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Auto-oxidation Products of Epigallocatechin Gallate Activate TRPA1 and TRPV1 in Sensory Neurons

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Abstract

The sensation of astringency is elicited by catechins and their polymers in wine and tea. It has been considered that catechins in green tea are unstable and auto-oxidized to induce more astringent taste. Here, we examined how mammalian transient receptor potential V1 (TRPV1) and TRPA1, which are nociceptive sensors, are activated by green tea catechins during the auto-oxidation process. Neither TRPV1 nor TRPA1 could be activated by any of the freshly prepared catechin. When one of the major catechin, epigallocatechin gallate (EGCG), was preincubated for 3 h in Hank's balanced salt solution, it significantly activated both TRP channels expressed in HEK293 cells. Even after incubation, other catechins showed much less effects. Results suggest that only oxidative products of EGCG activate both TRPV1 and TRPA1. Dorsal root ganglion (DRG) sensory neurons were also activated by the incubated EGCG through TRPV1 and TRPA1 channels. Liquid chromatography-mass spectrometry revealed that theasinensins A and D are formed during incubation of EGCG. We found that purified theasinensin A activates both TRPV1 and TRPA1, and that it stimulates DRG neurons through TRPV1 and TRPA1 channels. Results suggested a possibility that TRPV1 and TRPA1 channels are involved in the sense of astringent taste of green tea.

Key words: astringency, catechin, taste, theasinensin A

Introduction

In addition to 5 basic taste stimuli (sweet, umami, salty, sour, and bitter), the pungent stimulation of hot peppers is also recognized in the mouth. Such sensation is mainly mediated by TRPV1 (transient receptor potential V1) receptors (Caterina et al. 1997; Ishida et al. 2002). Further, in beverages such as tea, cider, and red wine, as well as in several types of fruits, nuts, and chocolate, a characteristic astringent sensation is elicited primarily by polyphenols. Of these polyphenols, catechin, epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) and their polymers are abundant in wine and tea. A most abundant green tea polyphenol is EGCG (Drewnowski and Gomez-Carneros 2000; Lesschaeve and Noble 2005). Currently, the sensation mechanism for astringent taste induced by green tea polyphenols such as EGCG is not well understood.

Green tea has been shown to have anticancer activity in many organs (Bettuzzi et al. 2006; Yang et al. 2006), and EGCG is the major polyphenol with the cancer preventive effects (Chung et al. 1999; Saeki et al. 2000). Tachibana et al.

have found that a 67-kDa laminin receptor (67LR) functions as a cell surface EGCG receptor inducing the anticancer action (Tachibana et al. 2004). EGCG has been shown to inhibit the growth of cancer cells through the 67LR (Umeda et al. 2005). 67LR is known to be overexpressed on the surface of various tumor cells (Ménard et al. 1997). Laminins are also known to play an important role in axonal growth and 67LR is present on various neurons such as dorsal root ganglion (DRG) neurons (Masuda et al. 2009). It is considered that the EGCG signaling using 67LR may not induce the astringent sensation in sensory terminals in the oral cavity.

We previously found that the mouse intestinal endocrine cell line STC-1 can respond to EGCG among 4 major tea catechins by the calcium-imaging technique. We further indicated that EGCG stimulates intestinal STC-1 cells by activating TRPA1 channels. Since TRPA1 is more likely to be expressed in nerve fibers in the tongue, we demonstrated that TRPA1 might play an important role in the astringency taste on the tongue (Kurogi et al. 2012). In the same report, we showed that TRPV1 is also activated by EGCG. On the other hand, it has been considered that green tea incubated for longer period after brewing tastes more astringent by auto-oxidation, and it was demonstrated that astringency increases with degree of polymerization of polyphenols (Peleg et al. 1999). EGCG is known to be auto-oxidized at neutral pH, and EGCG dimmers of theasinensins (A/D) and P2 (another dimer with Mr884) have been reported to be formed (Hong et al. 2002). Furthermore, several biological activities have been reported for theasinensins (Pan et al. 2000; Hou et al. 2005, 2010). How do these auto-oxidation products of EGCG affect TRPA1 and TRPV1 channels?

Here, we examined how TRPA1 and TRPV1 from rodents are activated by tea catechins in the course of auto-oxidation process. Interestingly, neither TRPA1 nor TRPV1 could be activated by any one of the freshly prepared catechins without preincubation. EGCG preincubated for 3 h in Hank's balanced salt solution (HBSS), however, significantly activated both TRP channels. The presence of ascorbic acid inhibited the preincubation effect on EGCG. In the previous experiments, the catechin solution was prepared before loading Ca^{2+} indicator dye into cells, and the solution was kept for about 30 min before assay. These results strongly suggested that only oxidative products of EGCG activate TRPA1 and TRPV1. Furthermore, we observed that DRG neurons are activated by the preincubated EGCG through TRPV1 and TRPA1 channels. Then, we found that EGCG dimmers, theasinensins, are present in the incubated auto-oxidized EGCG. Theasinensin A (TS-A) was synthesized and purified, and the activity to stimulate TRP channels was studied. Further, we studied the sensitivity to the auto-oxidized EGCG of TRPV1 and TRPA1 channels from other species. Then, experiments using the chimeras of the oxidized EGCG-sensitive and EGCG-nonsensitive channels demonstrated that the major important region for the sensitivity to the oxidized EGCG is located at the C-terminal half containing 6 transmembrane domains of rodent TRPV1 and TRPA1.

Materials and methods

Experimental animals

All 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 and the National Institute for Physiological Sciences.

Materials

(−)-EGCG, (−)-EC, (−)-ECG and (−)-EGC, and capsaicin (CAP) were from Wako. Allyl isothiocyanate (AITC) was from Nacalai Tesque. Ruthenium red (RR) and SB-366791 (SB) were from Sigma-Aldrich. 4-(4-Chlorophenyl)-3- methylbut-3-en-2-oxime (AP-18) was from Enzo Life Sciences. Fluo8-AM was from AAT Bioquest. Growth Factor Reduced MATRIGEL Matrix (matrigel) was from Becton Dickinson. The expression vector for mouse TRPA1 was previously described (Nagatomo and Kubo 2008), and the vectors for rat TRPV1, rattlesnake TRPV1, rattlesnake TRPA1, and chick TRPV1 were provided by Dr David Julius (UCSF, San Francisco, CA).

Chemical structures

The chemical structures of EC, ECG, EGC, EGCG, and TS-A/D are shown in Supplementary Figure 1.

Cell culture and calcium-imaging analysis

HEK293 cells were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 μg/mL kanamycin). For heterologous expression, HEK293T cells were transfected with the expression vector using Effectene transfection reagent (Qiagen). After 24–48 h, cells were examined by the calcium-imaging technique. For the expression of TRPA1, cells were incubated in 3 μM RR to increase viability for 24–48 h, then washed with HBSS and used for the calcium imaging.

