Molecular Basis Determining Inhibition/Activation of Nociceptive Receptor TRPA1 Protein

A SINGLE AMINO ACID DICTATES SPECIES-SPECIFIC ACTIONS OF THE MOST POTENT MAMMALIAN TRPA1 ANTAGONIST*

Received for publication, June 6, 2014, and in revised form, September 20, 2014. Published, JBC Papers in Press, September 30, 2014, DOI 10.1074/jbc.M114.586891

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The transient receptor potential ankyrin 1 (TRPA1) is a Ca²⁺-permeable, nonselective cation channel mainly expressed in a subset of nociceptive neurons. TRPA1 functions as a cellular sensor detecting mechanical, chemical, and thermal stimuli. Because TRPA1 is considered to be a key player in nociception and inflammatory pain, TRPA1 antagonists have been developed as analgesics. In recent years, utilizing species differences, we identified the molecular basis of the antagonistic action of A967079, one of the most potent mammalian TRPA1 antagonists. Here, we show a unique effect of A967079 on TRPA1 from diverse vertebrate species, i.e., as an agonist but not as an antagonist for chicken and frog TRPA1s. By characterizing chimeric channels of human and chicken TRPA1s, as well as point mutants, we found that a single specific amino acid residue located within the putative fifth transmembrane domain was involved in not only the stimulatory but also the inhibitory actions of A967079. AP18, structurally related to A967079, exerted similar pharmacological properties to A967079. Our findings and previous reports on species differences in the sensitivity to TRPA1 antagonists supply useful information in the search for novel analgesic medicines targeting TRPA1.

TRPA1² is a nonselective cation channel mainly expressed in sensory neurons and activated by electrophilic chemicals such as allyl isothiocyanate (AITC), cinnamaldehyde (CA), allicin, and acrolein (1–5). It is also activated by noxious cold, hyperosmosis, some metal ions, and oxidative stress (1, 6–13), although there is a debate about the noxious cold sensitivity (14, 15). TRPA1 activators can induce spontaneous, neuropathic, and inflammatory pain in vivo (16, 17), and TRPA1 is the first and only transient receptor potential channel mutation that is shown in humans to cause spontaneous pain (18). Therefore, TRPA1 provides a promising target for analgesics, and several antagonists have been developed. AP18 inhibits mammalian TRPA1 in vivo and in vitro (19, 20). A967079, the structure related to AP18, is known as the most potent mammalian TRPA1 antagonist and inhibits neuropathic and inflammatory pain in vivo (21).

Because nociception is a fundamental sensation for all animals, pharmacological properties of nociceptive receptors have been compared in a wide variety of species, and species diversity has been reported. For example, capsaicin, a transient receptor potential vanilloid 1 (TRPV1) agonist, activates human and rodent TRPV1 (22, 23). However, rabbit, western clawed frog, and chicken TRPV1s exhibit lower sensitivity to capsaicin (23–25). Regarding TRPA1, menthol activates mouse TRPA1 at low concentrations but blocks it at high concentrations, whereas it only activates human TRPA1 (26). Caffeine stimulates mouse TRPA1 but suppresses human TRPA1 (27). These species differences have been utilized to identify the specific amino acids involved in the ligand sensitivities (28).

For western clawed frog TRPA1, we previously reported that A967079 lacks an antagonistic action. By utilizing species differences, we identified two amino acid residues located within the putative fifth transmembrane (TM5) domain as critical determinants for the antagonistic action of A967079 (29). Quite recently, we also analyzed functional properties of chicken TRPA1, and we reported that it is a heat sensor, but not a cold one, unlike rodent TRPA1 (30).

In this study, we show that A967079 failed to antagonize chicken TRPA1 activity. In contrast, it exhibited an agonistic

* This work was supported, in whole or part, by a KAKENHI, Grants-in-aid for Scientific Research (B) 22380160 and 262922150 (to T. O.), and Grant-in-aid for Scientific Research (C) 24580426 (to K. T.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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2 The abbreviations used are: TRPA1, transient receptor potential ankyrin 1; AITC, allyl isothiocyanate; CA, cinnamaldehyde; DRG, dorsal root ganglia; [Ca²⁺]i, intracellular Ca²⁺ concentration; TRPV1, transient receptor potential vanilloid 1; TM, transmembrane.

Significance:
The present data provide novel insight in the search for analgesics targeting TRPA1.

Background: Nociceptive TRPA1 is an important drug discovery target for identification of analgesics.

Results: A967079, one of the most potent mammalian TRPA1 antagonists, showed marked species differences.

Conclusion: A single amino acid was responsible for inhibitory/stimulatory actions of A967079.

