The diversity in sensitivity of TRPA1 and TRPV1 of various animals to polyphenols

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ABSTRACT
The perception of tastes is sensed by the receptors that stimulate sensory cells. We previously reported that TRPA1 and TRPV1 channels expressed in the oral cavity of mammals, are activated by the auto-oxidized product of epigallocatechin gallate (oxiEGCG), a major astringent catechin in green tea. Here, we investigated and compared the sensitivity of TRPA1 and TRPV1 from various animals to astringent polyphenols. We selected three polyphenols, oxiEGCG, tannic acid and myricetin. HEK293T cells expressing TRPA1 or TRPV1 from mammal, bird, reptile, amphibian, and fish, were analyzed for their activation by the Ca\(^{2+}\)-imaging. We found the apparent diversity in the polyphenol-sensitivity among various animals. Mammalian TRPs showed relatively higher sensitivity to polyphenols, and especially, human TRPA1 and TRPV1 could be activated by all of three polyphenols at 20 μM. Reptile TRP channels, however, were insensitive to any polyphenols examined. Moreover, the polyphenol-sensitivity of zebrafish TRPA1 and TRPV1 was quite different from that of medaka TRP channels. Since many polyphenols are present in plants and the sensing of polyphenols using TRP channels in the oral cavity might cause astringent taste, the observed diversity of the polyphenol-sensitivity of TRP channels might be involved in the divergence in the food habit of various animals.

INTRODUCTION
Five basic taste stimuli (sweet, umami, salty, sour, bitter) are detected mainly by specialized cells in taste buds on the tongue surface. In addition, animals can detect the pungent stimulation of hot peppers in the mouth. Members of the TRP (Transient Receptor Potential) superfamily of cation channels, sharing the common feature of six transmembrane segments, are critically involved in transducing sensory signals and are activated by variety of stimuli. They are divided into seven subfamilies—TRPC, TRPV, TRPM, TRPA, TRPN, TRPP, and TRPML (Clapham 2003; Venkatachalam and Montell 2007). The first mammalian TRPV, TRPV1 was identified from sensory neurons by expression cloning in a search for channels activated by capsaicin, which gives spicy foods their characteristic hot taste (Caterina et al. 1997). It is well known that pungent taste is mainly mediated by TRPV1 channels in the oral cavity might cause astringent taste, the observed diversity of the polyphenol-sensitivity of TRP channels might be involved in the divergence in the food habit of various animals.

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Abbreviations: TRP, transient receptor potential; EGCG, (--)-epigallocatechin-3-gallate; TG, trigeminal ganglion; DRG, dorsal root ganglion.
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isothiocyanate in mustard oil (Venkatachalam and Montell 2007).

On the other hand, in several types of fruits and seeds, as well as in beverages such as tea, cocoa, and red wine, a characteristic sensation of astringent taste is induced primarily by polyphenols. The sensation mechanism for astringent taste induced by polyphenols is not well understood. Epigallocatechin gallate (EGCG) is a major component of the polyphenols in green tea, and astringency of EGCG is often described (Scharbert et al. 2004; Narukawa et al. 2010). We previously reported that TRPA1 and TRPV1 channels, which are expressed in the nerves on the tongue of rodents (Ishida et al. 2002; Nagatomo and Kubo 2008), are activated by auto-oxidized products of EGCG (oxiEGCG) in green tea (Kurogi et al. 2012, 2015). It is possible that the activation of these TRP channels by polyphenols such as oxiEGCG may be involved in the sense of astringent taste. On other hand, in recent years channel properties of TRPA1 and TRPV1 have been studied through variety of animal species, and the species diversity in the sensitivity of both channels to chemical stimuli has been shown (Saito and Tominaga 2015, 2017).

Animals are the most diverse group of organisms, and have adapted to their various environments in an endless number of ways, involving their behaviour, morphology and development. Moreover, diverse animal species have the most varied diets, and there is a great divergence in their food habits. Since the gustatory system is known to detect nutritive and beneficial compounds as well as harmful or toxic substances, diverse animals might must have different sensing ability of polyphenols derived from plants. Therefore, it is possible that animals might have own specific TRP channels with distinct sensitivity to polyphenols.

