Molecular Cloning and Functional Characterization of *Xenopus tropicalis* Frog Transient Receptor Potential Vanilloid 1 Reveal Its Functional Evolution for Heat, Acid, and Capsaicin Sensitivities in Terrestrial Vertebrates* 

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**Background:** TRPV1 is a thermosensitive channel in mammalian, but little information is available on amphibian TRPV1 function.

**Results:** Capsaicin, heat, and acid stimulated cloned and endogenous frog TRPV1 channels, and the former two stimuli evoked nocifensive behaviors.

**Conclusion:** Frog TRPV1 functions as a capsaicin-, heat-, and acid-sensitive channel.

**Significance:** TRPV1 may have acquired a physiological role for nocuous detection soon after its evolutionary origin.

The functional difference of thermosensitive transient receptor potential (TRP) channels in the evolutionary context has attracted attention, but thus far little information is available on the TRP vanilloid 1 (TRPV1) function of amphibians, which diverged earliest from terrestrial vertebrate lineages. In this study we cloned *Xenopus tropicalis* frog TRPV1 (xtTRPV1), and functional characterization was performed using HeLa cells heterologously expressing xtTRPV1 (xtTRPV1-HeLa) and dorsal root ganglion neurons isolated from *X. tropicalis* (xtDRG neurons) by measuring changes in the intracellular calcium concentration ([Ca2+]i). The channel activity was also observed in xtTRPV1-expressing *Xenopus* oocytes. Furthermore, we tested capsaicin- and heat-induced nocifensive behaviors of the frog *X. tropicalis* *in vivo*. At the amino acid level, xtTRPV1 displays ~60% sequence identity to other terrestrial vertebrate TRPV1 orthologues. Capsaicin induced [Ca2+]i increases in xtTRPV1-HeLa and xtDRG neurons and evoked nocifensive behavior in *X. tropicalis*. However, its sensitivity was extremely low compared with mammalian orthologues. Low extracellular pH and heat activated xtTRPV1-HeLa and xtDRG neurons. Heat also evoked nocifensive behavior. In oocytes expressing xtTRPV1, inward currents were elicited by heat and low extracellular pH. Mutagenesis analysis revealed that two amino acids (tyrosine 523 and alanine 561) were responsible for the low sensitivity to capsaicin.

Taken together, our results indicate that xtTRPV1 functions as a polymodal receptor similar to its mammalian orthologues. The present study demonstrates that TRPV1 functions as a heat- and acid-sensitive channel in the ancestor of terrestrial vertebrates. Because it is possible to examine vanilloid and heat sensitivities *in vitro* and *in vivo*, *X. tropicalis* could be the ideal experimental lower vertebrate animal for the study of TRPV1 function.

The genes of the transient receptor potential (TRP)family, including TRP vanilloid 1 (TRPV1), have been compared, and evolutionary differences among vertebrates have been demonstrated (1). Especially in mammalian and birds, TRPV1 orthologues have been cloned, and their channel properties have been characterized in considerable detail (2–7). In other distantly related species, however, the properties of TRPV1 orthologues are not well examined.

Recently, it has been reported that TRPV1 may contribute to somatic thermosensation in crocodiles (8) and snakes (9), although the channel activity at the molecular level has not been analyzed in detail. In amphibians, which are subject to thermal environmental variations (poikilothermal animals), the function of TRPV1 is also unknown. An early study reported that capsaicin did not affect substance P release from the spinal cord, although it changed nociceptive responses to cutaneous stimuli in *Rana esculenta* (10). It has also been reported that frogs are completely insensitive to capsaicin (11).

In addition, it is suggested that molecules of the heat sensor in the frog lack vanilloid sensitivity, as capsaicin does not produce any membrane current in *Rana pipiens* heat-sensitive dorsal root ganglion (DRG) neurons (12). TRPV1 is preserved in all terrestrial vertebrates examined so far (13). However, it has been reported that the channels do not always exhibit similar pharmacological properties. The most striking difference exists...
with respect to sensitivity to capsaicin; rabbits and chickens are less sensitive to it than other vertebrates (3, 6). Therefore, the frog TRPV1 molecule may lack sensitivity to capsaicin or have limited sensitivity to it.

There is no report that examines whether frog TRPV1 serves as a nociceptor against acid and heat stimuli. Moreover, phylogenetic analysis suggests that there is no apparent TRPV1 orthologue in teleost fish genomes (although genes similar to TRPV1 and TRPV2 exist) and only terrestrial vertebrates possess TRPV1 orthologues (1). Because amphibians diverged from amniotes in the early stage of the terrestrial evolutionary process, functional analysis of the amphibian TRPV1 orthologues may provide clues for understanding the functional evolution.