To establish primary cultures of DRG neurons, 6- to 10-week-old C57BL/6 mice were sacrificed 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), 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) and

changes of fluorescence intensity were analyzed by Image-Pro Plus imaging software (Media Cybernetics). The signals are expressed as relative fluorescence change: $\Delta F/F = (F - F_0)/F_0$. Various catechin samples were applied to cells at 6 s, and 10 μM CAP or 100 μM AITC was further applied at 240 or 120 s to confirm the channel expression. When effects of channel blockers were examined, the solution of CAP or AITC was similarly applied without blockers at the end of the imaging to cancel the blocker effect. All calcium-imaging experiments were repeated 2 or 6 times.

Electrophysiology

To examine the effects of oxidized EGCG and purified TS-A on TRP channels, macroscopic currents were recorded from HEK293T cells transfected with the TRP constructs (the TRPV1 or the TPRA1) and the successful transfection marker of fluorescent protein, as previously described (Tateyama and Kubo 2011). Briefly, whole-cell patch-clamp recordings were performed 24–48 h after transfection with Axopatch 200B amplifiers and the pClamp 9 software (Axon Instruments). The glass pipettes were filled with the internal solution composed of 140 mM KCl, 4 mM Na_2-ATP, 0.5 mM ethylene glycol tetraacetic acid, 0.1 mM $CaCl_2$, 10 mM HEPES, 5 mM $MgCl_2$, pH 7.4 adjusted with KOH. Cells expressing fluorescent protein were held at −20 mV and applied repetitive ramp pulses (from −60 to 60 mV for 400 ms) every 5 s. Cells were continuously perfused with HBSS supplemented with $CaCl_2$ (final 2 mM) and $MgCl_2$ (final 0.4 mM). The reagents were applied by using the fast solution exchange system (VC-77SP, Warner Instruments). The current amplitudes at 58–60 mV during ramp pulse were averaged and then normalized by the cell capacitance to calculate the current density. The density before the oxidative EGCG application (basal density) was subtracted from the maximal densities during the application of the reagents to calculate the reagents-induced current densities.

Molecular biology

Chick TRPA1 cDNA

Chick TRPA1 cDNA was cloned into the expression vector. Total RNA was isolated from DRG of 14-day chick embryo using TRIzol reagent (Invitrogen) and subjected to reverse transcription with random primers. The reverse-transcribed cDNA was used as a template of PCR. Based on the predicted sequence of chick (*Gallus gallus*) TRPA1 mRNA (XM_418294), we used the following primers to amplify 4 overlapping DNA fragments of cDNA (primer pairs: *XbaI*_chickTRPA1-f and 1r, 2f and 2r, 3f and 3r, 4f, and chickTRPA1_*XbaI*-r).

*XbaI*_chickTRPA1-f:
5′-TTTTTCTAGACTTAGTCCACCATGAAGCGCTC TCTGTGGC-3′
chickTRPA1_1r:
5′-TGCCAAATGAAGTGGACTGCACTTCC CATTATTGGT-3′
chickTRPA1_2f:
5′-ATAATGGGAAGTGCAGTCCACTTCATTTGG CAGTTC-3′
chickTRPA1_2r:
5′-CACAGAGAAAAAGGGCCCCTCTTT TCAGAAGAAACT-3′
chickTRPA1_3f:
5′-TCTTCTGAAAAGAGGGGCCCTTTTTCT CTGTGACTA-3′
chickTRPA1_3r:
5′-CAGTCCAGTAGATTGGAGTAATCCAA CAGATATTTC-3′
chickTRPA1_4f:
5′-TATCTGTTGGATTACTCCAATCTACTGGA CTGGACA-3′
chickTRPA1_*XbaI*-r:
5′-GGGTCTAGATCACATAGAAGTCTACAA TAAGC-3′

After subcloning and sequence confirmation of each cDNA fragment, a single full-length cDNA fragment of chick TRPA1 (3381 bp, AB986554) was amplified using these 4 overlapping DNA fragments. The resultant DNA was digested with *XbaI* and cloned into *XbaI*-digested pcDNA3.1Hygro(−). The orientation and the nucleotide sequence of cDNA were further confirmed by DNA sequencing.

Chimera constructs

Chimeras of mouse and chick TRPA1 were generated using standard overlapping PCR. The DNA fragment encoding the whole N-terminus cytoplasmic region containing ankyrin repeats (corresponding to amino acid 1–711) was amplified by PCR from mouse TRPA1 cDNA. The DNA fragment encoding the C-terminus containing 6 transmembrane domains (corresponding to amino acid 716–1126) was amplified by PCR from chick TRPA1 cDNA. By the overlapping PCR using these 2 DNA fragment as a template, the cDNA of mouse–chick TRPA1 chimera (MC TRPA1) was obtained. The cDNA of chick–mouse TRPA1 chimera (CM TRPA1) was obtained similarly. The DNA fragment of the N-terminal part of chick TRPA1 (corresponding to amino acid 1–715) was connected to the DNA fragment of C-terminal part of mouse TRPA1 (corresponding to amino acid 712–1125) using the overlapping PCR.

The DNA fragment encoding the N-terminal part containing whole ankyrin repeats was swapped between rat TRPV1 cDNA and rattlesnake TRPV1 cDNA. The cDNA fragment of the N-terminal part of rat TRPV1 (corresponding to amino acid 1–422) was connected to the DNA fragment of C-terminal part of rattlesnake TRPV1 (corresponding to amino acid 415–822) using the overlapping PCR

(RS TRPV1). Also, the DNA fragment of the N-terminal part of rattlesnake TRPV1 (corresponding to amino acid 1–414) was connected to the DNA fragment of C-terminal part of rat TRPV1 (corresponding to amino acid 423–828) (SR TRPV1). These chimera cDNAs were cloned into pcDNA3.1 Hydro(−) and their sequences were confirmed. Since the response of RS TRPV1 to CAP was not detected, the expression of RS TRPV1 was examined using the antibody which recognizes the peptide (25–75 aa) present near the N-terminus of rat TRPV1 (SC-12498, Santa Cruz Biotechnology). Although we easily observed the expression of rat TRPV1, the RS TRPV1 expression could not be detected.

Liquid chromatography-electrospray ionization mass spectrometric and liquid chromatography–tandem mass spectrometric analysis

Liquid chromatography-electrospray ionization mass spectrometric (LC-ESI-MS) and liquid chromatography–tandem mass spectrometric (LC–MS/MS) analyses were performed using a LCMS-IT-TOF (Shimadzu).

Samples (10 µl, 4 mM) were applied to a Cosmosil $5C_{18}$-ARII column (Nacalai Tesque Inc., 2.0 mm i.d. × 100 mm). To elute the column, in the first 15 min, the solvent was changed in a linear gradient from 90% A [0.1% formic acid] + 10% B [CH_3OH:CH_3CN = 3:2] to 75% A + 25% B at a flow rate of 0.2 mL/min. In the next 10 min, the solvent was changed in a linear gradient to 40% A + 60% B. Then, the solvent was changed back to 90% A + 10% B and maintained at 90% A + 10% B for another 15 min. The MS instrument was operated using an ESI source in negative ionization mode. Ionization parameters were as follows: probe voltage: 4.5 kV; nebulizing gas flow: 1.5 L/min; curved desolvation line temperature: 200 °C; block heater temperature: 200 °C.

