Analysis of Transient Receptor Potential Ankyrin 1 (TRPA1) in Frogs and Lizards Illuminates Both Nociceptive Heat and Chemical Sensitivities and Coexpression with TRP vanilloid 1 (TRPV1) in Ancestral Vertebrates

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Running title: TRPA1 Acts as a Heat and Chemical Sensor in Frog and Lizard

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Background: TRPA1 is involved in pain sensation in mammals.

Results: Characterization of the physiological function of TRPA1 in frogs and lizards revealed that it serves as a noxious heat and chemical sensor.

Conclusion: TRPA1 served as a noxious heat and chemical sensor in ancestral vertebrates.

Significance: Our findings provide a novel insight into the functional evolution of pain receptors in vertebrate evolutionary process.

SUMMARY

TRPA1 and TRPV1 perceive noxious temperatures and chemical stimuli and are involved in pain sensation in mammals. Thus, these two channels provide a model for understanding how different genes with similar biological roles may influence the function of one another during the course of evolution. However, the temperature sensitivity of TRPA1 in ancestral vertebrates and its evolutionary path are unknown as its temperature sensitivities vary among different vertebrate species. To elucidate the functional evolution of TRPA1, TRPA1s of western clawed (WC) frogs and green anole lizards were characterized. WC frog TRPA1 was activated by heat and noxious chemicals that activate mammalian TRPA1. These stimuli also activated native sensory neurons and elicited nocifensive behaviors in WC frogs. Similar to mammals, TRPA1 was functionally co-expressed with TRPV1, another heat and chemical sensitive nociceptive receptor, in native sensory neurons of WC frog. Green anole TRPA1 was also activated by heat and noxious chemical stimulation. These results suggest that TRPA1 was likely a noxious heat and chemical receptor and co-expressed with
TRPV1 in the nociceptive sensory neurons of ancestral vertebrates. Conservation of TRPV1 heat-sensitivity throughout vertebrate evolution could have changed functional constraints on TRPA1 and influenced the functional evolution of TRPA1 regarding temperature sensitivity, while conserving its noxious chemical sensitivity. In addition, our results also demonstrated that two mammalian TRPA1 inhibitors elicited different effect on the TRPA1s of WC frogs and green anoles, which can be utilized to clarify the structural bases for inhibition of TRPA1.

Nociceptive receptors enable animals to sense noxious stimuli such as cold, heat, and chemicals, in order to prevent tissue damage. Thus, nociceptive receptors play important roles influencing survival in changing environments during evolutionary adaptation. Thermal and chemical stimuli are perceived by several ion channels belonging to the transient receptor potential (TRP) superfamily (1-3). Among these, TRPA1 and TRPV1 serve as multimodal receptors by perceiving noxious temperatures and chemicals in mammals. In mammals, TRPA1 is activated by cold temperatures and several noxious chemicals such as allyl isothiocyanate (AITC), cinnamaldehyde (CA), carvacrol, and acrolein that are contained in wasabi, cinnamon, oregano, and exhaust gas, respectively (4-10). The sensitivity of TRPA1 to noxious chemical has been established, while some debate exists regarding cold sensitivity (1,3,5,8-10). In contrast, TRPV1 is activated by high temperatures, acids, and several chemicals, including capsaicin, in mammals (11-13). These traits have been conserved through terrestrial vertebrate evolution, as TRPV1 also exists in other vertebrate species, such as chicken and WC frogs and is activated by high temperatures and acidic stimuli (14,15). In rodents, expression of TRPV1 and TRPA1 shows considerable overlap in native sensory neurons of the dorsal root ganglion (DRG) and trigeminal ganglion, and functional interaction between them has been reported (9,16-20). These two channels thus provide a model for understanding how different genes with similar biological roles influence the function of one another during the course of evolution.

TRPA1 is conserved in insects, such as Drosophila, where it perceives warm temperatures and is involved in avoidance of unfavorable temperatures (21,22). Drosophila TRPA1 is also sensitive to chemicals that activate mammalian TRPA1 (23). Therefore, thermal and chemical sensitivity of TRPA1 was acquired during early stages of animal evolution. Recently, TRPA1 was reported to be activated by heat stimulation in several snake species (24), while zebrafish TRPA1 was reported to be insensitive to temperature stimulation (25). Variability of TRPA1 thermal sensitivity among mammals, snakes, and zebrafish raises questions as to the ancestral state and functional evolution of TRPA1 in vertebrate lineages. In order to elucidate the functional evolution of TRPA1 in vertebrate evolutionary processes, comparison of TRPA1 among various species is essential. In addition, to understand the coevolution between TRPA1 and TRPV1, elucidation of the evolutionary timing of coexpression in sensory neurons could be informative. In this respect, amphibians, which diverged earliest among terrestrial vertebrates, may provide novel information regarding the functional evolution of TRPA1 and TRPV1 in vertebrate lineages. In addition, examining TRPA1 channel properties in novel reptilian species distantly related to snakes could prove informative in understanding the general physiological role of TRPA1 in reptiles.

In the present study, we cloned TRPA1 from the WC frog *Xenopus tropicalis* and the green anole lizard *Anolis carolinensis*, and characterized their channel properties and physiological roles relating to chemical and thermal sensitivity. We found that WC frog TRPA1 was activated by noxious chemicals that elicited nocifensive
behaviors in the frog. Regarding temperature sensitivity, WC frog TRPA1 was activated by high temperatures. TRPA1 and TRPV1 were functionally co-expressed in native sensory neurons, suggesting that these two channels cooperatively serve as nociceptive receptors in WC frogs. In addition, green anole TRPA1 was also activated by heat and noxious chemicals. These results suggest that TRPA1 served as a heat sensor in the early stages of animal evolution, and TRPV1 emerged later and became co-expressed with the existing TRPA1 in ancestral vertebrates. Here we discuss the significance of the evolution of these two different nociceptive receptors during the course of vertebrate evolution.