In this study, we investigated and compared the sensitivity of main noxious chemical sensors, TRPA1 and TRPV1 from various animal species to astringent polyphenols. We selected three polyphenols, oxiEGCG, tannic acid, and myricetin. OxiEGCG is abundant in green tea, its main component is a EGCG dimer with four galloyl moieties, and it activates mammalian TRPA1 and TRPV1 channels (Kurogi et al. 2015). Tannic acid with five galloyl moieties presents in varying concentrations in plant foods and in relatively high concentrations in red wines and teas (Serrano et al. 2009; Imamura et al. 2015). Myricetin with one galloyl moiety is the compound that occurs naturally in grapes and red wine (McDonald et al. 1998). It has been known that these three polyphenols induce astringent taste (Scharbert et al. 2004; Schöbel et al. 2014). Since it has been suggested that the presence of galloyl moiety might be one of the features for astringents (Schöbel et al. 2014), we selected these three polyphenols with different number of galloyl moieties. HEK293T cells were transfected with the expression vectors for TRPA1 or TRPV1 from mammal, bird, reptile, amphibian, and fish, and the Ca^{2+}-imaging analysis was performed.

MATERIALS AND METHODS

**Experimental animals.** All of the animal experiments described below conformed to the institutional guidelines and were approved by the Animal Experiment Committee of Nagahama Institute of Bio-Science and Technology.

**Materials.** The expression vectors for mouse, chick, medaka, and axolotl TRPA1 were previously described (Nagatomo and Kubo 2008; Kurogi et al. 2015; Oda et al. 2017, 2019), and the vectors for rattlesnake and rat snake TRPA1 were provided by Dr. David Julius (UCSF, California, USA). The vectors for human and takifugu TRPA1 were provided by Dr. A. Patapoutian (Scripps Research Institute, California, USA). The vector for xenopus TRPA1 was provided by Dr. M. Tominaga (National Institute for Physiological Sciences, Aichi, Japan), and zebrafish TRPA1 cDNAs (zTRPA1a and zTRPA1b) were kindly from Dr. David Prober (Caltech, California, USA). Molecular cloning of axolotl TRPV1 was recently reported (Hori and Saitoh 2020) and the vectors for rat, chick, rattlesnake, and zebrafish TRPV1 were provided by Dr. David Julius (UCSF, California, USA). Zebrafish has one gene for TRPV1, but medaka has two TRPV1 genes (TRPV1a and b). Since Medaka TRPV1b is similar to zebrafish TRPV1, TRPV1b cDNA was kindly obtained from Miss M. Asano (Nagahama Institute of Bio-Science and Technology, Shiga, Japan) and human TRPV1 cDNA was provided by Dr. C. Reilly (University of Utah, Utah, USA).

**Cell culture and calcium imaging analysis.** Human kidney epithelial cell line HEK293T was maintained in the culture medium consisted of DMEM supplemented with 10% FBS and antibiotics (100 μg/mL kanamycin). For heterologous expression, HEK293T cells were transfected with the expression vector (TRPV1 or TRPA1) using Effectene transfection reagent (Qiagen, Chatsworth, California, USA). After 24–48 h, cells were examined by the calcium-imag-
ing technique. In experiments for the expression of TRPA1, cells were incubated in 3 μM ruthenium red to increase viability for 24–48 h, then washed with Hank’s balanced salt solution (HBSS) and used for the calcium-imaging.

To establish primary cultures of dorsal root ganglia (DRG), 6- to 10-week-old C57BL/6 mice were killed by cervical dislocation, after which the DRG were mechanically isolated. The isolated ganglia were dissociated and cultured as described (Dai et al. 2007).

Using cells grown on matrigel-coated μ-Slide 8 well (80826, ibidi; MPI für Infektionsbiologie, Berlin, Germany), the calcium-imaging analysis with Fluo8-AM was performed as previously described (Kurogi et al. 2012). Fluo8 fluorescence was recorded every 3 s using Axiovert 200 (Carl Zeiss, Göttingen, Germany) and changes of fluorescence intensity of 10 cells were analyzed by Image-Pro Plus imaging software (Media Cybernetics, Silver Springs, Maryland, USA). The signals are expressed as relative fluorescence change: ΔF / F = (F − F₀) / F₀. Each of three polyphenols was applied to cells at 6 s after the start of measurement. All calcium imaging experiments were repeated two or three times.

Statistical analyses. The values and error bars shown in the figures indicate mean and standard errors. The statistical significances of the differences of multiple groups were performed by the Tukey-Kramer method. To highlight the presence of the statistical significance, we indicated by *(P < 0.05) and **(P < 0.01) for the focused groups.