In this study we cloned TRPV1 from the western clawed frog *Xenopus tropicalis* (xtTRPV1) and expressed it in HeLa cells and *Xenopus laevis* oocytes to characterize its ion channel properties. These data showed that xtTRPV1 possessed a functional vanilloid binding site. Capsaicin induced nocifensive behavior in the *X. tropicalis* in vivo with potency similar to that observed in vitro. We found that xtTRPV1 was less sensitive to capsaicin due to the differences of two amino acids from mammalian orthologues. We also found that xtTRPV1 was activated by acidic and heat stimuli. Our data demonstrate that xtTRPV1 plays a role as a polymodal receptor like mammalian TRPV1.

**EXPERIMENTAL PROCEDURES**

**Cell Preparation—** *X. tropicalis* were kindly provided by the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT). They were housed in large plastic tanks, fed once every 2 days, and kept on a 12-h light/dark cycle. Room temperature was maintained at 22–25 °C. DRG neurons were obtained from *X. tropicalis* according to the procedure reported elsewhere with some modification (14). In brief, *X. tropicalis* (~5 cm) were sacrificed by decapitation. The spinal column was opened, and DRGs were detached with a dissecting microscope. The lumbar DRGs were removed, and attached fibers were trimmed in Ca$^{2+}$- and Mg$^{2+}$-free phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 12H$_2$O, 1.8 KH$_2$PO$_4$). Isolated DRGs were enzymatically dissociated in Liebowitz-15 (Invitrogen) tissue culture medium (diluted with a 3/13 volume of water) supplemented with collagenase P (2.5 mg/ml; Roche Applied Science), dispase II (2 mg/ml; Roche Applied Science), DNase (0.5 mg/ml; Roche Applied Science), penicillin (100 units/ml; Meiji Seika), and streptomycin (100 μg/ml; Banyu) for 30 min at 37 °C. After enzyme digestion, individual cells were mechanically dissociated by pipetting up and down using a fire-polished Pasteur pipette. Then the cell suspension was centrifuged (85 × g for 2 min), and the pellet-containing cells was resuspended in culture medium. Aliquots were plated onto glass coverslips coated with poly-L-lysine (Sigma) in a humidified atmosphere at ~25 °C. The cells were used for experiments within 1 day after preparation.

**Cloning of xtTRPV1—** Using total RNA extracted from peripheral nerves of the *X. tropicalis* as a template, the full-length complementary DNA (cDNA) of xtTRPV1 was amplified by RT-PCR using PCR primers (forward, 5′-ATGAAAAT-GAGAAATGGGAA-3′; reverse, 5′-TCACCTGTCCCTTG-GATGTC-3′) that were designed based on the genome sequence data base published by Ensembl. The xtTRPV1 gene was cloned into pENTR/D-Topo (Invitrogen) and then subcloned into the pVenus-NLS vector for transfection into HeLa cells or into the pOx (+) (15) vector for oocyte expression. pVenus-NLS was constructed by introducing five mutations (F46L/F64L/M153T/V163A/S175G) into the pVenus-NLS (Clontech) as previously described (16) and by inserting the oligonucleotide (TCCGGAGATCAGAGAGAGCTGATGTC-CAAAAAGAGAGAGATGAGTACAGCTCAAAGAAAGAAGA-GAAAGGTAGATCC) coding three copies of the nuclear localization signal of the simian virus 40 large T-antigen (17) between the BspEI and BgIII sites.

HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were transfected with the expression vector using a transfection reagent (Lipofectamine™ LTX Reagent, Invitrogen) and used 24 h after transfection. xtTRPV1-expressing HeLa cells were detected by GFP fluorescence (Venus) with appropriate filters. Single and double amino acid mutants of xtTRPV1 were made using a PrimeSTAR Mutagenesis Basal kit (Takara) with the following sets of primers: for Y523S, forward (5′-AGCTACTCTGAAATTTCTGTCSTATGCC-3′) and reverse (5′-AAATTCCAGATGTCAGCATCAA-3′); for A561T, forward (5′-AGCCTGGACAATGTGCTGTACTA-CAGC-3′) and reverse (5′-CACTGTTGACCTGCTTTTG-CTAAAAC-3′). All wild-type and mutant clones used were verified by sequencing.