In addition, we also found that HC-030031 and AP18, known mammalian TRPA1 inhibitors (26,27), were not capable of inhibiting WC frog TRPA1 activity. On the other hand, only AP18 failed to elicit an inhibitory effect on green anole TRPA1. This difference in the effects of TRPA1 inhibitors may contribute to our understanding of the structural bases for inhibition of TRPA1.

EXPERIMENTAL PROCEDURES

All procedures involving the care and use of animals were approved by the National Institute for Physiological Sciences (Japan).

**WC frog strain**—The Yasuda WC frog strain (28), kindly provided by the National Bio-Resource Project (NBRP) of the Ministry of Education, Culture, Sports, Science & Technology in Japan (MEXT), was used for cloning TRPA1, Ca^{2+}-imaging experiments for DRG neurons, and behavioral experiments.

**Two-electrode voltage-clamp method**—WC frog TRPA1 was heterologously expressed in *X. laevis* oocytes, and ionic currents were recorded using the previously described two-electrode voltage-clamp method (30). Fifty nl of WC frog TRPA1 complementary RNA (cRNA) (50-200 ng/µl) were injected into defolliculated oocytes and ionic currents were recorded 2-6 days post-injection. Oocytes were voltage-clamped at –60 mV. All chemical compounds were diluted in ND96 bath solution and applied to oocytes by perfusion. For thermal stimulation, heated or chilled ND96 bath solutions were applied by...
perfusion. The current-voltage relationship was obtained using 200 ms voltage-ramp pulses from -100 to +100 mV applied every 1.5 seconds.

**Ca\(^{2+}\)-imaging experiments**—The procedure for DRG neuron preparation and Ca\(^{2+}\)-imaging was previously described in detail (15). Briefly, the pcDNA3.1(+) or pVenus-NLS vectors containing WC frog or green anole TRPA1 were transfected into HEK293 cells using Lipofectamine reagent (Invitrogen) according to the supplier’s instructions. The transfected cells were incubated at 33°C or 37°C for WC frog TRPA1 or green anole TRPA1, respectively. Cells were used for Ca\(^{2+}\)-imaging experiments after incubation lasting approximately 24 hours. Fura-2 was loaded into cells by incubating at 33°C or 37°C for WC frog TRPA1 or green anole TRPA1, respectively, for 0.5-1 hours with fura-2 acetoxymethyl ester. Cells were transferred to recording chambers and chemical or thermal stimuli were applied by perfusion of bath solutions. To measure intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)), transfected cells were exposed to lights at 340 and 380 nm, intensities of fluorescent signals (at 500 nm) emitted by lights at 340 (F340) and 380 (F380) nm were recorded, and their ratios were calculated.

**Behavioral experiments**—Behavioral experiments were performed as previously described (15). Briefly, a single WC frog (about 5 cm long) was placed into a glass bottle with a small amount of water (50 ml) and allowed to habituate for 20 minutes. Jumping behavior was assessed by counting before and after addition of chemicals added directly to the water.

**Chemicals**—Capsaicin (Cap), 2-aminoethoxydiphenil borate (2-APB), ruthenium red (RR), and HC-030031 were purchased from Sigma. CA and carvacrol were purchased from Wako. AITC, acrolein monomer, and AP-18 were purchased from Kanto Chemical, Tokyo Chemical Industry, and TOCRIS Bioscience, respectively. Cap was dissolved in ethanol or dimethyl sulfoxide (DMSO) and AP18 was dissolved in ethanol as stock solutions (100 mM). RR and acrolein monomer were dissolved in DW as stock solutions at 10 mM and 2 M final concentrations, respectively. The remaining chemicals were dissolved in DMSO as stock solutions (0.1-2 M).

**Data analysis**—The data are presented as mean ± SEM (n = number of observations). Statistical significance was examined using the Tukey-Kramer test. EC\(_{50}\) was determined using Origin software (OriginLab). The amino acid sequences of vertebrate TRPA1s were aligned using ClustalW implemented in MEGA4 and amino acid similarities were calculated (31,32).