Preparation of TS-A

TS-A was synthesized from EGCG and purified according to the method described by Shii et al. (2011). A solution of 5 mg EGCG and 0.01 mmol $CuCl_2$ in 30% MeOH (2 mL) was vigorously mixed at 25 °C for 24 h. To the mixture, 50 mg of ascorbic acid was added and heated at 85 °C for 15 min. After cooling, the mixture was 2.5 times diluted with H_2O and applied to a preparative high-performance liquid chromatography (HPLC) using a Cosmosil $5C_{18}$-ARII column (Nacalai Tesque Inc., 20 mm i.d. × 250 mm). To elute the column, in the first 50 min, the solvent was changed in a linear gradient from 90% A [H_2O] + 10% B [CH_3OH:CH_3CN = 3:2] to 75% A + 25% B at a flow rate of 3 mL/min. In the next 20 min, the solvent was changed in a linear gradient to 40% A + 60% B and maintained at 40% A + 60% B for another 20 min. Then, the solvent was changed back to 90% A + 10% B and maintained at 90% A + 10% B for 60 min. LC chromatograms were obtained at UV 254 nm. The fraction (TS-A) was collected and concentrated by evaporation and kept at −80 °C. Purified TS-A was redissolved in HBSS containing 4 mM ascorbic acid to block further auto-oxidization.

Statistical analyses

The values and error bars shown in the figures indicate mean and standard errors. The n values are 8–10. 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

Auto-oxidized products of EGCG activate TRPV1 and TRPA1 channels

To examine how EGCG activates TRPV1 and TRPA1 channels in the course of auto-oxidation process, we compared effects of freshly prepared and incubated EGCG on both channels. HEK293T cells were transfected with the rat TRPV1 or mouse TRPA1 expression vector and Ca^{2+}-imaging analysis was performed. Since the amino acid sequence of rat TRPV1 is 95% identical with that of mouse TRPV1, rat cDNA was used instead of mouse cDNA. EGCG was dissolved in HBSS at 200 µM and incubated at 25 °C for 2–6 h or freshly prepared just before use. We did not observe a significant increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in HEK293 cells expressing TRPV1 or TRPA1 with the freshly prepared EGCG. However, following incubation, EGCG could induce an increase in $[Ca^{2+}]_i$ in cells expressing either TRP channels. The 3-h-incubated EGCG significantly activated TRPV1 and TRPA1 channels and it was used for further studies. When EGCG was incubated in the presence of an antioxidant, ascorbic acid (1 mM), the increase in $[Ca^{2+}]_i$ was not induced by the EGCG in HEK293T cells expressing TRPV1 or TRPA1 channels (Figure 1). In control nontransfected HEK293T cells, the increase in $[Ca^{2+}]_i$ was not observed with the incubated EGCG (Supplementary Figure 2). Results suggested that auto-oxidized products are the activators of TRPV1 and TRPA1 channels in the incubated EGCG. We further analyzed the sensitivity and selectivity to auto-oxidation products of 4 major catechins. Catechins were dissolved and incubated for 3 h, and then used to examine the effect to stimulate TRP channels. The effects on TRPV1(Figure 2) or TRPA1(Figure 3) were compared with those of catechins dissolved in 1 mM ascorbic acid. EGCG and auto-oxidized EGCG were applied to cells at 2–200 µM, and then 10 µM CAP or 100 µM AITC was further applied to confirm the channel expression. Time courses of individual cell recordings are shown in Figures 2 and 3. The averages of $[Ca^{2+}]_i$ response at 90 s with the 3-h-incubated EGCG was obtained and plotted against the EGCG concentration (Figures 2B

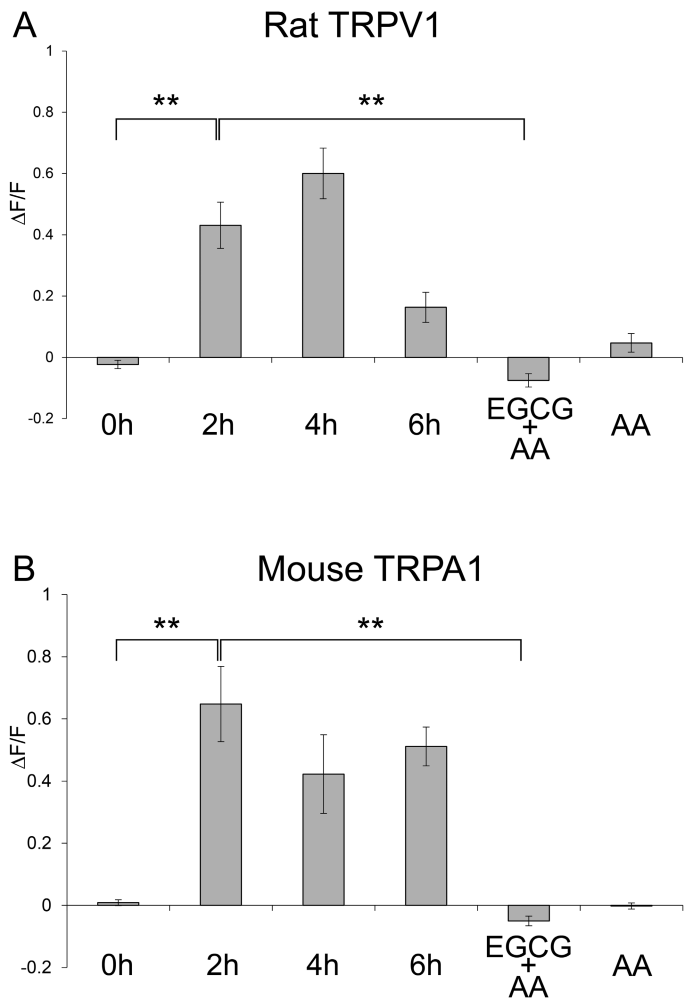

Figure 1 Auto-oxidized EGCG stimulates rat TRPV1 and mouse TRPA1 channels. (**A** and **B**) Effects of freshly prepared and incubated EGCG (200 μM) on $[Ca^{2+}]_i$ in HEK293T cells expressing rat TRPV1 (A) or mouse TRPA1 (B) were examined. After transfection with the expression vector, cells were loaded with 5 μM Fluo8-AM. The Fluo8 fluorescence was recorded every 3 s and the relative fluorescent change (Δ*F*/*F*) was determined. At 6 s, EGCG (fresh or incubated) was applied. At 240 s, 10 μM CAP (A) or 100 μM AITC (B) were further applied to the EGCG-containing bath solution to confirm the channel expression. EGCG (200 μM) in HBSS was freshly prepared (0 h), or prepared and incubated at 25 °C for 2, 4, and 6 h. EGCG (200 μM) in HBSS containing 1 mM ascorbic acid was prepared and incubated at 25 °C for 2 h (EGCG + AA). HBSS containing 1 mM ascorbic acid without EGCG (AA) was also prepared. They were used as a ligand solution. The average Δ*F*/*F* at 90 s was obtained and compared.