**Ca$^{2+}$ Imaging—** The intracellular calcium concentration ([Ca$^{2+}$]) in single cells was measured with a fluorescent calcium indicator, fura-2, by dual excitation using a fluorescent imaging system controlling illumination and acquisition with software (Aqua Cosmos; Hamamatsu Photonics, Japan) as described previously (18). To load fura-2, cells were incubated for 0.5–1 h at 37 °C with 10 μM fura-2-acetoxyethyl ester (Fura-2 AM; Molecular Probes) and 0.02% cremophore EL (Sigma) in HEPS-buffered solution: 134 mM NaCl, 6 mM KCl, 1.2 mM MgCl$_2$, 2.5 mM CaCl$_2$, 10 mM HEPS (pH 7.4 with NaOH). A coverslip with fura-2-loaded cells was placed in an experimental chamber mounted on the stage of an inverted microscope (IX71; Olympus) equipped with an image acquisition and analysis system. To measure [Ca$^{2+}$], cells were illuminated every 5 s with light at 340 and 380 nm, and the respective fluorescent signals of 505 nm were detected. The fluorescent light was projected to a charge-coupled device camera (ORCA-ER; Hamamatsu Photonics), and the intensities of fluorescent light at 340 and 380 nm were detected. The fluorescent light was projected to a charge-coupled device camera (ORCA-ER; Hamamatsu Photonics), and the intensities of fluorescent light at 340, 380, and their ratio (F340/F380) were stored on the hard disc of a computer (Endeavor pro2500; Epson). Cells were continuously superfused with the external solution through multibarreled tubes by gravity at a flow rate of 1.5 ml/min, and the bath level was adjusted so that the total bath volume was about 0.5 ml. Drugs were applied through multibarreled tubes of puffer pipettes. All experiments were carried out at...
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room temperature (22–25 °C) except for heat stimulation. For heat stimulation, heated HEPES-buffered solution was applied by perfusion. The temperature of the bathing solution was monitored with a temperature sensor (IT-21; AD Instrument), the tip of which was placed near the cell of interest. Heat stimulation was stopped when the chamber temperature reached the intended value.

Two-electrode Voltage Clamp Method—The TRPV1 channel of *X. tropicalis* was heterologously expressed in oocytes of *X. laevis*, and ionic currents were recorded using the two-electrode voltage-clamp method (13). xTRPV1 complementary RNA (cRNA) was synthesized using pOX (+) vectors containing xTRPV1 with an mMESSAGE MACHINE SP6 kit (Ambion) according to supplier’s instructions. Then 50 nl of xTRPV1 cRNA (50–200 ng/μl) was injected into defolliculated oocytes, and ionic currents were recorded for 1–6 days post-injection. The oocytes were voltage-clamped at −60 mV. Capsaicin was diluted into ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES, pH 7.6) and applied to the oocytes by perfusion. For thermal stimulation, heated ND96 solution was applied. For acidic stimulation, ND96 solution at pH 4.0 was applied.

Behavioral Experiment—*X. tropicalis* (~5 cm) was placed in water in a glass bottle (22–25 °C) and allowed to habituate to the environment for 20 min before tests to allow appropriate behavioral immobility. To observe capsaicin-induced jumping behavior, concentrated capsaicin was added directly to the water in which the *X. tropicalis* was immersed. Before the addition of capsaicin, the frog was mostly quiescent. After the addition of capsaicin, the frog began jumping along the wall of the glass bottle. We counted the number of jumps as nocifensive behavior for 5 min before and 20 min after the addition of capsaicin. The effect of a vehicle (dimethyl sulfoxide; DMSO) on jumping behavior was assessed by adding it to the water. To record the number and timing of the jumping behavior, we manually applied electrical noises to an AD convertor (PowerLab, AD Instrument). The effect of heat on *X. tropicalis* was observed in a similar way to that of capsaicin. In brief, after habituation, the water in the glass bottle was replaced with heated water at appropriate temperatures. Immediately, the glass bottle containing the frog was immersed in a water bath at the same temperature.

Chemicals—Capsaicin was purchased from Sigma. Capsaicin was dissolved in ethanol for the calcium imaging experiment, and for the behavioral test it was dissolved in DMSO at a high concentration as a stock solution. Amiloride and capsazepine from Sigma were dissolved in DMSO to make a stock solution (100 mM). The drugs were diluted to their final concentrations with external solution.

Data Analysis—The data are presented as the mean ± S.E. (*n* = number of observations). Statistical significance was tested using the unpaired Student's *t* test with *p* values <0.05. Values of the 50% effective concentrations (EC50) were determined using Origin software (OriginLab). These data were obtained from at least three different transfections or animals per experiment.

RESULTS

Cloning of *X. tropicalis* TRPV1—xTRPV1 was cloned from total RNA extracted from peripheral nerves. The cDNA nucleotide sequence coding for xTRPV1 consisted of 2520 bp, and the predicted protein of xTRPV1 had 840 amino acid residues. Like other TRPV1 members, the *X. tropicalis* orthologue contained six putative hydrophobic transmembrane and six N-terminal ankryin repeat domains. Upon alignment with vertebrate TRPV1 orthologues, the amino acid sequence similarities were as follows: 92% with *X. laevis*, 61% with the rattlesnake, 64% with the chicken, and 60–64% with mammals (Fig. 1).