RESULTS

Sensitivity of mouse DRG neurons to tannic acid and myricetin

It has been known that the nerve fibers in the taste papillae of tongue express TRPV1 (Ishida et al. 2002) and TRPA1 (Nagatomo and Kubo 2008). Primary afferent neurons are clustered in the dorsal root ganglion (DRG) and within cranial nerve ganglia such as the trigeminal ganglion (TG). It has been shown that DRG and TG neurons express TRPV1, TRPA1, and TRPM8 (Kobayashi et al. 2005). We previously showed that oxidized EGCG (oxiEGCG) activated TRPA1 and TRPV1 channels expressed in cultured DRG neurons, and demonstrated the possibility that the activation of TRPA1 and TRPV1 by polyphenols might be involved in the sensation of astringency (Kurogi et al. 2015). Using acutely dissociated sensory neurons of DRG analogous to TG, we examined the sensitivity to two astringent polyphenols, tannic acid and myricetin by the Ca²⁺-imaging technique. After isolation of DRG from mice, neurons were dissociated and cultured. Tannic acid was applied to DRG neurons at 20 μM. Time courses of individual cell recordings were shown in Fig. 1A. Many cells were activated by tannic acid. These cellular responses were inhibited by a general TRP blocker, ruthenium red and a TRPV1 blocker, SB-366791, but not significantly attenuated by a TRPA1 blocker, HC-030031. When myricetin was applied at 100 μM, cultured DRG neurons were also activated. This myricetin-induced activation was blocked by ruthenium red, but rather partially inhibited by HC-030031 and SB-366791 (Fig. 1). These observations demonstrated that DRG sensory neurons are activated by these two astringent polyphenols in addition to oxiEGCG through TRP channels. Further, as previously observed with oxiEGCG (Kurogi et al. 2015), the responses of individual DRG neurons were transiently induced and were not synchronized. Firing of DRG neurons are triggered by voltage-activated channels and are detected by Ca²⁺-imaging. Since the response of TRP channels to polyphenols such as oxiEGCG might be slow, the time to reach the threshold for firing is considered to differ in individual DRG neurons. Therefore, it appears that DRG neurons transiently and individually respond to polyphenols.

The sensitivity diversity of TRPA1 from various animals to three polyphenols

As shown in Fig. 1, it is apparent that sensory neurons of mice can recognize three astringent polyphenols. To investigate how differently three polyphenols, oxiEGCG, tannic acid, and myricetin activate TRPA1 channels from various animals, we compared effects of three polyphenols on TRPA1 of 10 animal species (mammals: human and mouse, other land animals: chicken, rat snake, rattlesnake, Xenopus and axolotl, fish: zebrafish, fugu (Takifugu) and medaka). HEK293T cells were transfected with each TRPA1 expression vector and Ca²⁺-imaging analysis was performed. Each polyphenol at 20 μM or 200 μM was applied to the transfected cells at 6 s after the start of measurement and the expression of each TRPA1 channels was further confirmed by the addition of allyl isothiocyanate to 100 μM at 90 s. We first investigated and compared effects of oxiEGCG on the activation of TRPA1 channels from various animal species. Activation by 20 μM oxiEGCG was only observed with human TRPA1, and 200 μM oxiEGCG activated mouse TRPA1 and zebrafish
the high sensitivity to polyphenols, and was activated by all of three polyphenols at 20 μM. No response, however, was observed for TRPA1s of reptiles such as rat snake and rattlesnake treated with any of polyphenols. Pungent ligand for TRPA1, allyl isothiocyanate, activated these reptile TRPA1s.

From results of Fig. 1, it seems that mouse sensory neurons can recognize three astringent polyphenols, and that mouse TRPV1 might be also involved in the recognition of these polyphenols. We further focused on the diversity of sensitivity of TRPV1 to three polyphenols, oxiEGCG, tannic acid, and TRPA1a in addition to human TRPA1. Other TRPA1 channels were not activated by 200 μM oxiEGCG (Fig. 2 upper). We next examined effects of tannic acid on various TRPA1 channels. Tannic acid at 20 μM activated human and chicken TRPA1s, and 200 μM tannic acid activated chicken TRPA1, axolotl TRPA1, and zebrafish TRPA1a (Fig. 2 middle). We further studied effects of myricetin on various TRPA1 channels. Addition of myricetin to 20 μM only activated human TRPA1, and 200 μM myricetin activated TRPA1s from human, mouse, Xenopus, axolotl, zebrafish (TRPA1a), fugu, and medaka (Fig. 2 lower). Thus, it is evident that any of three astringent polyphenols differentially activated TRPA1s from various animals. Especially, human TRPA1 showed