NOVEMBER 14, 2014 • VOLUME 289 • NUMBER 46

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 46, pp. 31927–31939, November 14, 2014
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Species-specific Actions of the Most Potent TRPA1 Antagonist

TABLE 1
Sequences of primers used for construction of human TRPA1 mutants

| Mutation | Forward primer | Reverse primer |
|----------|----------------|----------------|
| S725I | TTAGGAAATTACCTGGCTGCTGCTCAT | AACAGATTTATCTGACTATCATATG |
| L782L | TCGAGCTTACCTGGCTGCTGCTCAT | ACCCAAAATGCTGATAAAGACACTAA |
| C856Y | GAATTTGAAATTAGTTGTTGTTGGAAG | AATTTGACATTTTCTATGTTAGG |
| L811I | TTTCTTATTCCGCTGCTGCTGCAGC | ATGCAAATTTTGTAGTAAGAACCAC |
| L811M | TTTCTTATTCCGCTGCTGCTGCAGC | ATGCAAATTTTGTAGTAAGAACCAC |
| L811V | TTTCTTATTCCGCTGCTGCTGCAGC | ATGCAAATTTTGTAGTAAGAACCAC |
| L882L | TTTCTTATTCCGCTGCTGCTGCAGC | ATGCAAATTTTGTAGTAAGAACCAC |
| D896Q | TTCACGACACACACTGACCTCCTCAATTG | GAGGTTTCTCCCTTTAATAAGGAGCT |
| R928S | TATCTTTCAAGATATTGAGCAGATACCTCA | TCTATTTGGCTTTACGAGTCAGAAT |
| N929S | CTGGAAAGCTGTTGCTGACATACCTCATT | GAAACTTCTCTTCTTACGAGTCTCA |
| V935F | CACCTCTTCTGCTCTTCTGCAACTCTT | GAGCAAAATGTTGGGGCTCACTATTT |

TABLE 2
List of primers for construction of expression vector of chimeric TRPA1

| Primer name | Direction | Sequence |
|-------------|-----------|----------|
| Vector-F | Forward | GTAATCATGTTGCTGCTGCTG |
| TRPA1(H-C)-1F | Forward | CAATATCTTTGCTGCTGCTG |
| TRPA1(C-H)-1R | Reverse | CAATATCTTTGCTGCTGCTG |
| TRPA1(C-H)-1F | Forward | TATATCTTTGCTGCTGCTG |
| TRPA1(C-H)-1R | Reverse | TATATCTTTGCTGCTGCTG |
| TRPA1(H-C)-2F | Forward | ATGTGGAGCAGATACCTGACCA |
| TRPA1(C-H)-2R | Reverse | ATGTGGAGCAGATACCTGACCA |
| Vector-R | Reverse | GCATATTTCCGCTGCTGCTG |

Effect on chicken TRPA1. Moreover, A967079 was capable of inducing of nociception in the chicken in vivo. Through analyses of chimeric and mutant TRPA1 channels, we have identified a single amino acid residue located within the putative TM5 domain that is necessary for both inhibition and activation by A967079. Our data provide an important insight into the molecular basis of TRPA1 inhibition and for the development of new analgesic drugs related to TRPA1 function.

EXPERIMENTAL PROCEDURES

All protocols for experiments on animals were approved by the Committee on Animal Experimentation of Tottori University (Japan).

Chicken Dorsal Root Ganglion Neurons—Fertilized chicken eggs were incubated at 37 °C until they reached the desired stages (embryonic day 20 to postnatal day 1). Chicken dorsal root ganglion (DRG) neurons were obtained from chickens according to the procedure reported previously (30). In brief, chickens were sacrificed by decapitation. The lumbar DRGs were removed, and isolated ganglia were enzymatically digested for 30 min at 37 °C with collagenase (1 mg/ml, type II, Worthington) and DNase I (1 mg/ml) for 15 min at 37 °C. After enzyme digestion, the ganglia were washed with culture medium (Dulbecco’s modified Eagle’s medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (Sigma), penicillin G (100 units/ml), streptomycin (100 μg/ml), and DNase I (1 mg/ml, Roche Applied Science). Subsequently, the ganglia were further treated with trypsin (5 mg/ml, Sigma) and DNase I (1 mg/ml) for 15 min at 37 °C. After enzyme digestion, the ganglia were washed with culture medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (Sigma), penicillin G (100 units/ml), and streptomycin (100 μg/ml). DRG cells were obtained by gentle trituration with a fine-polished Pasteur pipette.

Expression Vectors—We used expression vectors of chicken CHC-TRPA1, human TRPA1, green anole TRPA1, and mouse TRPA1 due to their heat sensitivities and at 37 °C for human and mouse TRPA1s, human/chicken chimeric TRPA1, and human TRPA1 mutant channels. The cells were used for Ca²⁺ imaging and current recording after incubation lasting 24h.