**RESULTS**

**Chemical sensitivities of WC frog and green anole TRPA1s**—To clone WC frog TRPA1, RT-PCR was performed and the cDNA fragment containing the entire coding region of TRPA1 was amplified. The coding region of TRPA1 consisted of 3432 bp, resulting in 1144 amino acid residues (Supplemental Fig. 1). WC frog TRPA1 exhibited 56%, 56%, 63%, 56%, 52%, and 49% amino acid sequence similarity to human TRPA1, mouse TRPA1, chicken TRPA1, rattlesnake TRPA1, zebrafish TRPA1a and zebrafish TRPA1b, respectively. The WC frog TRPA1 cDNA fragment was cloned into expression vectors to characterize its channel properties. We first expressed WC frog TRPA1 in *Xenopus laevis* oocytes by injecting its cRNA and then recorded the ionic currents with a two-electrode voltage-clamp method. Stimulation of oocytes injected with WC frog TRPA1 cRNA with CA, a chemical known to activate mammalian TRPA1 through covalent modification of the cytosolic cysteine residues (33,34), produced clear inward currents in a dose-dependent manner with a half-effective concentration (EC\(_{50}\)) of 0.39 ± 0.12.
mM (Fig. 1, A and B), but not in water-injected oocytes (Supplemental Fig. 2A). We then examined the effect of carvacrol, another mammalian TRPA1 agonist that acts through non-covalent modification of the receptor, on WC frog TRPA1. Carvacrol also produced inward currents in a dose-dependent manner with an EC$_{50}$ of 0.75 ± 0.11 mM in oocytes expressing WC frog TRPA1 (Fig. 1, C and D), while oocytes injected with water did not respond to carvacrol stimulation (Supplemental Fig. 2B). WC frog TRPA1 was also activated by AITC, acrolein, and 2-APB, all of which are known mammalian TRPA1 activators (Fig. 1, E-G) (5-8,33). We further confirmed that CA stimulation increased [Ca$^{2+}$], in a dose-dependent manner in HEK293 cells that expressed WC frog TRPA1 (Fig. 2, A and B). In HEK293 cells the EC$_{50}$ for CA was 0.025 ± 0.002 mM, much lower than that of the *X. laevis* oocyte expression system (Fig. 1B). Stimulation with carvacrol, AITC or acrolein also increased [Ca$^{2+}$], in HEK293 cells expressing WC frog TRPA1 (Fig. 2, C-E). Thus, WC frog TRPA1 was activated by five different mammalian TRPA1 agonists, suggesting that noxious chemical sensitivity is conserved in WC frog TRPA1.

The ion channel properties of TRPA1s have been reported in several snake species (24). To further investigate the general characteristics of TRPA1 in reptiles, we cloned TRP A1 from the green anole which is distantly phylogenetically related to snakes within the reptilian class. Green anole TRPA1 consisted of 1112 amino acid residues (Supplemental Fig. 1) and showed amino acid sequence similarities of 63%, 65%, 82%, 74%, 61%, 52%, and 49% to human TRPA1, mouse TRPA1, chicken TRPA1, rattlesnake TRPA1, WC frog TRPA1, zebrafish TRPA1a, and zebrafish TRPA1b, respectively. Stimulation with CA, carvacrol, AITC, acrolein or 2-APB elicited clear inward currents in *X. laevis* oocytes injected with green anole TRPA1 cRNA (Fig. 3, A-G), indicating that noxious chemical sensitivity is also conserved. The EC$_{50}$ of CA for green anole and human TRPA1s were approximately 3.95 ± 0.50 mM (Fig. 3B) and 0.10 ± 0.01 mM, respectively. Thus, the order of CA sensitivities of TRPA1s was human > WC frog > green anole. The EC$_{50}$ of carvacrol for green anole TRPA1 was 1.03 ± 0.02 mM (Fig. 3D), which was similar to that of WC frog TRPA1 (Fig. 1D).

**Temperature sensitivity of WC frog and green anole TRPA1s**—Mammalian TRPA1 has been reported to be activated by cold, while snake TRPA1 is activated by heat (4,5,9,10,24). Thus, we examined the temperature sensitivity of WC frog TRPA1. Only heat stimulation, and not cold, elicited clear inward currents with desensitization upon repetitive stimulation in oocytes expressing WC frog TRPA1 (Fig. 4, A and B). The average temperature threshold for activation by the first heat stimulation (calculated with Arrhenius plots) was 39.7 ± 0.7°C (n = 12; Fig. 4C). Heat stimulation also increased [Ca$^{2+}$], in HEK293 cells expressing WC frog TRPA1 (Fig. 4D). The currents produced by heat or AITC exhibited an outwardly-rectifying current-voltage relationship characteristics of various TRP channels (Fig. 4A, inset). Heat stimulation did not elicit any clear currents in water-injected oocytes (Supplemental Fig. 2C) or increase [Ca$^{2+}$] in vector-transfected HEK293 cells (Supplemental Fig. 2D). No response to cold or heat stimulation could be detected in water-injected oocytes (Supplemental Fig. 2E).

We next examined the temperature sensitivity of green anole TRPA1. Heat stimulation also elicited clear inward currents in *X. laevis* oocytes expressing green anole TRPA1 with repeated heat stimulation resulting in desensitization (Fig. 4E). Cold stimulation did not produce currents in oocytes expressing green anole TRPA1, although subsequent heat stimulation elicited clear inward currents in the same oocytes (Fig. 4F). The average temperature threshold for activation by the first heat stimulation was 33.9 ± 0.8°C (n = 11; Fig.
Heat stimulation also increased \([\text{Ca}^{2+}]_i\) in HEK293 cells expressing green anole TRPA1 (Fig. 4H).

The \(Q_{10}\) values of the first heat stimulation for WC frog and green anole TRPA1s were calculated using data obtained from the \(X.\ laevis\) oocytes expression system. They were 59.24 ± 17.99 (n = 12) and 45.71 ± 6.04 (n = 11), respectively, which are somewhat larger than those of rattlesnake and rat snake TRPA1s (13.7 and 8.8, respectively) (24).

These observations clearly indicate that WC frog and green anole TRPA1s are heat-activated ion channels having similar properties, although sensitivities to CA and temperature thresholds for activation were somewhat different.