and 3B, right). In HEK293T cells expressing TRPV1, a significant increase in $[Ca^{2+}]_i$ was detected at 20 μM of the incubated EGCG. In TRPA1-expressing HEK293T cells, a major response of $[Ca^{2+}]_i$ was observed from 100 μM. Using Ca^{2+}-free HBSS as the bath solution, however, no increase in $[Ca^{2+}]_i$ was observed in HEK cells expressing TRPV1 nor TRPA1 (Supplementary Figure 3). Results suggested that the incubated EGCG induced the Ca^{2+} influx through TRP channels. For other 3 major catechins (EC, ECG, and EGC), the same experiments were performed and results are summarized in Figures 2B and 3B, right. When compared among the 4 catechins, it is evident that the incubated EGCG most effectively induced the $[Ca^{2+}]_i$ response in HEK293T cells expressing TRPV1 or TRPA1.

As a next step, we examined whether blockers for TRP channels might block the increase in $[Ca^{2+}]_i$ induced by EGCG. A TRPA1-specific blocker, 4-(4-Chlorophenyl)-3-methylbut-3-en-2-oxime (AP-18), and a TRPV1-specific blocker, SB-366791 (SB), were used. First, we confirmed that CAP-induced activation of TRPV1 was specifically blocked by SB, but not significantly by AP-18 (Figure 2C, left). We also confirmed that AITC-induced activation of TRPA1 was specifically blocked by AP-18, but not significantly by SB (Figure 3C, left). Then, as indicated in Figure 2C, right, SB completely blocked the $[Ca^{2+}]_i$ response in HEK293T cells expressing TRPV1 induced with the auto-oxidized EGCG, but AP-18 did not show a significant inhibitory effect. Further, in TRPA1-expressing HEK293T cells, AP-18 completely inhibited the increase in $[Ca^{2+}]_i$ (Figure 3C, right). Thus, it is evident that the $[Ca^{2+}]_i$ responses induced by the incubated EGCG are mediated through specific TRP channel activation.

Sensitivity of DRG neurons to auto-oxidized products of EGCG

It has been known that the nerve fibers in the tongue express TRPV1 (Ishida et al. 2002), and it has been suggested that TRPA1-positive nerve fibers are present in the tongue (Nagatomo and Kubo 2008). Primary afferent neurons are clustered in the DRG and within cranial nerve ganglions such as the trigeminal ganglion (TG). It has been shown that DRG and TG neurons express TRPV1, TRPA1, and TRPM8 (Kobayashi et al. 2005). It is of interest to know whether TRPV1 or TRPA1 channels in the nerves innervating the tongue are involved in the perception of the astringent taste of green tea. We examined the sensitivity of the acutely dissociated sensory DRG neurons to the auto-oxidized EGCG using the calcium-imaging technique. After the DRG was isolated from mice, neurons were dissociated and cultured. The freshly dissolved EGCG and the auto-oxidized EGCG were applied to DRG neurons at 200 μM. Time courses of individual cell recordings are shown in Figure 4A. The mean maximum response in individual neurons during the first 120 s stimulation was compared with control. When the fresh EGCG was used, no significant increase in $[Ca^{2+}]_i$ was observed. On the other hand, auto-oxidized EGCG induced a $[Ca^{2+}]_i$ response in DRG neurons. When EGCG was incubated in the presence of 1 mM ascorbic acid, the increase in $[Ca^{2+}]_i$ was not induced by the EGCG. Depletion of Ca^{2+} from the bath solution (HBSS) abolished the Ca^{2+} response in DRG neurons (Supplementary Figure 3, right). Further, the response of DRG neurons to the auto-oxidized EGCG