The full length of chimeric TRPA1 cDNA was amplified by a three-step polymerase chain reaction (PCR). In the first step, the DNA fragment of human type TRPA1, Hn (hTRPA1(1–707)), Hnm (hTRPA1(1–968)), Hm (hTRPA1(969–968) (with N699S)), Hmc (hTRPA1(699–1112) (with N699S)), or Hc (hTRPA1(960–1112)) was amplified by PCR with the expression vector of human TRPA1 cDNA as a template and the combination of primer vector-F/TRPA1(H-C)-1R, vector-F/TRPA1(H-C)-2R, TRPA1(C-H)-1F/TRPA1(C-H)-2R, TRPA1(C-H)-1F/vector-R, or TRPA1(C-H)-2F/vector-R listed in Table 2. The lower-case letters m, n, and c in DNA fragment names stand for the N terminus, transmembrane, and C terminus, respectively. The DNA fragment of chicken TRPA1 cDNA, Cn (cTRPA1(1–713)), Cnm (cTRPA1(1–969)), Cm (cTRPA1(705–969)), Cnc (cTRPA1(705–1112)), or Cc (cTRPA1(961–1112)) was also amplified by PCR with the expression vector of chicken TRPA1 as a template and the combination of primer vector-F/TRPA1(C-H)-1R, vector-F/TRPA1(C-H)-2R, TRPA1(C-H)-1F/TRPA1(C-H)-2R, TRPA1(C-H)-1F/vector-R, or TRPA1(C-H)-2F/vector-R, respectively. At the second step, PCR was carried out with a mixture of DNA fragments Hn/Cmc, Hnm/Cc, Hnm/Cmc, Hm/Cmc, Hmc/Cc, and Cn/Hm/Cc for chimeric HCC, HHC, HCH, CHH, CCH, and CHC-TRPA1, respectively. At the third step, the full-length chimeric TRPA1 cDNA was amplified by PCR with the second PCR product as a template and the combination of primer vector-F and vector-R. The third PCR product was inserted into an expression vector (pVenus-NLS) as described previously (25). When these chi-
meric TRPA1s were expressed in HEK293 cells, chimeric HCC-TRPA1 and HHC-TRPA1 were insensitive to AITC (0.3 mM).

**Ca**\(^{2+}\)/**H**\(^{11001}\) Imaging—The procedure for **Ca**\(^{2+}\) imaging was described previously (32). HEK293 cells heterologously expressing TRPA1 and DRG neurons were loaded with the **Ca**\(^{2+}\)/**H**\(^{11001}\)-sensitive fluorescent dye fura-2. To load fura-2, the loading temperature was set at 33 °C for chicken, western clawed frog, and green anole TRPA1s expressed in HEK293 cells and chicken DRG neurons. For cells expressing mouse and human TRPA1s and human mutant channels, the loading temperature was set at 37 °C. The cells were incubated with fura-2 acetoxymethyl ester (10 \(\mu\)M, 40 min) in HEPES-buffered solution (in mM: 134 NaCl, 6 KCl, 1.2 MgCl\(_2\), 2.5 CaCl\(_2\), and 10 HEPES, pH 7.4). Fura-2 loaded cells were transferred into a recording chamber, and chemical stimuli were applied using a perfusing bath solution. To measure the intracellular **Ca**\(^{2+}\) concentration ([**Ca**\(^{2+}\)]\(_i\)), the cells were exposed to light at 340 and 380 nm. The intensities of fluorescent signals at 500 nm emitted by each excitation light were monitored, and their ratios (**F**\(_{340}\)/**F**\(_{380}\)) were calculated using a fluorescent imaging system (Aqua Cosmos, Hamamatsu Photonics, Japan). Experiments were carried out at room temperature (22–25 °C).

**Electrophysiology**—TRPA1 channel activity was investigated using whole-cell and inside-out patch clamp recordings. For whole-cell recording, cells were superfused with HEPES-buffered solution as for **Ca**\(^{2+}\) imaging experiments (standard bath solution). The pipette solution contained (in mM) 140 KCl, 5 EGTA, and 10 HEPES, pH 7.4, adjusted with KOH. For inside-out recording, the bath solution was the pipette solution for whole-cell recording, and the pipette solution was the standard bath solution. The resistance of patch electrodes ranged from 4 to 5 megohms. The membrane currents were sampled at 10 kHz and filtered at 2 kHz using a patch-clamp amplifier (Axopatch 200B; Molecular Devices, Sunnyvale, CA) in conjunction with an A/D converter (Digidata 1322A; Molecular Devices). For whole-cell recording, the membrane potential was clamped at −60 mV and voltage ramp pulses from −100 mV to +80 mV for 100 ms were applied every 5 s. For inside-out recording, the membrane potential was clamped at −60 mV. Data were analyzed using clamp fit 10.3 software. Single channel unitary cur-