**Sensitivities to inhibitors differ among WC frog, and green anole TRPA1s**—We then examined the pharmacological properties of WC frog TRPA1 using known TRP inhibitors. RR, a broad TRP channel blocker, completely inhibited the CA-induced inward current that was recovered after washing out RR in oocytes expressing WC frog TRPA1 (Fig. 5A). Such RR-induced inhibition was also observed in \(\text{Ca}^{2+}\)-imaging experiments in HEK293 cells expressing WC frog TRPA1 that responded to carvacrol (Fig. 5B). We then assessed the responses to chemicals known to inhibit mammalian TRPA1 with relatively high specificity compared to other TRP channels (26,27). HC-030031 did not inhibit the WC frog TRPA1-mediated currents elicited by CA in the oocytes expressing WC frog TRPA1 (Fig. 5C), and similar results were obtained from \(\text{Ca}^{2+}\)-imaging experiments in HEK293 cells expressing WC frog TRPA1 (Fig. 5D). In addition, AP18 also failed to inhibit WC frog TRPA1-mediated responses in oocytes or HEK293 cells expressing WC frog TRPA1 (Fig. 5E and F). Thus, WC frog TRPA1 retains RR sensitivity while lacking sensitivity to two mammalian TRPA1 inhibitors (Fig. 5G).

We also examined the effects of mammalian TRPA1 inhibitors on green anole TRPA1. HC-030031 partially inhibited TRPA1-mediated responses in \(X.\ laevis\) oocytes expressing green anole TRPA1 (Fig. 5H, 63 ± 7% inhibition, n =3), while AP18 showed no inhibitory effect (Fig. 5I). Therefore, inhibitory effects of two mammalian TRPA1 blockers varied among TRPA1s from three vertebrate species that are distantly phylogenetically related each other.

**Functional expression of TRPA1 and TRPV1 in WC frog sensory neurons**—To investigate the role of TRPA1 in WC frog native sensory neurons, we examined the responses of primary cultured DRG neurons to various stimuli by measuring changes in \([\text{Ca}^{2+}]_i\). Application of CA (0.3 mM) increased \([\text{Ca}^{2+}]_i\) in WC frog DRG neurons (Fig. 6, A and B), suggesting functional expression of TRPA1. The CA-sensitive DRG neurons also responded to Cap (0.3 mM; Figs. 6, A and B), which was reported to activate WC frog TRPV1 (15), consistent with the previous observations in mammals that TRPA1-expressing neurons also express TRPV1 (9,16,18,19). A total of 313 DRG neurons from 8 WC frogs were examined and 93 neurons responded to CA and/or Cap. Among these, 82 DRG neurons responded to both chemicals (88.2%), while 6 DRG neurons responded to Cap and 5 to CA, alone (Fig. 6B). This expression pattern was recapitulated with another TRPA1 agonist, AITC (Fig. 6C). Thus, the expression of TRPA1 and TRPV1 largely overlapped in WC frog DRG neurons.

Given that WC frog TRPA1 was activated by heat stimulation when heterologously expressed (Fig. 4, A-D), we sought to examine the endogenous temperature sensitivity of WC frog DRG neurons. Heat stimulation was applied to DRG neurons followed by CA and Cap applications (Fig. 6D). Among the heat-sensitive DRG neurons (61 neurons from 4 frogs), 52 DRG neurons responded to both CA and Cap, and only 8 neurons (four of each) responded to only a single chemical (Fig. 6D). Therefore, among the heat-sensitive DRG neurons, 85.2% (52/61)
possessed both TRPA1 and TRPV1 and this value is highly similar to the value obtained from all DRG neurons (Fig. 6B). Only one heat-sensitive DRG neuron responded to neither of the two chemicals (Fig. 6D). Furthermore, DRG neurons (15 neurons from 2 frogs) which responded to heat, Cap and CA did not demonstrate cold sensitivity (Fig. 6E) and only two neurons responded to both CA and cold stimuli. In contrast, a cold-sensitive but CA-insensitive DRG neuron responded to menthol, an activator of cold-sensitive TRPM8 in the WC frog (Fig. 6F) (35). Thus, most of the heat-sensitive, but not cold-sensitive, DRG neurons expressed TRPA1 and TRPV1 in WC frogs.

Chemical sensitivities of native sensory neurons and nocifensive behavior in WC frogs—We next examined the responses of WC frog DRG neurons to several chemicals that activated WC frog TRPA1 in heterologous expression systems (Figs. 1 and 2). CA increased [Ca$^{2+}$], in DRG neurons in a dose-dependent manner with an EC$_{50}$ of 0.031 ± 0.003 mM (Fig. 7, A and B), similar to results from HEK293 cells expressing WC frog TRPA1 (Fig. 2B) but much smaller than that of oocytes expressing WC frog TRPA1 (Fig. 1B). A similar apparent difference in sensitivity to chemicals among the expression systems was found for WC frog TRPV1 and channels other than TRP (15,36). One plausible explanation for these findings may be that chemicals become trapped within the viscous yolk of the X. laevis oocytes resulting in a reduction of chemical sensitivity (36). Although we decided to utilize X. laevis oocytes for TRPA1 current measurements in this study because frogs and lizards are ectothermic vertebrates, HEK293 cells likely exhibit sensitivity of WC frog TRPA1 to agonists more similar to those found in native conditions. [Ca$^{2+}$] also increased upon stimulation with carvacrol, AITC or acrolein (Fig. 7, C-E) within the same concentration range that activated HEK293 cells expressing WC frog TRPA1 (Fig. 2, C-E). These observations indicate that TRPA1 also serves as a chemical sensor in WC frog native DRG neurons.