**Fig. 1** Effects of tannic acid and myricetin on mouse DRG neurons. (A) Ca\(^{2+}\) responses of dorsal root ganglion (DRG) to tannic acid and myricetin were examined. DRG sensory neurons were isolated from mice and cultured. On culture day 1, the Ca\(^{2+}\)-imaging analysis was performed. Ten μM tannic acid or 100 μM myricetin in HBSS was used as a ligand solution at 6 s after the start of measurement, 100 μM allyl isothiocyanate was applied at 120 s, and 10 μM capsaicin was further applied at 150 s. Time courses of ΔF/F of individual cell recordings were shown. When effects of blockers were examined, cells were pretreated with 10 μM ruthenium red (RR), 100 μM HC-030031 (HC), or 10 μM SB-366791 (SB) for 15 min, and then ligand with a blocker was applied. The solution of capsaicin or allyl isothiocyanate was similarly applied without blockers at the end of the imaging to cancel the blocker effect. (B) From results in A, the average of the highest response in individual neurons during the first 120 s stimulation was obtained and compared. Differences were judged to be significant by the Tukey-Kramer method (**P < 0.01).
myricetin among various animals, and we compared effects of three polyphenols on TRPV1 of 7 animal species (mammals: human and rat, other land animals: chicken, rattlesnake and axolotl, fish: zebrafish and medaka). HEK293T cells were transfected with each TRPV1 expression vector and Ca$^{2+}$-imaging analysis was performed. Each polyphenol at 20 μM or 200 μM was applied to the transfected cells at 6 s after the start of measurement and polyphenol-induced cellular activation was analyzed as described in the previous section. First, effects of oxiEGCG on the activation of TRPV1 channels from various animal species were examined. Activation by 20 μM oxiEGCG was observed with human and rat TRPV1, and 200 μM oxiEGCG activated TRPV1s from human, rat, chicken, and zebrafish. Other TRPV1 channels were not activated by 200 μM oxiEGCG (Fig. 3 upper). We next examined effects of tannic acid on various TRPV1 channels. Tannic acid at 20 μM activated human, rat, axolotl, and zebrafish TRPV1s, and 200 μM tannic acid further activated chicken TRPV1 in addition to TRPV1s of human, rat, axolotl, and zebrafish (Fig. 3 middle). We further studied effects of myricetin on various TRPV1 channels. Addition of myricetin to 20 μM only activated human TRPV1, and 200 μM myricetin activated TRPV1s of human, rat, and chicken (Fig. 3 lower).

These results apparently indicated that TRPV1s from some animals have the sensitivity to polyphenols as TRPA1, and TRPV1s of various animals differentially respond to polyphenols. Similarly to human TRPA1, human TRPV1 showed the highest

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**Fig. 2**  The sensitivity diversity of TRPA1s to three polyphenols. Effects of three polyphenols (oxidized EGCG (ovi-EGCG), tannic acid and myricetin, 20 μM or 200 μM) on [Ca$^{2+}$] in HEK293T cells expressing TRPA1 from various animals were examined. After transfection with the expression vector of each TRPA1, cells were loaded with 5 μM Fluo8-AM. The Fluo8 fluorescence was recorded every 3 s and the relative fluorescent change (ΔF/F) was determined. At 6 s after the start of measurement, ligand was applied. At 90 s, 100 μM allyl isothiocyanate was further applied to confirm the channel expression. The average ΔF/F at 90 s was obtained and compared. Differences were judged to be significant by the Tukey-Kramer method (*P < 0.05, **P < 0.01). Time course of Ca$^{2+}$ response of various TRPA1s was indicated in Supplementary Fig. 1.
analysis of HEK293T cells transfected with the expression vectors for TRPA1 or TRPV1 from mammal, bird, reptile, amphibian, and fish, we found the apparent diversity in the sensitivity to polyphenols of TRPA1 and TRPV1 channels of various animals. Results were summarized in Table 1. Mammalian TRPs showed relatively higher sensitivity to polyphenols, and especially, human TRPA1 and TRPV1 could be activated by all of three polyphenols at 20 μM. In contrast, any polyphenols could not activate reptile (rattlesnake) and medaka TRPV1 were not significantly activated by any of three polyphenols at the examined concentration. Typical ligand for TRPV1 (capsaicin or 2-aminoethoxydiphenylborane) activated reptile or medaka TRPV1.