Characterization of Recombinant *X. tropicalis* TRPV1 Receptor—xTRPV1 was transiently expressed in HeLa cells (xTRPV1−HeLa) to characterize the channel properties. Because the TRPV1 channel has high Ca2+ permeability (19), we examined whether capsaicin could stimulate TRPV1 channels using Ca2+ imaging with fura-2. Fura-2-loaded xTRPV1−HeLa were stimulated with various concentrations of capsaicin (Fig. 2A). In xTRPV1−HeLa, capsaicin elicited a tiny [Ca2+]i increase at 30 μM. At 100 μM, [Ca2+]i peaked during its application and returned to the resting level after washout. The concentration-response relationship for capsaicin is depicted in Fig. 2B. We found that the capsaicin sensitivity of xTRPV1 was extremely low compared with mammalian TRPV1 orthologues (2, 4, 5, 7, 20). The EC50 of capsaicin was estimated to be ~85.4 μM, although we could not obtain maximal responses to capsaicin. At higher concentrations of capsaicin (>1 mM), nonspecific action was evoked in xTRPV1-untransfected HeLa cells, which prevented us from observing maximal responses. In xTRPV1-untransfected HeLa cells, capsaicin did not evoke [Ca2+]i increases up to 300 μM (data not shown).

It has been reported that the mammalian TRPV1 channel is activated by extracellular low pH and heat (20). We compared the TRPV1 amino acid residues that were reported to be
responsible for proton sensitivity (Fig. 3A) (21) and found that they were conserved in X. tropicalis TRPV1. To examine whether acidic stimuli evoked [Ca^{2+}], increases in xTRPV1-HeLa, they were stimulated with various concentrations of protons and subsequently with capsaicin at 300 μM (Fig. 3B). Protons evoked [Ca^{2+}], increases in a concentration-dependent manner (Fig. 3C). Very few xTRPV1-untransfected HeLa cells (only 4 of 58 cells) responded to pH 5.0, at which xTRPV1-HeLa cells responded considerably. Next we assessed whether xTRPV1 responded to heat. Heat also evoked [Ca^{2+}], increases in xTRPV1-HeLa (Fig. 3D). Temperature-response relations in xTRPV1-HeLa and untransfected HeLa cells are shown in Fig. 3E. At 38 °C or higher temperatures, significant increases of [Ca^{2+}], were induced by heat stimulation in xTRPV1-HeLa compared with untransfected HeLa cells.

Next, the ion channel properties of xTRPV1 were examined by expressing it in oocytes of X. laevis and measuring ionic currents using the two-electrode voltage-clamp method. We first examined whether the oocytes expressing xTRPV1 were activated by heat. Heat stimuli induced inward currents, the amplitudes of which were gradually increased by repetition (data not shown). Fig. 4A shows a representative membrane current at the third heat stimulus. No current was induced by heat stimulation in water-injected oocytes (Fig. 4B). When analyzed with Arrhenius plots, the average temperature threshold for activation was 38.33 ± 0.75 °C (n = 6) (Fig. 4C), the value of which did not change regardless of the repetition of heat stimuli. The threshold temperature for activation was essentially consistent with that of xTRPV1-HeLa. Capsaicin stimulation did not elicit observable currents in the oocytes expressing xTRPV1, although a high concentration of capsaicin (300 μM) that activated xTRPV1-HeLa was applied (Fig. 4D). Oocytes injected with xTRPV1 cRNA responded to acidic stimulation (Fig. 4E). Water-injected oocytes also responded to acidic stimulation (Fig. 4F), but the current amplitudes were much smaller than those elicited by oocytes injected with xTRPV1 cRNA.

[Ca^{2+}], Responses to Polymodal Stimuli in Isolated X. tropicalis DRG Neurons—To compare the properties of recombinant xTRPV1 with endogenous xTRPV1, the effects of capsaicin, extracellular low pH, and heat on [Ca^{2+}], were examined in DRG neurons isolated from X. tropicalis (xDRG neurons). xDRG neurons were stimulated with various concentrations of capsaicin and subsequently with KCl (80 mM, Fig. 5A). At 30 μM or higher, capsaicin evoked [Ca^{2+}], increases in xDRG neurons. Unlike the recombinant xTRPV1 receptor, [Ca^{2+}], increases induced by capsaicin rapidly returned to the basal level after its washout even when a high concentration (300 μM) was used. Fig. 5B shows the concentration–dependence relationship for capsaicin in xDRG neurons. The EC_{50} for capsaicin was estimated to be 49.8 μM, the value of which was similar to that in xTRPV1-HeLa. Consistent with our previous reports on rodents (22, 23), capsaicin-sensitive DRG neurons in X. tropicalis were distributed in relatively small neurons (Fig. 5C). The average sizes of capsaicin-responding xDRG neurons (389 ± 22.6 μm²) was significantly smaller than those of non-responding ones (620 ± 14.1 μm², p < 0.05). The capsaicin-induced [Ca^{2+}], increase was reversibly inhibited by capsazepine (50 μM, Fig. 5D), although capsazepine itself elicited [Ca^{2+}], increases in a few neurons.