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**FIGURE 1. Effects of A967079 on HEK293 cells expressing chicken TRPA1 and chicken DRG neurons.** A and D, representative traces of [**Ca**\(^{2+}\)]\(_i\) responses to CA (0.3 mM, 4 min) in the absence (panel a) and presence of A967079 (A96; panel b, 1 \(\mu\)M; panel c, 30 \(\mu\)M) for HEK293 cells expressing chicken TRPA1 (A) and chicken DRG neurons (D). A967079 was applied 2 min (panel b) or 10 min (panel c) prior to the application of CA. B and C, quantification of the increases in [**Ca**\(^{2+}\)], induced by CA alone, A967079 alone, and CA with inhibitors in chicken TRPA1-expressing HEK293 cells (CA alone, n = 16; A967079 (1 \(\mu\)M) alone and CA + A967079, n = 11; A967079 (30 \(\mu\)M) alone and CA + A967079, n = 12; AP18 (1 \(\mu\)M) alone and CA + AP18, n = 7; AP18 (10 \(\mu\)M) alone and CA + AP18, n = 6). E and F, quantification of the increases in [**Ca**\(^{2+}\)], induced by CA alone, A967079 alone, and CA with inhibitors in chicken DRG neurons (CA alone, n = 10; A967079 (1 \(\mu\)M) alone and CA + A967079, n = 11; A967079 (30 \(\mu\)M) alone and CA + A967079, n = 14; AP18 (1 \(\mu\)M) alone and CA + AP18, n = 13; AP18 (10 \(\mu\)M) alone and CA + AP18, n = 11). Each bar represents the mean ± S.E.
rent was determined from the Gaussian distribution of the amplitude histograms.

For recording ionic current from *Xenopus laevis* oocytes expressing chicken TRPA1, the two-electrode voltage clamp method was used as mentioned previously (30). Complementary RNA (cRNA) of chicken TRPA1 was synthesized using an expression vector designed for *X. laevis* oocytes as a template, and 50 nl of chicken TRPA1 cRNA (50 ng/µl) was injected into defolliculated oocytes. Ionic currents were recorded 6 days post-injection. Oocytes were voltage-clamped at -110 mV, and currents were recorded using an OC-725C amplifier (Warner Instruments) with a 1-kHz low pass filter and digitized at 5 kHz by a Digidata 1440 (Axon Instruments). Chemical compounds were diluted in ND96 bath solution and applied to oocytes by perfusion.

**Behavioral Experiment**—Chickens (postnatal day 1) were placed in cages for 30 min before experiments. When TRPA1 agonists were administered intraplantarly, they showed licking, biting, and flicking behaviors that were similar to the pain-related behaviors in mice (13, 16). Before the injection of CA, a TRPA1 agonist, chickens were mostly quiescent. After intraplantar injection of CA (1 µmol), the chickens began pecking and flicking the injected foot. Therefore, we interpreted pecking and flicking as nociceptive behaviors and counted the number of these behaviors for the injected foot for 5 min before and 10 min after the injection of CA. A967079 (1 µmol) was applied intraplantarly and then behavioral responses were counted. Dimethyl sulfoxide (DMSO, vehicle; 10 µl) was injected intraplantarly as a vehicle control. HC-030031 (50 µM), a TRPA1 antagonist, was injected intraperitoneally 15 min before the intraplantar injection of CA or A967079. To record the numbers and timing of the nociceptive behaviors, we manually provided electrical signals to an AD converter (Power Lab, AD Instrument).

**Chemicals**—CA, A967079, and AITC were purchased from Wako (Tokyo, Japan), Santa Cruz Biotechnology, and Nakalai

**FIGURE 2. Activation of chicken TRPA1 by A967079 and AP18 in HEK293 cells and DRG neurons.** A and C, representative traces of changes in [Ca$^{2+}$], induced by increasing concentrations of A967079. B and D, concentration-response curve for A967079-induced [Ca$^{2+}$], increases in HEK293 cells expressing chicken TRPA1 (B) and chicken DRG neurons (D). Each data point represents the mean ± S.E. (B, n = 15; D, n = 25–41). E and H, [Ca$^{2+}$] responses induced by repetitive application of A967079 (20 µM, 2 min) with an interval of 10 min. HC-030031 (50 µM) was applied 2 min before, during, and 2 min after the second application of A967079 (E, HEK293 cell expressing chicken TRPA1; H, chicken DRG neurons). F and I, quantification of the increases in [Ca$^{2+}$], induced by A967079 (20 µM) in the presence and absence of HC-030031 (F, HEK293 cell expressing chicken TRPA1, n = 15; I, chicken DRG neurons, n = 33). G and J, quantification of [Ca$^{2+}$], induced by AP18 (20 µM) in the presence and absence of HC-030031 (50 µM; G, HEK293 cell expressing chicken TRPA1, n = 15; J, chicken DRG neurons, n = 10). Each bar represents the mean ± S.E. **, p < 0.01.
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(Tokyo, Japan), respectively. AP18 and HC-030031 were purchased from Sigma. All chemicals were dissolved in DMSO as stock solutions (0.01–1M).

Data Analysis—The data using HEK293 cells or DRG were obtained from at least three different transfections or three different chickens per experiment, respectively. The data are presented as mean ± S.E. (n = number of observations). Values of the 50% effective concentration (EC_{50}) were determined using Origin version 9.0 J (Origin-Lab). Comparison of the two groups was done with Student's t test. For multiple comparisons, one-way analysis of variance was performed following the Tukey-Kramer test. A p value of less than 0.05 was considered significant.