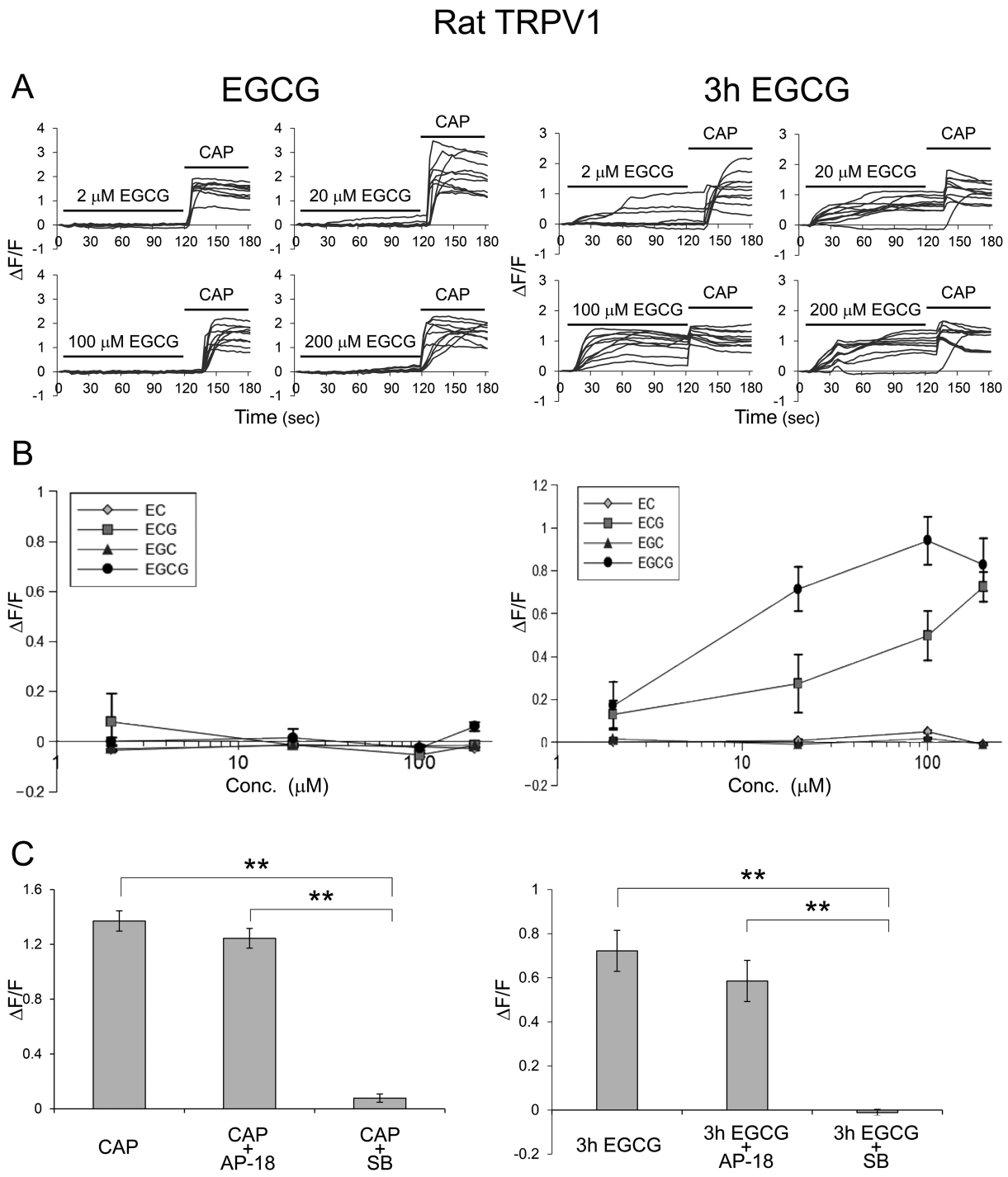

Figure 2 Effects of the incubated catechins on rat TRPV1 channels. (**A**) Effects of EGCG or incubated EGCG on $[Ca^{2+}]_i$ in HEK293T cells expressing rat TRPV1 were examined. HEK293T cells expressing rat TRPV1 were loaded with 5 μM Fluo8-AM. The Fluo8 fluorescence was recorded every 3 s and Δ*F*/*F* was determined. At 6 s, ligand was applied. At 120 s, 10 μM CAP was further added to the bath solution containing EGCG to monitor the channel expression. EGCG was prepared in HBSS containing 1 mM ascorbic acid (EGCG), or prepared in HBSS without 1 mM ascorbic acid and incubated at 25 °C for 3 h (3-h EGCG). They were used as a ligand solution. Time courses of Δ*F*/*F* of individual cell recordings were shown. (**B**) Effects of catechins or incubated catechins on $[Ca^{2+}]_i$ in HEK293T cells expressing rat TRPV1 were examined. The calcium-imaging analysis of HEK293T cells expressing rat TRPV1 was similarly performed as in A, except for ligand solutions. EC, EGC, ECG, or EGCG was prepared in HBSS containing 1 mM ascorbic acid (left), or prepared in HBSS without 1 mM ascorbic acid and incubated at 25 °C for 3 h (right). They were used as a ligand solution. The average Δ*F*/*F* at 90 s was obtained and plotted against the catechin concentration. (**C**) The calcium-imaging analysis of HEK293T cells expressing rat TRPV1 was performed, and effects of channel blockers on TRPV1 activation induced by the incubated EGCG were examined. First, the specificities of channel blockers were analyzed. The cells were activated singly by 0.1 μM CAP, 0.1 μM CAP with 100 μM AP-18, or 0.1 μM CAP with 0.1 μM SB-366791 (SB) at 6 s. The average Δ*F*/*F* at 90 s was obtained and compared (left). EGCG (20 μM) was prepared in HBSS and incubated at 25 °C for 3 h. This 20 μM EGCG (3-h EGCG) was singly, with 100 μM AP-18 or with 0.1 μM SB applied to cells at 6 s. The average Δ*F*/*F* at 90 s was obtained and compared (right).

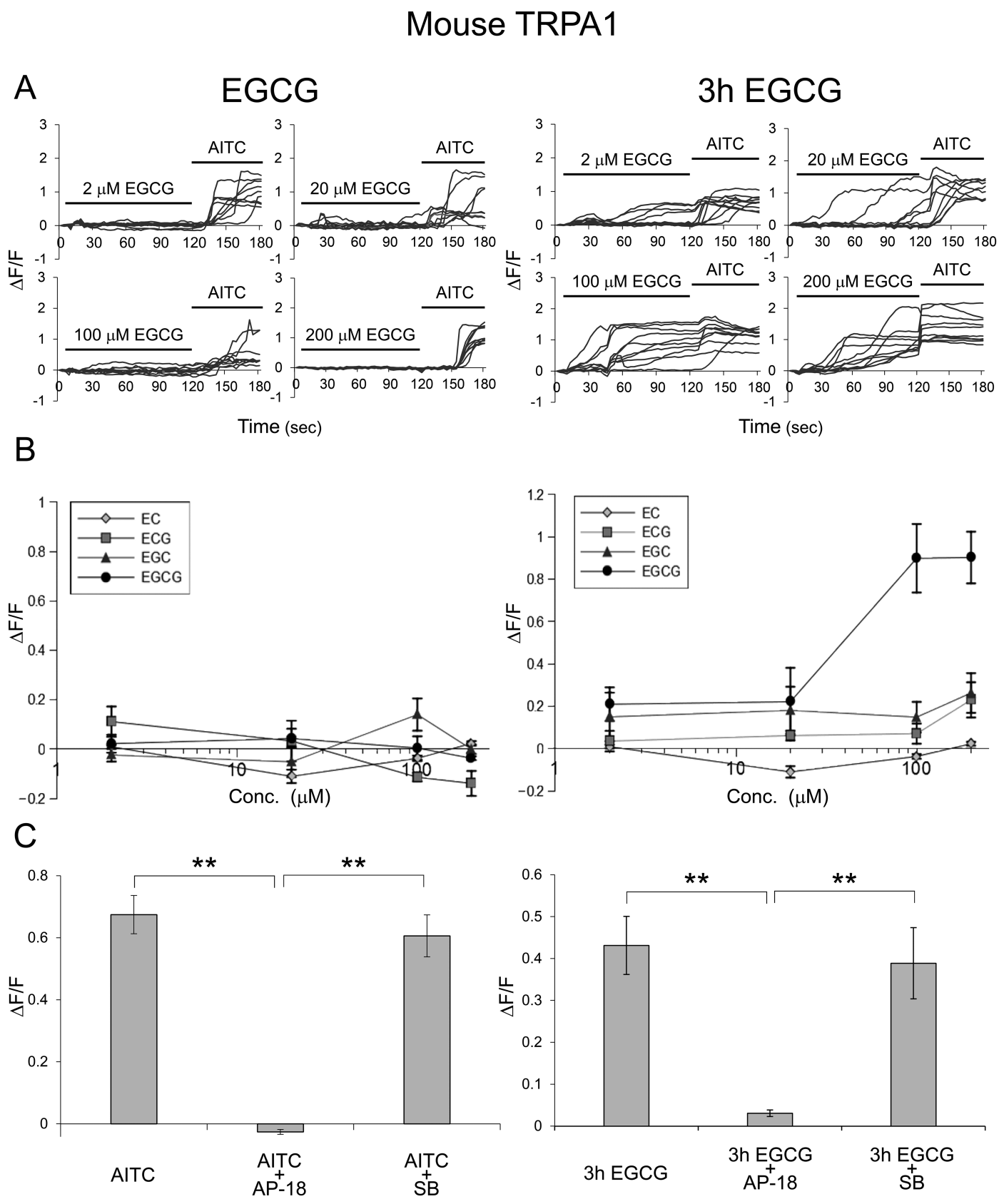

Figure 3 Effects of the incubated catechins on mouse TRPA1 channels. Data of mouse TRPA1. The experiments and presentations are almost same as those of rat TRPV1 in Figure 2. (**A** and **B**) AITC (100 μM) was used instead of 10 μM CAP. (**C**) AITC (10 μM) was used instead of 0.1 μM CAP and 100 μM 3-h EGCG was used instead of 20 μM 3-h EGCG.