Like xTRPV1-HeLa, protons evoked [Ca^{2+}], increases in capsaicin-responding xDRG neurons in a concentration-dependent manner (Fig. 6, A and B). However, the threshold pH was slightly higher in xDRG neurons (pH 6.5) than that of recombinant TRPV1, pH 6.0. It has been reported that acid-sensitive ion channels blocked by amiloride are expressed in frog DRG neurons (12). Therefore, we examined the effect of amiloride (100 μM) on the proton-induced [Ca^{2+}], increase in xDRG neurons. In approximately half (6 of 11) of the xDRG...
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FIGURE 4. Current responses of xtTRPV1 to heat, capsaicin, and acid. Responses of oocytes injected with xtTRPV1 cRNA (A) or water (B) to heat stimulation are shown. Repeated heat stimuli (4–5 times) were applied to the oocytes. The representative traces of the third heat stimulus are shown in A and B. C, shown is a representative current trace after acid applications to oocytes injected with xtTRPV1 cRNA. Shown are representative current traces after acidic applications to oocytes injected with xtTRPV1 cRNA (E) or water (F). Acidic responses were observed for four and two oocytes injected with xtTRPV1 cRNA and water, respectively. Capsaicin responses were observed for four oocytes injected with xtTRPV1 cRNA. For heat stimulation, six and three oocytes injected with xtTRPV1 cRNA and water, respectively, were observed. In each stimulation, similar responses were observed, as shown in representative traces.

neurons, amiloride inhibited the \([Ca^{2+}]_i\), increases induced by pH 6.5 (data not shown).

Heat also activated xtDRG neurons in a temperature-dependent manner (Fig. 6, C and D). The thermal threshold in xtDRG neurons was 38 °C, which was consistent with that of recombinant TRPV1. Heat (>42 °C) also evoked \([Ca^{2+}]_i\), increases in a small number (3 of 26) of capsaicin-non-responding xtDRG neurons. These data demonstrated that recombinant XT1 channels possessed polymodal sensitivity similar to endogenous TRPV1-expressing xtDRG neurons.

Nocifensive Behavior in Response to Capsaicin and Heat—
We next examined the effects of capsaicin and heat on X. tropicalis in vivo. X. tropicalis were placed in glass bottles filled with water and challenged with various concentrations of capsaicin dissolved in water in which the X. tropicalis were immersed. Capsaicin evoked a nocifensive behavior (jumping), which is indicated as vertical lines in Fig. 7A. The numbers of the behavior for each 1-min interval before and after the application of capsaicin (100 μM) are shown in Fig. 7C. At 100 μM, the nocifensive behavior started just after the application of capsaicin and continued intermittently. Fig. 7E shows the number of

FIGURE 5. Capsaicin increases \([Ca^{2+}]_i\), in xtDRG neurons. A, shown are representative \([Ca^{2+}]_i\), increases in response to capsaicin at different concentrations and KCl (K, 80 mM) applied for 1 min. B, shown is a concentration-response relationship for capsaicin in xtDRG neurons (3 μM, n = 10; 10 μM, n = 14; 30 μM, n = 10; 100 μM, n = 9; 300 μM, n = 12). Each data point represents the mean ± S.E. C, the histogram summarizes the numbers and sizes of cells that were sensitive (filled bars; n = 89) or not sensitive (open bars; n = 361) to capsaicin. Note that capsaicin-sensitive neurons are distributed in relatively small neurons. D, shown are representative \([Ca^{2+}]_i\), increases induced by capsaicin before, during, and after the application of capsazepine (50 μM). Similar results were obtained for 5 other neurons.