RESULTS

A967079 Lacked Antagonistic Effect on Chicken TRPA1—First, we examined the effect of A967079 on CA (0.3 mM)-induced [Ca^{2+}]_i increase in HEK293 cells expressing chicken TRPA1 or chicken DRG. A967079 at 1 μM, the concentration which completely blocks human TRPA1 activity (20), failed to inhibit the [Ca^{2+}]_i increase induced by CA (Fig. 1, A and D). Even at a high concentration (30 μM), A967079 was not capable of inhibiting the CA-induced [Ca^{2+}]_i increase (Fig. 1, A, panel c, and D, panel c). Surprisingly, A967079 (30 μM) itself evoked increases of [Ca^{2+}]_i in both cellular systems. The effects of A967079 on the [Ca^{2+}]_i responses to CA in HEK293 cells expressing chicken TRPA1 and chicken DRG are summarized in Fig. 1, B and E. AP18 (1 and 10 μM), which has a structure related to A967079 (20), also failed to inhibit the [Ca^{2+}]_i increase induced by CA in chicken TRPA1-expressing HEK293 cells and chicken DRG (Fig. 1, C and F).

Activation of Chicken TRPA1 by the Mammalian TRPA1 Antagonist A967079—As shown in Fig. 1, A967079 (30 μM) elicited a [Ca^{2+}]_i increase rather than inhibiting the [Ca^{2+}]_i increase induced by CA. Next, we examined the effects of A967079 on HEK293 cells expressing chicken TRPA1 and chicken DRG at various concentrations. A967079 increased [Ca^{2+}]_i in a dose-dependent manner with a half-effective concentration (EC_{50}) of 11.3 ± 2.7 μM in HEK293 cells expressing chicken TRPA1 (Fig. 2, A and B). The dose-dependence for chicken DRG neurons was also assessed, and a similar EC_{50} (14.6 ± 0.5 μM) value was obtained compared with the heterologous expression system using HEK293 cells (Fig. 2, C and D). Because the inclination of the dose-response relation in DRG neurons was steeper than that in HEK293 cells expressing chicken TRPA1, some different factors, such as voltage-gated calcium channels (33), may also be involved in the stimulatory action of A967079 in DRG neurons.

We have previously reported that chicken TRPA1 activity is inhibited by HC-030031 (30), another TRPA1 antagonist structurally different from A967079. Therefore, we assessed whether

![Figure 3](image-url)
the chicken TRPA1 activity induced by A967079 was suppressed by HC-030031. The A967079 (20 μM)-induced [Ca$^{2+}$]i increase was inhibited by HC-030031 (50 μM) in chicken TRPA1-expressing HEK293 cells (Fig. 2, A and F) and chicken DRG (Fig. 2, H and I), indicating that A967079 specifically activated chicken TRPA1. The [Ca$^{2+}$]i increases induced by AP18 (10 μM) were also suppressed by HC-030031 (50 μM) in both HEK293 cells expressing chicken TRPA1 and chicken DRG (Fig. 2, G and F).

To obtain direct evidence for TRPA1 channel activation induced by A967079, we recorded ionic currents with whole-cell and inside-out patch configurations from HEK293 cells expressing chicken TRPA1. Fig. 3A shows representative whole-cell currents evoked by A967069 (30 μM) and AITC (0.1 mM, a TRPA1 agonist) in chicken TRPA1-expressing HEK293 cells. Functional expression of chicken TRPA1 was confirmed by the response to AITC. The current elicited by A967079 exhibited an outward rectifying current-voltage relationship similar to that observed for AITC stimulation (Fig. 3B). In inside-out patch clamp recordings, A967079 caused an increase in the single-channel activity in the excised membrane (Fig. 3, C and D). In this study, no inorganic polyphosphate was added into the pipette solution. Different from the report by Kim and Cavanaugh (34), no marked rundown was observed. It may be due to the short recording time or the differences of species used (35). The application of AITC (0.1 mM) to the same patch membrane also caused increased single-channel activity, confirming the functional expression of chicken TRPA1. Fig. 3E shows open channel histograms for A967079 and AITC. The calculated unitary conductance for A967079 was 57.6 ± 3.8 picoisemens ($n = 12$), which was almost the same as that for AITC (56.0 ± 4.5 picoisemens, $n = 12$). When chicken TRPA1 was expressed in X. laevis oocytes, A967079 also elicited inward currents that were completely inhibited by HC-030031, demonstrating that activation of TRPA1 by A967079 was not a cell type-specific phenomenon (Fig. 3F).

