case for cultured porcine DRG neurons. These data indicate that pTRPV1 is pharmacologically more akin to human TRPV1 than rodent TRPV1. It has recently been suggested that a leucine (L) at 547 is essential for capsazepine antagonism of proton activation [12]. This was not the case in pTRPV1, because this portion of the amino acid is methionine (M) instead of leucine (L) as in rodent TRPV1 [10,13]. It has also been demonstrated that capsazepine becomes effective in suppressing pH-induced responses after mutation of some series of residues (I514M, V518L and M547L) in rat TRP [43]. As mentioned above, a number of studies have been reported about different pharmacology for the TRPV1 channels expressed by different species. However, there are very few reports concerning the porcine orthologue of this channel. Only the presence of [^{3}H] RTX-binding sites in capsaicin-sensitive neurons in pig spinal cord and dorsal root ganglia has been indicated [21]. In the anesthetized pig, it has been reported that capsaicin evokes vasodilatation via release of a calcitonin-gene-related peptide [22–24]. These biological responses may be mediated by the activation of the TRPV1 channel, which was characterized in the present study.

In conclusion, this is the first study of the cloning, functional expression and pharmacological characterization of pTRPV1 and comparison with native DRG. The present results indicate that pTRPV1 may be a suitable target molecule for studying TRPV1 functions. Moreover, some pharmacological similarities to the human orthologue suggest that the pig might be a good large animal model for human pain research in vivo.

Acknowledgments

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