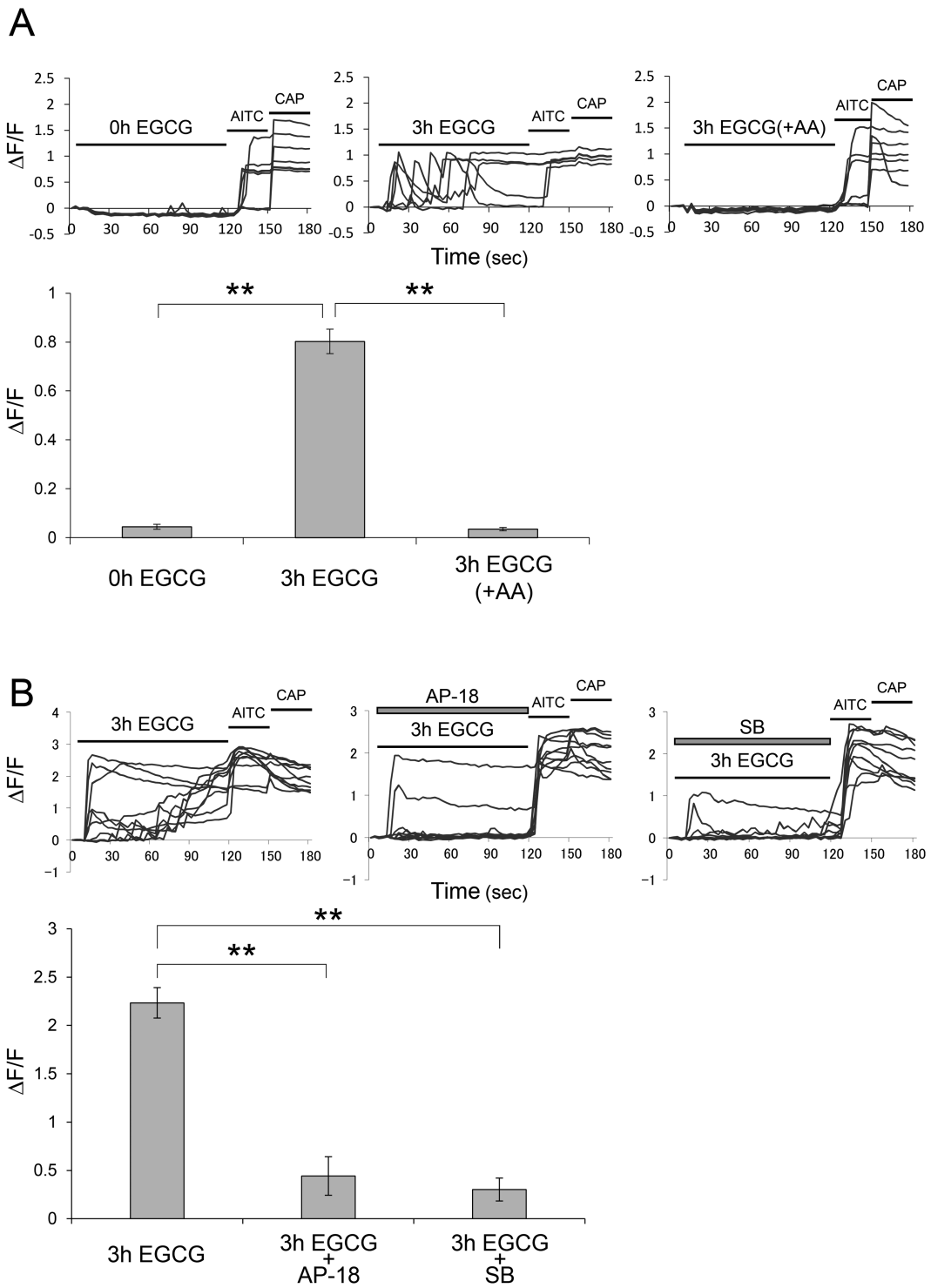

Figure 4 Effects of the incubated EGCG on mouse DRG neurons. (**A**) Ca^{2+} response of DRG neurons to the incubated EGCG was examined. DRG sensory neurons were isolated from mice and cultured. On culture day 1, the calcium-imaging analysis was performed. EGCG (200 μM) in HBSS was freshly prepared (0 h EGCG), or prepared and incubated at 25 °C for 3 h (3-h EGCG). EGCG (200 μM) in HBSS containing 1 mM ascorbic acid was prepared and incubated at 25 °C for 3 h (3-h EGCG [+AA]). They were used as a ligand solution at 6 s, 100 μM AITC was added to the bath solution containing EGCG at 120 s, and 10 μM CAP was further added at 150 s. Time courses of Δ*F*/*F* of individual cell recordings were shown. The average of the highest response in individual neurons during the first 120 s stimulation was obtained and compared. (**B**) Effects of blockers on the Ca^{2+} response of DRG neurons induced by the incubated EGCG were examined. On culture day 1 of DRG sensory neurons, the calcium-imaging analysis was performed. EGCG (200 μM) in HBSS was prepared and incubated at 25 °C for 3 h (3-h EGCG). EGCG (3 h) was mixed with 100 μM AP-18 or with 0.1 μM SB. They were used as a ligand solution at 6 s, 100 μM AITC was added to the bath solution at 120 s, and 10 μM CAP was further added at 150 s. The average of the highest response in individual neurons during the first 120 s stimulation was obtained and compared.

was significantly attenuated by AP-18 or SB (Figure 4B). Cross-talk between TRPA1 and TRPV1 might be present in DRG neurons. We further examined effects of the incubated EGCG on neurons not expressing TRPV1 or TRPA1. Dissociated neurons from cerebral hemisphere of 15-day mouse embryo were cultured. Although these cells were activated with glutamate, no clear response to CAP or AITC were observed. When these cells were exposed to the oxidized EGCG, no responses were observed (Supplementary Figure 4). These results clearly demonstrated that the responses to the auto-oxidized EGCG but not to EGCG itself were confirmed in DRG sensory neurons, and that they were considered to be mediated through TRPV1 and TRPA1 channels.