We next examined the chick behavioral responses to CA and A967079 in vivo. Chicks were injected with CA or A967079 intraplantarly, and the numbers of pecking and flicking behaviors were measured. CA (1 μmol) elicited pecking and flicking of the injected foot (Fig. 4A), whereas the vehicle control (DMSO) alone did not elicit such behaviors. These behaviors began after injection and ceased within 10 min. Intraperitoneal pretreatment with HC-030031 (5 μmol) suppressed these nociceptive behaviors induced by CA (Fig. 4B). As shown in Fig. 4E, the total numbers of behavioral responses to CA were significantly decreased by HC-030031, indicative of induction of TRPA1-dependent nociceptive behavior by CA in chickens. Next, we examined the behavioral responses of chickens to A967079. The application of A967079 (1 μmol) elicited pecking and flicking (Fig. 4C), which was suppressed by intraperitoneal pretreatment with HC-030031 (5 μmol) (Fig. 4, D and F). These results suggested that A967079 elicited nociception through TRPA1 activation in chickens.

Transmembrane Domain Is Involved in A967079-induced Activation of Chicken TRPA1—To identify the molecular basis for the agonistic action of A967079, we made human-chicken TRPA1 chimeras by systematically introducing N-terminal
transmembrane (TM) and C-terminal domains of chicken TRPA1 into the human TRPA1 background (Fig. 5). Functional expression of the chimeras was confirmed by the AITC (0.3 mM)-induced response in the Ca\(^{2+}/\)H\(^{11001}\) imaging assay. Wild type human TRPA1 (HHH in Fig. 5 B) was not activated by A967079 (50 \(\mu\)M) stimulation, whereas in wild type chicken TRPA1 (CCC), A967079 induced a \([\text{Ca}^{2+}/\text{H}^{11001}\]) increase as already mentioned (Fig. 2). When the N-terminal and TM domains of chicken TRPA1 (CCH) or the TM domain of chicken TRPA1 (HCH) were introduced, A967089 stimulation successfully activated the chimeric channels, whereas when only the N-terminal domain of chicken TRPA1 (CHH) or both the N- and C-terminal domains of chicken TRPA1 (CHC) were introduced, it failed to activate the chimeric channels (Fig. 5 B). Therefore, chimeric channel analyses indicated that the critical domain for TRPA1 activation by A967079 resided within the TM domain.

**A Single Specific Amino Acid Residue Changed the Antagonist A967079 to an Agonist**

We recently reported that A967079 and AP18 failed to inhibit the activity of green anole and western clawed frog TRPA1s (29, 31). Therefore, we examined whether TRPA1s of both species could be activated by A967079. A967079 (30 \(\mu\)M) increased \([\text{Ca}^{2+}/\text{H}^{11001}\]) in HEK293 cells expressing western clawed frog and green anole TRPA1s. A967079 was not capable of activating mouse TRPA1 (Fig. 6 B).

As described above, the agonistic action of A967079 against TRPA1 varied among species. Next, we searched for candidate amino acid residues involved in the agonistic action of A967079 by comparing the amino acid sequences of TRPA1s from the five species examined in the present study. Based on analysis of the chimeric channels (Fig. 5 B), the potential action sites for A967079 seemed to be present in the TM domain in chicken TRPA1. Therefore, we investigated the amino acids that satisfied the following formula: (human - mouse) = (chicken - western clawed frog) ≠ green anole TRPA1 within the TM domain. The five amino acid positions indicated in the schematic structure of TRPA1 in Fig. 7 A corresponded to the above requirement.

We hypothesized that these amino acid(s) might be related to the agonistic action of A967079. To test this hypothesis, we constructed single mutants in which the targeted amino acid was mutated to the chicken type amino acid in human TRPA1.
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A, schematic structure of the TRPA1 channel showing the positions of amino acid mutations introduced in this study. Amino acid alignment of TRPA1s for five different vertebrate species (human, mouse, chicken, green anole, and western clawed frog) analyzed in this study. Only the positions where mutations were introduced are shown. Numbering above the alignment is based on human TRPA1. The amino acid positions involved with A967079 are shown in the box. B, quantification of the increases in [Ca\(^{2+}\)] induced by CA (panel a, 0.3 mM) or A967079 (panel b, 30 \(\mu M\)) in five different human TRPA1 mutants (n = 12–20). For human TRPA1 (C856Y), 1 mM CA was used. C, representative traces of [Ca\(^{2+}\)] induced by CA alone and CA with A967079 (CA alone, n = 20; CA + A967079 1 \(\mu M\), n = 6; CA + A967079 30 \(\mu M\), n = 10) in HEK293 cells expressing human TRPA1 (L881I). Each bar represents the mean ± S.E.

(S725I, I782L, C856Y, L881I, and V935F). All mutant channels were sensitive to CA (Fig. 7B, panel a). Among the five mutant channels, only human TRPA1 (L881I) showed an obvious stimulatory effect of A967079 (30 \(\mu M\)) (Fig. 7B, panel b). Remarkably, this mutant also lost the antagonistic action of A967079 (1 \(\mu M\), 30 \(\mu M\)) (Fig. 7C). The effects of A967079 on [Ca\(^{2+}\)] responses to CA in human TRPA1 (L881I) are summarized in Fig. 7C, panel d. In the other human TRPA1 mutants (S725I, I782L, C856Y, and V935F), [Ca\(^{2+}\)] responses to CA were inhibited by A967079 (1 \(\mu M\)), and these mutants showed no agonistic activity, like wild type human TRPA1 (Fig. 7B, panel b).