**DISCUSSION**

Here, we focused on the diversity of the astringent-taste sensing of animals and compared the polyphenol-sensitivity of main noxious chemical sensors, TRPA1 and TRPV1 to astringent polyphenols among various animal species. As an astringent, we used three polyphenols, oxidized EGCG, tannic acid, and myricetin. By the Ca^{2+}-imaging sensitivity to three polyphenols, and it was activated from 20 μM of each polyphenol. In addition, rat and chicken TRPV1s have the broad sensitivity to polyphenols and all of three polyphenols could activate them. On the other hand, reptile (rattlesnake) and medaka TRPV1 were not significantly activated by any of three polyphenols at the examined concentration. Typical ligand for TRPV1 (capsaicin or 2-aminoethoxydiphenylborane) activated reptile or medaka TRPV1.

![Fig. 3](image_url) The sensitivity diversity of TRPV1s to three polyphenols. Effects of three polyphenols (oxidized EGCG(oxiEGCG), tannic acid and myricetin, 20 μM or 200 μM) on [Ca^{2+}] in HEK293T cells expressing TRPV1 from various animals were examined. After transfection with each TRPV1 vector, cells were loaded with 5 μM Fluo8-AM. The Fluo8 fluorescence was recorded and the relative fluorescent change (ΔF/F) was determined. Each polyphenol was applied at 6 s after the start of measurement. To confirm the channel expression, 10 μM capsaicin, 100 μM tannic acid, or 1 mM 2-aminoethoxydiphenylborane was further applied to the imaging chamber. The average ΔF/F at 90 s was obtained and compared. Differences were judged to be significant by the Tukey-Kramer method (*P < 0.05, **P < 0.01). Time course of Ca^{2+} response of various TRPV1s was shown in Supplementary Fig. 2.
be involved in the divergence in the food habit of various animals.

Concerning the relationship between the diversity of taste receptors expressed in gustatory cells and the food habit of each animals, Jiang et al. (2012) reported very important observation. Mammalian sweet taste is known to be primarily mediated by Tas1r2 (T1R2) and Tas1r3 (T1R3) (Chandrashekar et al. 2006). Jiang et al. (2012) sequenced the entire coding region of T1R2 from various animal species and showed that seven species, which are exclusive meat eaters, have independently pseudogenized T1R2 caused by ORF-disrupting mutations. Their data demonstrated that loss of taste receptor function in mammals is widespread and related to food habit. On the other hand, bitter taste reception in taste receptor cells is mediated by a family of divergent receptors to distinct compounds, T2Rs (Chandrashekar et al. 2006). Diverse bitter compounds are present in plant than in animal tissue, and therefore herbivores are expected to encounter more bitter substances than carnivores. Hu and Shi (2013) characterized the functional gene repertoire of T2Rs by searching the genome sequences from ten different species of mammals. Their results indicated that carnivores have few T2R genes, herbivores an intermediate number, and omnivores the largest T2R gene repertoire. Thus, it is considered that the function of taste receptors expressed in gustatory cells on the tongue is strongly related to the feeding behavior of each animals.

In case of the connection between the sensitivity of TRP channels expressed in the nerves in the taste papillae of tongue and the food habit of various animals, only limited studies were reported. Chili peppers produce capsaicin, the pungent compound which offers protection from predatory mammals. Birds are indifferent to capsaicin and serve as vectors for dispersal of seeds. Jordt and Julius (2002) cloned a chick TRPV1 cDNA, and their functional analysis revealed that chick TRPV1 has functional properties similar to those of its mammalian counterpart with the exception its significantly reduced capsaicin sensitivity, thereby providing a molecular explanation for insensitivity of birds to chili peppers. In this paper, we showed that human, which eat plants, meat and many kinds of food, has the TRP channels with the highest sensitivity to three astringent polyphenols. Reptiles such as snakes, which feeds on flesh, express polyphenol-insensitive TRP channels. Quite interestingly, these observation is very close to bitter taste receptor (T2Rs) gene repertoire in omnivores, herbivores and carnivores. Food habit is one of the essential parts supporting the diversity of animals living in the various environments on the earth. Therefore, as one approach to understand the animal diversity, we must determine the molecular basis for food habit of various animals. From this point of view, this paper provided a new concept of the diversity of polyphenol sensitivity of TRP channels involved in feeding behavior. As a next step, we will further study the polyphenol-sensitivity of TRP channels of omnivores, herbivores and carnivores from each animal class (mammals, birds, reptiles, amphibians, and fish).