Preparation of an EGCG dimer, TS-A

Our results suggested that EGCG is auto-oxidized during the 3-h incubation in HBSS buffer, and only the resultant products can stimulate TRPV1 and TRPA1 channels. To study the biochemical changes in the EGCG solution, HPLC analysis was performed. Figure 5A shows the HPLC chromatogram of the freshly prepared EGCG. A large peak with the retention time of 11.7 min corresponded to EGCG. During the incubation for 3 h in HBSS, several additional peaks appeared in addition to the peak of EGCG on the HPLC chromatogram. High-resolution electrospray ionization–mass spectrometric (HRESI–MS) analysis demonstrated that 2 different EGCG dimers were present in the auto-oxidized products. One peak with the retention time of 10.0 min showed a molecular ion of *m/z* 913.1492 (ESI negative, $[M\text{-}H]^-$). Another peak with the retention time of 13.5 min also showed a molecular ion of *m/z* 913.1470 (ESI negative, $[M\text{-}H]^-$) (Figure 5B). Based on the calculated value ($C_{44}H_{33}O_{22}$ of 913.1463), the above 2 peaks were suggested to represent the isomeric dimers of EGCG, theasinensins A and D. This was further confirmed by LC–MS/MS analysis. The MS/MS spectra of their molecular ion $[M\text{-}H]^-$ were consistent with those of the reported TS-A (591.2, 743.0, and 761.1, Hong et al. 2002; Sang et al. 2005). LC-MS analysis further indicated that some dimmers with different structures might be also formed in the other 3 major catechin in HBSS during 3-h incubation (Supplementary Figure 5). The auto-oxidized products of EC, ECG, and EGC did not induce the high level of increase in $[Ca^{2+}]_i$ in the TRPV1- or TRPA1-expressing HEK293T cells, but only the incubated ECG was partially active to rat TRPV1 relative to EGCG (Figures 2B and 3B). Thus, it is possible that theasinensins present in the auto-oxidized products of EGCG might contribute to the activity to stimulate TRPV1 and TRPA1 channels. To examine this possibility, we synthesized and purified TS-A from EGCG (Figure 6) by the biomimetic method developed by Shii et al. (2011). On the HPLC chromatogram of the purified product, one major peak with the retention time of 10.0 min was observed. HRESI–MS analysis showed a molecular ion of *m/z* 913.1414 (ESI negative, $[M\text{-}H]^-$), which is the same as the calculated for TS-A, $C_{44}H_{33}O_{22}$ (913.1463). It was further confirmed by LC–MS/MS to be TS-A. Namely, the MS/MS spectra were similar to the reported spectrum of TS-A showing mass fragments of 591.2, 743.0, and 761.1 (Hong et al. 2002; Sang et al. 2005.). Thus, we successfully obtained highly pure TS-A. In addition, we clarified that on the HPLC chromatogram of the auto-oxidized EGCG, a peak with the retention time of 10.0 min in Figure 5B contained TS-A and another peak with the retention time of 13.5 min in Figure 5B corresponded to TS-D.

An EGCG dimer, TS-A, activates TRPA1 and TRPV1 channels

We next examined whether or not an EGCG dimer, TS-A, activates TRPA1 and TRPV1 channels. The expression vector carrying TRPA1 or TRPV1 was transfected into HEK293T cells and calcium-imaging analysis was performed using prepared TS-A (Figure 7). The increase in $[Ca^{2+}]_i$ was induced by TS-A in HEK293T cells expressing TRPV1 channels starting at 4 μM, and the response increased up to 200 μM (Figure 7B, left). On the other hand, in case of TRPA1, the $[Ca^{2+}]_i$ response was first detected at 2 μM TS-A. A significant but slow increase in $[Ca^{2+}]_i$ was observed at 4 μM, and the response decreased at 40 and 200 μM (Figure 7B, right). The relationship between the averages of the response at 90 s and the concentration of TS-A is shown (Figure 7B). Results suggested that a lower dose of TS-A more efficiently stimulates TRPA1 than TRPV1. Further, although it was not easy to conclude, based on results of calcium-imaging analysis, it seemed that TRPA1 might be inhibited by high concentration of TS-A. A similar bimodal sensitivity of TRPA1 to menthol was reported. Namely, low concentrations of menthol cause channel activation, but higher concentrations lead to channel block (Karashima et al. 2007). We next examined whether or not blockers for TRP channels might block the increase in $[Ca^{2+}]_i$ induced with TS-A. Although AP-18 had little effect, SB completely inhibited the $[Ca^{2+}]_i$ response induced with TS-A in HEK293T cells expressing TRPV1 (Figure 7C, left). Conversely, the response in TRPA1-expressing HEK293T cells evoked by TS-A was completely blocked by AP-18 (Figure 7C, right). SB did not decrease the response. These observations clearly demonstrate that EGCG itself cannot activate TRPV1 and TRPA1 channels, but that one of auto-oxidized products of EGCG, TS-A indeed activates both TRP channels.

TS-A activates DRG neurons

The synthesized and purified TS-A (80 μM) was applied to DRG neurons, and the responses were recorded by calcium-imaging analysis (Figure 8). Time courses of representative recordings are shown (Figure 8A). The averages of peak level of the responses in individual neurons during the first 120 s stimulation are shown with and without blockers (Figure 8B). The increase in $[Ca^{2+}]_i$