In human TRPA1 (L881I), A967079 elicited [Ca\(^{2+}\)] increases in a dose-dependent manner with an EC\(_{50}\) of 26.5 ± 1.1 \(\mu M\) (Fig. 8, A and B). In this mutant channel, similar effects were observed with AP18. AP18 (20 \(\mu M\)) increased [Ca\(^{2+}\)]\(_{p}\) and AP18 (1 \(\mu M\) and 20 \(\mu M\)) failed to inhibit the [Ca\(^{2+}\)] increase induced by CA (Fig. 8, E and F). The [Ca\(^{2+}\)] increase induced by AP18 in human TRPA1 (L881I) was also suppressed by HC-030031 (Fig. 8G). These results suggested that leucine 881 located within the putative TM5 domain was involved in the antagonistic action of A967079.

Furthermore, substitution of this amino acid residue changed the antagonistic action of A967079 to an agonistic one.

Next, we examined the effect of A967079 on reverse mutation, i.e. a chicken TRPA1 was replaced with human TRPA1 in the corresponding position (I1882L). However, the agonistic action of A967079 did not disappear in HEK293 cells expressing chicken TRPA1 (I882L) (Fig. 9), suggesting that amino acid residues other than isoleucine 882 on chicken TRPA1 were also involved in the agonistic action of A967079. Therefore, in the predicted loop between TM5 and TM6 on TRPA1, we looked for the additional candidate amino acid positions, which were satisfied with the following relationship (human = mouse ≠ chicken ≠ green anole ≠ western clawed frog), based on the assumption that the agonistic action of A967079 on the chicken and western clawed frog TRPA1 was maintained by different amino acids. Accordingly, three other human TRPA1 mutant channels (D896Q, R928S, and N929S) were constructed and functionally expressed in HEK293 cells. In these mutant channels, however, A967079 did not show agonistic action and its antagonistic action remained unchanged (Fig. 10).

We finally examined the mechanistic insight into how the change in a single amino acid from leucine to isoleucine was so critical. Because isoleucine is structurally similar to methionine and valine from a viewpoint of a side chain of the amino acid,
two mutated human channels (L881M and L881V) were constructed and analyzed. However, A967079 failed to activate both mutant channels (Fig. 11).

**DISCUSSION**

A967079 is one of the most potent antagonists of mammalian TRPA1 (21); however, we found that it activated heterologously or endogenously expressed chicken TRPA1 (Fig. 1). In addition, A967079 had no antagonistic effect on chicken TRPA1. Similar pharmacological properties were observed for AP18, a chemical structurally related to A967079 (20). Application of a high concentration of A967079 (30 μM) caused rapid increases in \([Ca^{2+}]_i\), after which \([Ca^{2+}]_i\) gradually decreased and almost returned to the basal level even in its presence. Under these conditions, subsequent application of CA was capable of eliciting \([Ca^{2+}]_i\) increases in both HEK293 cells expressing chicken TRPA1 and chicken DRG neurons. These data suggested that the site(s) of agonistic action for A967079 would be different from that of CA, an electrophilic compound (36). This phenomenon was confirmed by the chimeric and mutagenesis analyses described below.

For the first time, we found nociceptive behaviors mediated via TRPA1 in chickens (Fig. 4). As in rodents, intraplantar injection of CA into chickens evoked pain-related behaviors that were suppressed by pretreatment with the TRPA1 antagonist HC-030031. Because we recently found that A967079 had an agonistic effect on chicken TRPA1, we assessed whether A967079 caused nociceptive behaviors in chickens.
As expected, intraplantar injection of A967079 evoked such behaviors, which were significantly suppressed by preadministration of HC-030031, indicating that A967079 acted through TRPA1 activation. Many studies have been carried out to investigate the function of TRPA1 in vivo using mice and rats as experimental animals (13, 16, 37). We recently reported that TRPA1 agonists such as CA, AITC, and acrolein induced nociceptive behaviors in the western clawed frog (31) and emphasized the importance of this species as an alternative experimental animal. Here, we demonstrated that a TRPA1 agonist caused clear nociceptive behaviors in chickens, which share homeothermy with mammals. Thus, chickens can also be utilized as alternative experimental animals to study TRPA1.