We further examined WC frog behavioral responses to TRPA1 activators. To do so, a WC frog was placed into a small amount of water in a grass bottle, chemicals were then dissolved in water for cutaneous application, and jumping behavior (interpreted as nocifensive behavior) was assessed via counting. Application of CA elicited jumping behavior in a dose-dependent manner with an EC$_{50}$ of 0.040 ± 0.005 mM similar to those of HEK293 cells expressing TRPA1 and DRG neurons (Fig. 8, A and B), while vehicle control (dimethyl sulfoxide, DMSO) alone did not elicit such responses (Fig. 8A). Jumping behavior was also elicited by stimulation with carvacrol, acrolein, and AITC (Fig. 8C), all of which activated WC frog TRPA1 (Figs. 1 and 2). These results indicate that TRPA1 is involved in perception of noxious chemicals and causes nocifensive behavior in WC frogs.

DISCUSSION

Channel properties and physiological roles of WC frog and green anole TRPA1s—In the present study, we show that TRPA1 serves as a heat and chemical receptor in WC frogs. Characterization of the channel properties of WC frog TRPA1 in heterologous expression systems revealed that it was activated by the same noxious chemicals that activate mammalian TRPA1 such as CA, AITC, acrolein, carvacrol and 2-APB (Figs. 1 and 2). These chemicals also activated a subset of DRG neurons (Fig. 7) and elicited nocifensive behaviors in WC frogs (Fig. 8). These observations clearly show that WC frog TRPA1 serves as a noxious chemical receptor similar to mammalian TRPA1. Regarding temperature sensitivity, we found that WC frog TRPA1 was activated by heat, but not cold stimuli in heterologous expression systems (Fig. 4) unlike mammalian TRPA1 (5,9). The temperature threshold for activation of WC frog TRPA1 was about 40°C when expressed in X.
laevis oocytes (Fig. 4C), suggesting that TRPA1 serves not only as a chemical but also a high temperature receptor in WC frogs. We recently reported that WC frog TRPV1 was also activated by heat stimulation and that the temperature threshold for activation was about 38°C when expressed in the X. laevis oocytes (15). In WC frog DRG neurons, functional TRPA1 expression largely overlapped TRPV1 expression and most of the heat-sensitive neurons responded to both TRPA1 and TRPV1 agonists (Fig. 6, B-E). Therefore, our previous observation that WC frogs exhibited nocifensive behaviors to heat stimuli above 38 °C (15) could be explained by activation of both TRPA1 and TRPV1. Hence, TRPA1 and TRPV1 could act cooperatively as heat receptors in WC frogs.

Green anole TRPA1 was also activated by heat stimulation with an average temperature threshold for activation of about 34°C (Fig. 4, E-H), which is higher than the preferred body temperature or selected ambient temperatures for green anoles (37,38). Taking into account that TRPA1 serves as a nociceptive receptor, differences in the temperature thresholds for activation between TRPA1s of WC frogs and green anoles may reflect temperatures that are perceived as noxious by these species. Indeed, WC frogs inhabit the tropics, exhibit high tolerance to heat and can survive even when they are exposed to water at 42°C (15).

Diversity of channel properties among TRPA1s from distantly related vertebrate species—We showed that TRPA1s were activated by heat stimulation, however, their temperature thresholds for activation were different among species. Recently, ankyrin repeat domains, residing approximately in the N-terminal half of TRPA1, were shown to be capable of modulating the temperature sensitivity of TRPA1 by using chimeric channels between human and snake TRPA1s (39). The difference in temperature thresholds for activation among TRPA1s could provide a clue towards identifying structural determinants that modulate TRPA1 temperature sensitivity.

We also found that sensitivities to CA varied among three divergent vertebrate species such that the order of sensitivities was human > WC frog > green anole. In addition, the EC50 of human TRPA1 was approximately 40 times lower than that of green anole TRPA1. CA, as well as other electrophiles, such as AITC and acrolein etc., is known to activate TRPA1 through covalent modification of several cytosolic cystein residues (33,34). All of these cystein residues are conserved in WC frog and green anole TRPA1s (Supplemental Fig. 1), therefore, there may be additional mechanisms that modify the sensitivity of TRPA1 to CA. Recently indeed, rattlesnake TRPA1 has been reported to exhibit lower AITC sensitivity compared to human TRPA1, and several ankyrin repeat domains have been implicated in this difference (39).

Two mammalian TRPA1 antagonists, HC-030031 and AP18, did not inhibit WC frog TRPA1 whereas RR, a broad TRP channel blocker, effectively inhibited WC frog TRPA1 (Fig. 5A-G). Furthermore, AP18 also failed to inhibit green anole TRPA1 (Fig. 5I). In the case of AP18, mutation of two amino acid residues (S876V and T887L in mouse TRPA1) located in the 5th putative transmembrane domain has been reported to reduce this inhibitory effect (40). Amino acid alignment of vertebrate TRPA1s shows that the two amino acid residues in the corresponding positions are different in TRPA1s of WC frog (I898 and V899) and green anole (I871 and A872) from those of mouse TRPA1 (Supplemental Fig. 1). Substitutions of these amino acids may have caused the difference in the inhibitory effect of AP18 between mammalian and WC frog/green anole TRPA1s. On the other hand, the structural determinants for TRPA1 inhibition by HC-030031 have yet to be identified. In this study, we found that HC-030031 was able to inhibit green anole TRPA1, but not WC frog TRPA1, while AP18 had no inhibitory effect on TRPA1 in either species. As HC-030031 and AP18 are structurally different,
this observation may reflect the difference in structural bases of TRPA1 for these two inhibitors. Amino acid comparison among mammalian, green anole, and WC frog TRPA1s and the experiments with chimeric mutants could contribute to the identification of the residues critical for HC-030031 inhibition.