### Table 1 Summary of polyphenol-sensitivity of TRPA1 and TRPV1 of various animals

| classification | Species | Polyphenols | Oxi-EGCG | Tannic acid | Myricetin |
|----------------|---------|-------------|----------|-------------|----------|
|                |         | A1 | V1 | A1 | V1 | A1 | V1 |
| Mammal         | Human   | ○ | ○ | ○ | ○ | ○ | ○ |
|                 | Rodent  |△ | ○ | × | ○ | △ | △ |
| Bird           | Chicken | × | △ | ○ | △ | × | △ |
| Reptile        | Snake   | × | × | × | × | × | × |
| Amphibian      | Axolotl | × | × | △ | ○ | △ | × |
| Fish           | Zebrafish | △ | △ | △ | ○ | △ | × |
|                | Medaka  | × | × | × | × | △ | × |

○: significantly sensitive to 20 μM polyphenol
△: significantly sensitive to 200 μM polyphenol
×: insensitive
amphibians, fish). On the other hand, it has been demonstrated that cultured TG neurons of mice can be activated by variety of astringent polyphenols derived from plants and its activation is mediated through G protein-coupled signaling (Schöbel et al. 2014). This system should be also compared among various animal species.

Although overall protein sequences are closely related between bird and reptile and between zebrafish and medaka on phylogenetic trees in both cases of TRPA1 and TRPV1 (Oda et al. 2019; Hori and Saitoh 2020), our study indicated that their polyphenol-sensitivity was apparently distinct from such overall similarity. Hence, the short specific site for polyphenol-recognition might be present. Both TRP channels are composed of the N-terminal cytoplasmic domain containing ankyrin repeats and the C-terminus containing six transmembrane domains. In case of TRPA1, the four residues (C619, C639, C663, K708) within the cytoplasmic N-terminus are important for the activation by allyl isothiocyanate (Hinman et al. 2006). The capsaicin-recognition region of TRPV1 has been reported to be located within the transmembrane 3-4 region (Jordt and Julius 2002). Concerning the region for the polyphenol-sensitivity, we previously indicated that the region providing the sensitivity to oxiEGCG is located at the C-terminus part of 6 transmembrane domain within mouse TRPA1, and the region for oxiEGCG-sensitivity of rat TRPV1 is also present within the C-terminal transmembrane domain (Kurogi et al. 2015). In future, based on the observed diversity of the polyphenol-sensitivity, we will determine the recognition site for oxiEGCG, tannic acid, and myricetin on TRP channels.

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Supplementary Fig. 1
The time course of Ca\(^{2+}\) response of various TRPA1s to three polyphenols. After transfection of HEK293T cells with the expression vector of each TRPA1, cells were loaded with 5 μM Fluo8-AM. The Fluo8 fluorescence was recorded every 3 s and the relative fluorescent change (ΔF/F) was determined. At 6 s, each polyphenol was applied. At 90 s, 100 μM allyl isothiocyanate was further applied to confirm the channel expression. Time course of the average fluorescent change for various TRPA1 was indicated.
The time course of Ca\(^{2+}\) response of various TRPV1s to three polyphenols. After transfection of HEK293T cells with the expression vector of each TRPV1, cells were loaded with 5 μM Fluo8-AM. The Fluo8 fluorescence was recorded every 3 s and the relative fluorescent change (ΔF/F) was determined. At 6 s, each polyphenol was applied. For human, rat, or rattlesnake TRPV1, 10 μM capsaicin was further applied to confirm the channel expression at 120 s. Since chicken TRPV1 has been known to have less sensitivity to capsaicin (Jordt and Julius 2002), 100 μM tannic acid was added at 120 s to confirm the expression of chicken TRPV1. For similar reason, to confirm the expression of zebrafish TRPV1, 100 μM tannic acid was added at 120 s. For confirmation of the expression of axolotl or medaka TRPV1, we checked the activation by 10 μM capsaicin or 1 mM 2-aminoethoxydiphenylborane using cells transfected at the same time. Time course of the average fluorescent change for various TRPV1 was indicated.