FIGURE 6. Protons and heat increase \([Ca^{2+}]_i\), in xtDRG neurons. A, representative \([Ca^{2+}]_i\), increases in response to protons at pH 7.0, pH 5.5, capsaicin (cap, 300 μM) and KCl (K, 80 mM) for 1 min are shown. B, concentration-response relationships for stimulation by protons in capsaicin-responding xtDRG neurons (filled circles, pH 7.0, n = 6; pH 6.5, n = 13; pH 6.0, n = 11; pH 5.5, n = 9; pH 5.0, n = 8) and capsaicin-non-responding neurons (open circles, pH 7.0, n = 8; pH 6.5, n = 8; pH 6.0, n = 8; pH 5.5, n = 6; pH 5.0, n = 6) are shown. Each data point represents the mean ± S.E. * p < 0.05, when compared with capsaicin-non-responding xtDRG neurons. C, shown are representative \([Ca^{2+}]_i\), increases in response to heat of 38 and 42 °C, capsaicin (cap, 300 μM), and KCl (K, 80 mM) for 1 min. D, shown is the temperature-response relationship in capsaicin-responding xtDRG neurons (filled circles, 34 °C, n = 11; 38 °C, n = 15; 42 °C, n = 9; 46 °C, n = 7) and capsaicin-non-responding ones (open circles, 34 °C, n = 11; 38 °C, n = 11; 42 °C, n = 12, 46 °C, n = 14). Each data point represents the mean ± S.E. * p < 0.05, when compared with capsaicin-non-responding xtDRG neurons.
times of the behavior for the first 3 min of the capsaicin application for different concentrations. The concentration-response relationship for capsaicin essentially corresponded to the data for xtTRPV1-HeLa and xtDRG neurons.

We also observed nocifensive behavior in *X. tropicalis* induced by heat. Frogs began jumping just after the application of water at 38 °C (Fig. 7B). The nocifensive response to heat was remarkable during the first 1 min; thereafter the movement of *X. tropicalis* became sluggish (Fig. 7D). Fig. 7F shows the counts of the behavior for 1 min after heat stimulation at different temperatures. The temperature-response relationship was almost identical to those of xtTRPV1-HeLa and xtDRG neurons. We could not obtain stable data on nocifensive behavior induced by acid stimulation, as acid elicited behavioral changes with different timings and magnitudes in different animals. Similar discrepancies have been reported by Kuffler et al. (12). They explained that this occurred because frog skin has rich blood circulation that may greatly dilute the protons to prevent them from reaching a concentration that would activate peripheral endings of the afferent fibers.

**Characterization of the Capsaicin Responses in xtTRPV1 Mutant Channels**—In the present experiment, we found that xtTRPV1 was actually sensitive to capsaicin, although its sensitivity was extremely low compared with the majority of mammalian orthologues such as that of the human (2). It has been suggested that Ser-512 (3) and Thr-550 (6), located in the third and fourth putative transmembrane domains in capsaicin-sensitive TRPV1 (in humans and rodents), interact with vanilloid ligands. Comparison of the amino acid sequences revealed that these two amino acids changed to Tyr-523 and Ala-561 in *X. tropicalis* TRPV1, corresponding to Ser-512 and Thr-550 in the human, respectively (Fig. 8A). Therefore, to investigate whether these amino acids (one or both) were related to low vanilloid sensitivity in *X. tropicalis* TRPV1, we studied the effects of capsaicin on single (Y523S, A561T) and double (Y523S/A561T) point mutants of TRPV1. These mutant channels were transiently expressed in HeLa cells, which were stimulated by various concentrations of capsaicin (Fig. 8B). As expected, the sensitivity to capsaicin was increased by mutation of the above two-amino acid residues (Fig. 8C). The EC50 values for capsaicin in wild, Y523S, A561T, and Y523S/A561T point mutants of TRPV1. These mutant channels were transiently expressed in HeLa cells, which were stimulated by various concentrations of capsaicin (Fig. 8B). As expected, the sensitivity to capsaicin was increased by mutation of the above two-amino acid residues (Fig. 8C). The EC50 values for capsaicin in wild, Y523S, A561T, and Y523S/A561T channels were 85.4, 42.1, 0.82, and 0.18 μM, respectively. The capsaicin sensitivity to Y523S/A561T channels was almost identical to that for the human orthologue (EC50 = 0.19 μM (24)).

The above observations were further confirmed by capsaicin-induced currents and the relative concentration-relation for capsaicin in *Xenopus* oocytes expressing *X. tropicalis* TRPV1 mutant channels (Fig. 9). As expected, substitutions of these two amino acid residues increased the sensitivity to capsaicin. The concentrations of capsaicin that elicited observable
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Amino acid sequence alignments of terrestrial vertebrate TRPV1s within transmembrane domain 3 (TM3) and TM4 are shown. The mutations generated in xTRPV1 are indicated at the bottom. Amino acid residues (Tyr-523, Ala-561) reported to be responsible for vanilloid sensitivity in rodents and rabbits are shown with arrows. Representative [Ca\textsuperscript{2+}] responses to capsaicin at different concentrations for 1 min in wild and three kinds of mutant channels are shown. Symbols are circles for wild-type, triangles for Y523S, and squares for A561T mutant channels. Responses at respective concentrations are normalized to the maximal response in each channel. Point mutants of xTRPV1 (Y523S, A561T, and Y523S/A561T) show increased capsaicin sensitivity.