Functional evolution of TRPA1 in vertebrates—Nociceptive receptors perceive noxious temperatures and chemicals capable of causing tissue damage and are thus essential for animal survival in natural environments. In this respect, elucidation of the evolutionary process of nociceptive receptors would provide fundamental information towards understanding adaptive evolution. The functional properties of TRPA1s have been reported in several vertebrate species. Zebrafish have two paralogous TRPA1 genes (TRPA1a and TRPA1b) and both TRPA1s are activated by the same noxious chemicals which activate mammalian TRPA1s in heterologous expression systems, but only TRPA1b is involved in detection of noxious chemicals in zebrafish larvae (25). Sensitivity to noxious chemicals is further shared by Drosophila (23). In the present study, we also showed that noxious chemicals which activate mammalian TRPA1 also activated WC frog and green anole TRPA1s (Figs. 1-3). Therefore, it can be concluded that chemical sensitivity of TRPA1 was acquired during an early stage of animal evolution and has been conserved throughout the vertebrate lineages.

Mammalian TRPA1 was reported to serve as a cold sensor, although some debate about this temperature sensitivity exists (Fig. 9) (1,3,5,8-10). In several snake species, TRPA1 has been reported to be activated by heat stimulation (24), while zebrafish TRPA1s are reported to be insensitive to temperature stimulation (24,25). Activation of Drosophila and mosquito TRPA1s by heat stimulation suggests that TRPA1 acquired temperature sensitivity in an early stage of animal evolution (41,42). However, whether TRPA1 possessed heat sensitivity in ancestral vertebrates has remained enigmatic. In the present study, we showed that green anole TRPA1 was activated by heat stimulation (Fig. 4, E-H). Thus, in general, TRPA1 likely serves as a heat receptor in reptiles. In addition, we also demonstrated heat-sensitivity of TRPA1 in WC frogs (Fig. 4, A-D), which diverged earliest among the terrestrial vertebrate lineages. Taking into consideration that insect TRPA1 is also activated by heat stimulation, TRPA1 is likely to have acquired heat-sensitivity during an early stage in the course of animal evolution and maintained its heat-sensitivity in ancestral vertebrates (Fig. 9).

Evolutionary relationships of nociceptive receptors TRPA1 and TRPV1 in vertebrates—Channel properties of TRPV1s have been examined in several vertebrate species that are distantly related to one another, such as mammals, chickens, and frogs. In all vertebrate species examined, TRPV1s are activated by heat stimulation (11,12,14,15), indicating that heat-sensitivity of TRPV1 has been conserved (Fig. 9). Phylogenetic analysis showed that the TRPV1 and TRPV2 genes emerged in ancestral vertebrates through gene duplication from the proto-TRPV1/2 gene (30,43). In the case of teleost fishes, apparent TRPV1 orthologs do not exist in genomic sequences, however, genes closely related to terrestrial vertebrate TRPV1 and TRPV2 are present (named as TRPV1/2) (43). None of the orthologs for these three genes were found in genomic sequences of Drosophila, sea squirt, or nematode (44). Recently, zebrafish TRPV1/2 was reported to possess heat sensitivity (45), thus proto-TRPV1/2 in ancestral vertebrates is likely to have possessed heat sensitivity. In the present study, we showed that TRPV1 and TRPA1 are highly co-expressed in the same DRG neurons of WC frogs (Fig. 6). In mammals, expression of these two channels partially overlaps in DRG neurons (9,16,18,19). These observations suggest that TRPV1 became co-expressed with the
pre-existing TRPA1 and served as a nociceptive receptor in the common ancestor of tetrapods at the latest (Fig. 9).

The emergence of TRPV1 as a novel heat receptor in ancestral vertebrates may have significantly influenced the functional evolution of TRPA1 resulting in different evolutionary consequences in the respective vertebrate lineages. In the case of zebrafish, TRPA1 likely lost heat sensitivity due to the relaxed functional constraint on temperature sensitivity caused by the acquisition of TRPV1/2. In contrast, in the case of WC frogs, both TRPA1 and TRPV1 may have maintained their heat sensitivity and cooperatively act as heat receptors (Figs. 4 and 6). This may be due to functional compensation whereby: WC frog heat-evoked TRPA1 currents were desensitized by repetitive stimulation (Fig. 4A and 4B) while WC frog TRPV1 was sensitized upon repeated heat stimulation (15). Thus, both TRPA1 and TRPV1 may be complementarily required for heat sensation in the WC frog. In the case of snake species, TRPA1 was co-opted by a novel heat sensory organ, the pit organ, where it is used for infrared detection (24). TRPA1 has been reported to be expressed specifically in the trigeminal ganglia which innervate pit organs, and is not expressed in the DRG neurons that convey somatosensory information in the trunk. Moreover, temperature thresholds for TRPA1 activation are reduced in pit-harboring snakes (about 28°C for the rattlesnake TRPA1), while TRPA1 is activated by relatively high temperatures (about 37°C) and is expressed in both trigeminal ganglia and DRG neurons in snakes not possessing a pit organ, such as rat snakes in which TRPA1 is likely involved in somatic thermosensation. Therefore, co-option of TRPA1 in pit organs could be attributed to the emergence of TRPV1 as a novel heat receptor in ancestral vertebrates.