We found striking species-specific differences in the effects of A967079 on TRPA1. A967079 activated chicken and western clawed frog TRPA1s, although it inhibited human and mouse TRPA1s. Green anole TRPA1 was insensitive to A967079. The unique responses of various TRPA1 orthologues to A967079 enabled us to determine the specific amino acid residue responsible for the agonistic action of A967079 by chimeric and mutagenetic approaches. Consequently, our data suggested that the amino acid residue (leucine 881) located within TM5 on human TRPA1 was involved not only in the agonistic but also the antagonistic action of A967079. It has been shown that two amino acid residues located in the predicted inner site of TM5 are critical for the antagonistic action of AP18 (26). Similarly, we recently reported that these two amino acid residues (serine 873 and tyrosine 874) on human TRPA1 were also important for the antagonistic effect of A967079 (29). Furthermore, by analyses using a homology model of TRPA1 based on Kv1.2, it has also been reported that phenylalanine 944, valine 948, and isoleucine 950 in the predicted loop between TM5 and TM6 are involved in the antagonistic action of A967079 (38). Xiao et al. (26) proposed that AP18 and menthol share the binding pocket that makes up the TM domains. Thus, this report and previous reports suggest that the antagonistic actions of A967079 and AP18 are controlled by multiple amino acid residues in the TM domains of TRPA1, although it is unclear how A967079 interacts with these identified amino acid residues.

In this study, reverse mutation (chicken TRPA1-I882L) did not cause loss of the agonistic action of A967079, suggesting that amino acid residues other than leucine 881 on human
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TRPA1 were also involved in the agonistic action of A967079. This might be the reason that the EC50 value for A967079 in human TRPA1 (L881I) was slightly higher than that in wild type chicken TRPA1. To identify for the additional candidate the amino acid positions responsible for the agonistic action of A967079, three other human TRPA1 mutant channels (D896Q, R928S, and N929S) were constructed and functionally expressed in HEK293 cells. Unfortunately, in these mutant channels, A967079 did not show agonistic action, and its antagonistic action remained unchanged. Therefore, at present, we cannot demonstrate the difference of the effects of A967079 in human and chicken TRPA1s in both directions. Similar observations of species-specific differences of TRPA1 activities induced by activators such as thioaminals, caffeine, cold, and protons were explained by mutations in only one direction (15, 39–41). Nevertheless, as far as we know this is the first report to show that a single amino acid residue is involved in the functional change from antagonistic to agonistic action in transient receptor potential channels.

Comparison of the pharmacological properties of TRPA1 among diverse animal species provides a great opportunity for understanding the channel properties. The molecular determinants of TRPA1 with regard to several chemicals have been identified by analyses of mutants. For example, CMP1 activates rat TRPA1 but suppresses human TRPA1. Two amino acid residues (alanine 946 and methionine 949) located in the putative TM6 on rat TRPA1 have been reported to be involved in the activation of CMP1 (39). Menthol activates human TRPA1, although it blocks mouse TRPA1 at high concentrations. The menthol recognition sites (serine 873 and threonine 874) are located in the putative TM5 on human TRPA1 (26). Protons activate and sensitize human TRPA1 but inhibit mouse TRPA1. At least two amino acid residues (valine 942 and serine 943) located in TM5 and TM6 are involved in this species specificity for the effects of protons (41). Thus, slight differences in the amino acid residues on TRPA1 channels among various animal species might result in unexpected effects. In this study, we found that mutation of only a single amino acid (from leucine to isoleucine at position 881 of human TRPA1) changed the antagonistic action of A967079 into a stimulatory one. To provide in the way of mechanistic insight into how single amino acid was so critical, two mutated human channels, human TRPA1s (L881M and L881V), were analyzed (Fig. 11). However, A967079 failed to activate both mutant channels. Isoleucine is a structural isomer of leucine, and the difference between the two amino acids is the position of the methyl groups. This deduction may be supported by the characterization of the chemical structure of the TRPA1 ligand, because the deletion of a C3 methyl group in AP18 causes the loss of antagonistic action but results in an agonistic one (20). These results suggested that the methyl group in Leu-881 was located near the C3 methyl group in AP18 or A967079 and that the steroidal hindrance between these two methyl groups inhibited the conformational change to open the channel. Therefore, we should pay attention to species specificity and the chemical structures of TRPA1 antagonists to develop analgesics.

Many reports show that TRPA1 is a potentially interesting target for controlling pain (42). Systemic injection of A967079 decreases spontaneous and mechanically evoked firing of spinal neurons in uninjured, osteoarthritic, and inflamed rats (43). Oral administration of A967079 to rats decreases the AITC-induced nociceptive response and osteoarthritic pain (21). Pretreatment with AP18 significantly blocks AITC-induced nociception and mechanical hyperalgesia (37). HC-030031 reduces inflammatory and neuropathic pain (44, 45). Thus, our identification of the action site of TRPA1 antagonists should significantly contribute to the development of more specific and potent analgesics in the future.

In summary, we have demonstrated that the TM5 domain determines the sensitivity of TRPA1 to the agonistic action of A967079 and that a single specific residue is involved in species-specific gating of TRPA1. Further research on TRPA1 antagonists by utilizing species differences should help to develop new pain therapeutics.

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