Increased capsaicin sensitivities in oocytes expressing xTRPV1 harboring Y523S and/or A561T mutations. Representative current traces after capsaicin stimulation in the oocytes expressing xTRPV1 harboring A561T (A), Y523S (B), and Y523S/A561T (C) mutations are shown. Similar responses were observed in 3, 8, and 5 oocytes for A561T, Y523S, and Y523S/A561T mutants, respectively.

DISCUSSION

The cloning of TRPV1 from various species has provided substantial information on the structure-function relationships of TRPV1. Here we reported the cloning of the amphibian orthologue of TRPV1 and its characterization. Heterologous expression studies and characterization of native channels expressed in sensory neurons showed that xTRPV1 was sensitive to capsaicin, acid, and heat. In the behavioral test in vivo, capsaicin and high temperatures evoked nocifensive responses. These results indicated that xTRPV1 plays a role as a multimodal receptor similar to those of mammalian orthologues.

The amino acid sequence of xTRPV1 showed ~60% similarity to various TRPV1 orthologues (Fig. 1). xTRPV1 contains six putative membrane spanning domains with six N-terminal ankyrin repeats (data not shown). In the present study, to characterize this newly cloned TRPV1 orthologue, X. tropicalis TRPV1 was transiently expressed in HeLa cells and Xenopus oocytes. Then they were stimulated with polyomodal stimuli such as capsaicin, protons, and heat. It has been reported that heat-sensitive sensory neurons of R. pipiens lack capsaicin sensitivity (12). However, using a Ca\textsuperscript{2+} imaging assay, we observed that capsaicin actually increased [Ca\textsuperscript{2+}], in xTRPV1-HeLa. We also found [Ca\textsuperscript{2+}] increases in xTRPV3 neurons. The threshold concentrations of capsaicin were consistent in the two experimental systems, suggesting that endogenous TRPV1 responded to capsaicin, although its sensitivity was extremely low as discussed below. We could not observe current responses to capsaicin in oocytes expressing xTRPV1, although this was not due to the oocyte expression system because we observed clear current responses when xTRPV1 mutant channels were used (described below). In the case of mutant channels, capsaicin sensitivity in oocytes was lower than that in xTRPV1-HeLa. Thus it may be possible that the threshold for capsaicin activation of wild-type xTRPV1 became higher than 300 \textmu M when expressed in oocytes, which made it difficult to examine as much higher concentrations of capsaicin are difficult to dissolve in bath solution. It is, however, clear that capsaicin activates amphibian TRPV1 channels as it does in HeLa cell and DRG neurons.

Heat stimulated xTRPV1-HeLa, oocytes expressing xTRPV1, and xTRPV3 neurons in similar temperature ranges, but the value was somewhat lower than those for mammalian orthologues (25). We also observed heat-evoked [Ca\textsuperscript{2+}], increases in some capsaicin-non-responding xTRPV3 neurons, suggesting that other calcium-permeable heat-sensing channels were expressed in xTRPV3 neurons such as TRPV3 and TRPV4, other thermosensitive TRP channels whose thermal thresholds are lower than for TRPV1 (26, 27). Quite recently, however, our group reported that the heat-sensitive TRPV3 channel in mammals was activated by cold in X. tropicalis (13). Moreover, Gracheva et al. (9) have demonstrated that transient receptor potential ankyrin1 (TRPA1), a cold sensor in mammals, functions as a heat-sensitive detector in snakes. Therefore, in X. tropicalis, thermosensitive TRP channels whose temperature sensitivity is different from those in mammalian orthologues may be expressed in sensory neurons.
Regarding acid sensitivity, the threshold pH for xTRPV1 activation in xTRPV1-HeLa was lower (pH 6.0) than in sensory neurons (pH 6.5). This may be due to the expression of acid-sensitive ion channels (ASICs), because the [Ca\(^{2+}\)] response to pH 6.5 was partly inhibited by amiloride, a blocker of ASICs. Acid (pH 6.5) has been reported to evoke inward currents that are decreased by amiloride in frog DRG neurons (12). Additionally, it is suggested that TRPV1 receptor activity is modulated by the host cell expression system, as acid responses are different when TRPV1 is expressed in 1321 cells and HEK 293 cells (28).

We examined whether capsaicin and heat evoked nocifensive behaviors in X. tropicalis. It was reported that there were no signs of pain behavior when capsaicin (100 μM) was dropped into the eyes of R. pipiens (12). In the present study, however, we succeeded in quantification of nocifensive behaviors induced by capsaicin in X. tropicalis. This may be due to the differences in the sensitivities of TRPV1 to capsaicin among frog species or in the administration methods, as we used X. tropicalis and applied capsaicin systemically through the skin. The potency of capsaicin-induced nocifensive behaviors was almost identical to the stimulatory effect in the in vitro experimental systems (Figs. 2 and 5). Heat also evoked nocifensive behavior in X. tropicalis with similar thermal thresholds observed in calcium imaging and membrane current analyses.