In summary, we demonstrated that WC frog and green anole TRPA1s are noxious heat and chemical receptors. Given that this characteristic is shared between vertebrates and insects, the role for TRPA1 in chemical nociception may have been conserved throughout the course of animal evolution. Additionally, it is likely that TRPA1 already possessed heat sensitivity in ancestral vertebrates. Emergence of TRPV1 as a novel heat receptor in ancestral vertebrates could have influenced the functional evolution of TRPA1 during vertebrate evolution. TRPV1 and TRPA1 thus provide a model for understanding how different genes with similar biological roles may influence each other’s function during the course of evolution. Finally, diversity of channel properties among vertebrate TRPA1s can be utilized for examining the structure-function relationship of TRPA1, which in turn provides novel insights into the molecular mechanisms controlling TRPA1 activity.

References


TRPV1-TRPA1 interaction to the single channel properties of the TRPA1 channel. *J Biol Chem* **285**, 15167-15177


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FOOTNOTES
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The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank with accession numbers AB693189 and AB693190.

This article contains supplemental figures 1 and 2.

The abbreviations used are: TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1; TRP, transient receptor potential; WC frog, western clawed frog; AITC, allyl isothiocyanate; CA, cinnamaldehyde; DRG, dorsal root ganglion; [Ca\(^{2+}\)], intracellular Ca\(^{2+}\) concentrations; Cap, capsaicin; 2-APB, 2-aminoethoxydiphenyl borate; RR, ruthenium red; DMSO, dimethyl sulfoxide;

FIGURE LEGENDS

Figure 1. Noxious chemical sensitivities of WC frog TRPA1 expressed in X. laevis oocytes.  

A, A representative current trace for CA-evoked responses in X. laevis oocytes injected with WC frog TRPA1 cRNA.  

B, A dose-response curve for activation of WC frog TRPA1 by CA. Currents were normalized to the values at 2 mM. Each data point represents the mean ± SEM (n = 4-5).  

C, A representative current trace for carvacrol-evoked responses in X. laevis oocytes injected with WC frog TRPA1 cRNA.  

D, A dose-response curve for activation of WC frog TRPA1 by carvacrol. Currents were normalized to the values at 2 mM. Each data point represents the mean ± SEM (n = 4-7).  

E-G, Representative current traces for AITC (E), acrolein (F), and 2-APB (G) stimulation in oocytes injected with WC frog TRPA1 cRNA (AITC, n = 4; acrolein, n = 3; 2-APB, n = 7).

Figure 2. Noxious chemical sensitivities of WC frog TRPA1 expressed in HEK293 cells.  

A and B, Representative traces of [Ca\(^{2+}\)], changes in response to CA in HEK293 cells transfected with the pVenus-NLS vector harboring WC frog TRPA1 (A) and its dose-response curve (B). Each data point
represents the mean ± SEM (n = 10-17). C-E, Representative traces of [Ca^{2+}]i changes in response to carvacrol (C), AITC (D), and acrolein (E) in HEK293 cells transfected with the pVenus-NLS vector harboring WC frog TRPA1 (carvacrol, n = 23; AITC, n = 13; acrolein, n = 11).

Figure 3. Noxious chemical sensitivities of green anole TRPA1 expressed in X. laevis oocytes. A-G, All data were obtained by using X. laevis oocytes injected with green anole TRPA1 cRNA. A, A representative current trace for CA-evoked responses. B, A dose-response curve for activation of green anole TRPA1 by CA. Currents were normalized to the values at 4 mM. Each data point represents the mean ± SEM (n = 5-10). C, A representative current trace for carvacrol-evoked responses. D, A dose-response curve for activation of green anole TRPA1 by carvacrol. Currents were normalized to the values at 2 mM. Each data point represents the mean ± SEM (n = 4-8). E-G, Representative current traces for AITC (E), acrolein (F) 2-APB (G) (AITC, n = 3; acrolein, n = 3; 2-APB, n = 4).

Figure 4. Heat sensitivity of WC frog and green anole TRPA1s. A and B, Representative current (upper) and temperature (lower) traces for heat and AITC stimulation in X. laevis oocytes injected with WC frog TRPA1 cRNA. Current-voltage relationships of heat- and AITC-evoked responses in X. laevis oocytes injected with WC frog TRPA1 cRNA (A, inset). B, A representative current trace of X. laevis oocytes injected with WC frog TRPA1 cRNA stimulated sequentially with cold, heat, and CA (n = 4). C, An Arrhenius plot of the current elicited by the first heat stimulation in panel A. The average temperature threshold for heat stimulation was 39.7 ± 0.7°C (n = 12). D, Average changes in [Ca^{2+}]i (solid line) in response to heat, CA and ionomycin (Ion) and a temperature trace (dashed line) in HEK293 cells transfected with the pcDNA3.1(+) vector containing WC frog TRPA1. Each data point represents the mean ± SEM (n = 64). E and F, Representative current (upper) and temperature (lower) traces for heat (n = 11) or cold (n = 4) stimulation in X. laevis oocytes injected with green anole TRPA1 cRNA. G, An Arrhenius plot of the current elicited by the first heat stimulation in panel E. The average temperature threshold for heat stimulation was 33.9 ± 0.8°C (n = 11). H, Average changes in [Ca^{2+}]i (solid line) in response to heat, CA and Ion and a temperature trace (dashed line) in HEK293 cells transfected with the pcDNA3.1(+) vector containing green anole TRPA1.

Figure 5. Effects of mammalian TRPA1 inhibitors on WC frog and green anole TRPA1s. A, Inhibition of WC frog TRPA1 activity by RR. The CA-induced current in oocytes injected with WC frog cRNA was inhibited by RR even in the presence of CA. The CA-induced current was recovered after washing out RR for about 2 min (n = 3). B, RR inhibited the increase in [Ca^{2+}]i elicited by CA application in HEK293 cells transfected with pVenus-NLS vector containing WC frog TRPA1. First, carvacrol was applied to show the functional expression of WC frog TRPA1 and then CA was applied (upper trace). To examine the effect of RR, the drug was applied prior to and during the application of CA (lower trace). C and E, HC-030031 (C) or AP18 (E) were applied prior to and during CA stimulation in X. laevis oocytes injected with WC frog TRPA1 cRNA (HC-030031, n = 3; AP18, n = 3). D and F, HEK293 cells transfected with pVenus-NLS vector containing WC frog TRPA1 were stimulated with CA (D, upper). HC-030031 (D, lower) or AP18 (F) were applied prior to and during CA application. G, The average increase in [Ca^{2+}]i in HEK293 cells transfected with the pVenus-NLS vector containing WC frog TRPA1. HC and AP represent HC-030031 and AP18, respectively. Each bar represents the mean ± SEM (CA, n = 17; CA with RR, n =15; CA with HC, n = 13; CA with AP, n =18). RR significantly suppressed the average increase in [Ca^{2+}]i elicited by CA.
stimulation (*P < 0.01). H and I, HC-030031 (H) or AP18 (I) were applied prior to and during CA stimulation in X. laevis oocytes injected with green anole TRPA1 cRNA (HC-030031, n = 3; AP18, n = 3).

**Figure 6. Functional expression of TRPA1 and TRPV1 in WC frog DRG neurons.** A and B, Pseudocolor images (A) and a representative trace (B) of \([\text{Ca}^{2+}]\), changes in WC frog DRG neurons stimulated with CA, Cap, and 80 mM of K\(^+\) (80K). Viability of DRG neurons was confirmed by activation with high-concentration K\(^+\) stimulation (80K). B, The number of DRG neurons that responded to CA and/or Cap are summarized (313 DRG neurons from 8 frogs). The number of neurons that did not respond to both chemicals is indicated at lower right (box). C, The numbers of DRG neurons that responded to AITC (0.3 mM) and/or Cap (0.3 mM) are summarized (70 DRG neurons from 3 frogs). D, A representative trace of \([\text{Ca}^{2+}]\), (upper) and temperature (lower) changes in WC frog DRG neurons stimulated with heat, CA, Cap, and 80K. The number of DRG neurons that responded to CA and/or Cap among the total heat-sensitive DRG neurons (n = 61, from 4 frogs) are summarized. Note that only one heat-sensitive DRG neuron did not respond to CA and Cap. E, Representative traces of \([\text{Ca}^{2+}]\), (upper) and temperature (lower) changes of WC frog DRG neurons stimulated with cold, heat, Cap, CA, and 80K (n = 15, from 2 frogs). F, Representative traces of \([\text{Ca}^{2+}]\), (upper) and temperature (lower) changes of WC frog DRG neurons stimulated with cold, CA, menthol, and 80K.

**Figure 7. Chemical sensitivity of DRG neurons in the WC frog.** A, A representative trace of \([\text{Ca}^{2+}]\), changes in DRG neurons from WC frogs stimulated with increasing concentrations of CA. B, A dose-response curve for \([\text{Ca}^{2+}]\), changes by CA in DRG neurons. Each data point represents the mean ± SEM (n = 10-17, from 3 frogs). C-E, Representative traces for \([\text{Ca}^{2+}]\), changes in DRG neurons from WC frogs stimulated with carvacrol (C), AITC (D) or acrolein (E), and sequentially stimulated with CA and 80K (carvacrol, n = 9; AITC, n = 12; acrolein, n = 10; 3 frogs were used for each chemical).

**Figure 8. TRPA1 agonists evoked nocifensive behaviors in the WC frog.** A, Number of jumps induced by CA with different concentrations. Each bar indicates jumping during the 1-minute interval before and after application of CA (n = 3-4). B, A concentration-response relationship for the jumps elicited by CA application. The average number of jumps per minute during the 10-minute interval after CA application was plotted for each concentration. Each data point represents the mean ± SEM (n = 3-4). C, Number of jumps during the 1-minute interval before and after application of carvacrol (upper), acrolein (middle), and AITC (lower). Three frogs were examined for each chemical.

**Figure 9. The evolutionary scenario for nociceptive receptors TRPA1 and TRPV1 within the vertebrate lineages.** Temperature sensitivities of TRPA1 and TRPV1 in examined vertebrate species are listed. Note that cold sensitivity of mammalian TRPA1 is under debate. The channels that have not been examined are indicated with dashes. Zebrafish have only one gene that is similar to terrestrial TRPV1 and TRPV2 (indicated as TRPV1/2). Each deduced evolutionary event occurring in the respective branch is indicated by an arrow. The gene duplication event producing TRPV1 and TRPV2 was not clearly resolved, thus alternative possibilities are indicated by arrows with dashed lines.
Figure 1
Figure 2
Figure 3
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Figure 5
Figure 6
Figure 7
Figure 8
Figure 9

Gene duplication producing TRPV1 & TRPV2

Emergence of proto-TRPV1/2

TRPA1 heat & chemical sensitivities

- Heat-sensitivities of TRPV1
- Coexpression of TRPV1 and TRPA1

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Analysis of Transient Receptor Potential Ankyrin 1 (TRPA1) in Frogs and Lizards Illuminates Both Nociceptive Heat and Chemical Sensitivities and Coexpression with TRP vanilloid 1 (TRPV1) in Ancestral Vertebrates
Shigeru Saito, Kazumasa Nakatsuka, Kenji Takahashi, Naomi Fukuta, Toshiaki Imagawa, Toshio Ohta and Makoto Tominaga

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