These data revealed that the sensitivity to capsaicin of X. tropicalis TRPV1 was very low in recombinant xTRPV1, native sensory neurons, and in a behavioral test in vivo. Similarly, low sensitivity of capsaicin to sensory neurons in the chicken (29) and the lack of a binding site for a radiolabeled TRPV1 agonist, \([^{3}H]\) resiniferatoxin, to the dorsal root ganglia membrane in the rabbit (30) have been reported. Gavva et al. (6) reported the molecular basis for decreased capsaicin sensitivity in the rabbit. We found that two amino acids critical for capsaicin binding in mammalian TRPV1 were different in X. tropicalis and thus conducted point mutation studies. In single and double mutants of xTRPV1, the capsaicin sensitivity increased ~2-fold (A561T), 100-fold (Y523S), and 1000-fold (Y523S/A561T) (Fig. 8). Increased capsaicin sensitivity was also observed in mutant channels expressed in Xenopus oocytes (Fig. 9). Accordingly, Ser-512 was more important for capsaicin sensitivity than Ala-561. These results indicated that the properties of amino acids were closely connected with functional vanilloid binding. Gavva et al. (6) suggested that H-bonding potential was important for agonist activity. Thrreonine has an H-bonding-capable hydroxyl group, whereas alanine is similar in size but lacks the H-bonding functional side-chain group. Serine also interacts with capsaicin via a hydrogen bond (6). We confirmed that Tyr-523 and Ala-561 were the critical amino acids responsible for the low sensitivity to capsaicin in xTRPV1. Jordt and Julius (3) have reported that Ser-523 is essential for capsaicin sensitivity. However, the conclusion was only drawn from mutations introduced into rat TRPV1, and they did not demonstrate that mutations in the corresponding 523 position affected capsaicin sensitivity in native TRPV1 as this position of chicken TRPV1 is serine (Fig. 8A). Thus, the present study is the first report to show the importance of Tyr-523 in a native channel.

Capsaicin sensitivity of TRPV1 varies among vertebrate species (Fig. 10). Here we showed that the capsaicin sensitivity of X. tropicalis TRPV1 was low. The capsaicin sensitivity of chicken TRPV1 is also low, and Tewksbury and Nabhan (31) suggested the phenomenon of directed deterrence in chicken TRPV1. That is, for the hot pepper plant, birds are favored as vectors for seed dispersal, whereas mammalian predators are repelled. In some cases such as in mammalians, the sensitivity of TRPV1 to capsaicin may relate to adaptation. However, X. tropicalis are not likely to be exposed to capsaicin in the natural environment as they are fully aquatic anurans. Thus amino acid substitutions in their capsaicin binding sites may be neutral (that is, substitutions regarding capsaicin sensitivity would not influence the fitness of the X. tropicalis).

On the other hand, with respect to temperature, TRPV1 is likely to be involved in adaptation to the thermal environment. The activation temperature threshold for the cloned X. tropicalis TRPV1 channels was slightly lower than that of human channels. X. tropicalis inhabit tropical areas, and their optimal ambient temperature range is 22–28 °C. Because temperatures beyond the appropriate range have detrimental effects (32, 33), the activation temperature threshold for xTRPV1 may be lower than that of mammalian TRPV1, which is several degrees higher than the core body temperature. To support this, it is also reported that there are striking correlations between species core body temperatures and the temperature ranges that activate transient receptor potential melastatin 8 (TRPM8) (34). Therefore, thermal activation of TRPV1 may also be tuned to the most appropriate temperature ranges for respective species.

Phylogenetically, amphibians diverged the earliest among the terrestrial vertebrates (Fig. 10). Thus, investigating the physiological role of the TRPV1 channel may provide clues for understanding its functional evolution. In this study we clearly...
showed that TRPV1 was involved in detection of high temperature and acidic stimuli as in mammalians and chickens, indicating that TRPV1 functioned as a nocuous receptor more than 350 million years ago (35). Phylogenetic analysis of the TRPV subfamily revealed that TRPV1 and TRPV2 were produced by gene duplication around the time of divergence of teleost fishes and terrestrial vertebrates (Fig. 10) (1, 13); thus TRPV1 may have acquired a physiological role for nocuous detection soon after its emergence.

In this study we obtained consistent data in three different experimental systems: recombinant xtTRPV1-expression systems, xTDGR neurons, and nocicensive behavior in vivo. X. tropicalis are ideal experimental animals because they have a short generation time and small diploid genome (36) together with whole-genome sequence data (37). Taken together, the present results indicate that X. tropicalis may be suitable for the study of TRPV1 channel-mediating pain.

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