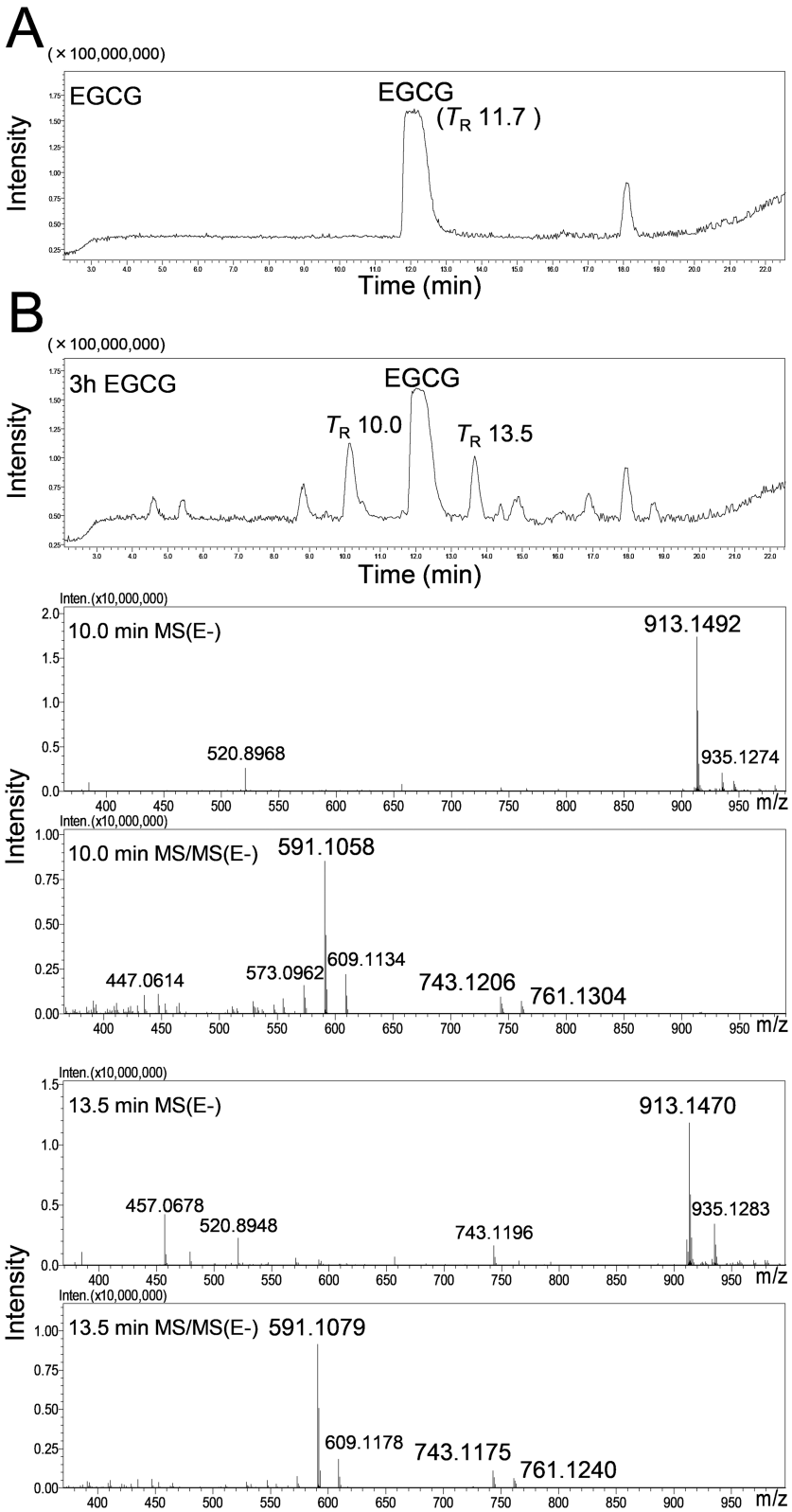

Figure 5 Identification of EGCG dimmers in the EGCG incubated for 3 h. (**A**) LC of EGCG. EGCG (4 mM) was dissolved in HBSS containing 1 mM ascorbic acid and analyzed by HPLC. (**B**) LC, MS, and MS/MS spectra of the incubated EGCG. EGCG (4 mM) was dissolved in HBSS, incubated for 3 h, and analyzed by LC–MS/MS. ESI-negative MS and MS/MS spectra of one major peak (T_R10.0) were shown. The peak with T_R10.0 showed a major molecular ion of *m/z* 913.1492. ESI-negative MS and MS/MS spectra of another major peak (T_R13.5) were also shown. The peak with T_R13.5 showed a major molecular ion of *m/z* 913.1470. Calculated exact mass of theasinensin A or D is 913.1463. These 2 peaks contained theasinensins A and D, isomeric dimers of EGCG.

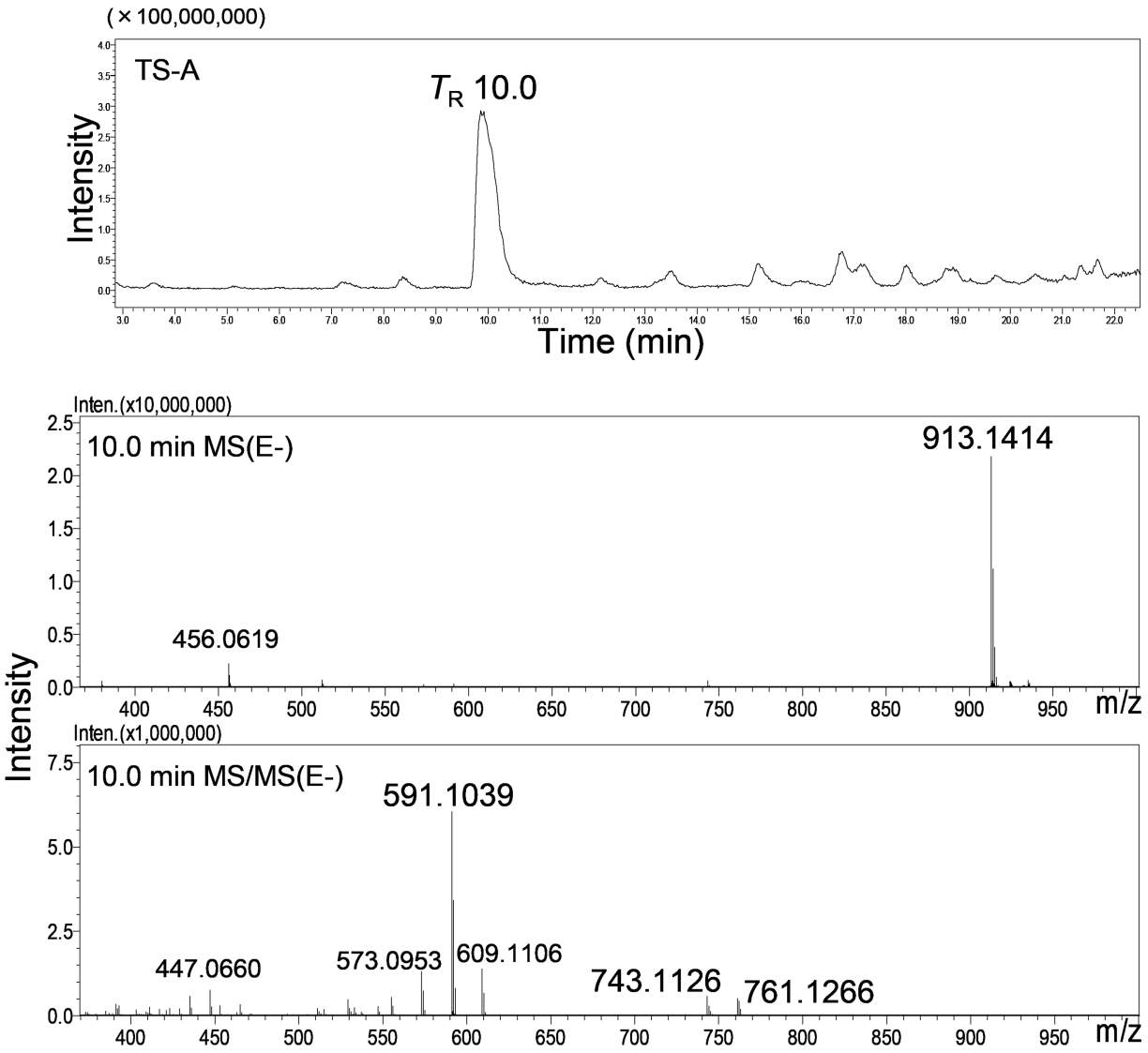

Figure 6 Preparation of TS-A. As described in Materials and methods, TS-A was synthesized and purified according to the method of Shii et al. (2011). The main peak from the preparative HPLC of the reaction mixture was analyzed by LC–MS/MS. ESI-negative MS and MS/MS spectra of the main peak (T_R10.0) were shown. The peak with T_R10.0 showed a major molecular ion of *m/z* 913.1414. Calculated exact mass of TS-A is 913.1463.

in several DRG neurons was apparently observed with TS-A. It was significantly inhibited by AP-18 and SB. In contrast, dissociated cultures of cerebral neurons, which do not contain CAP-responsive and AITC-responsive cells, were not activated by TS-A (Supplementary Figure 4). These results clearly show that TS-A, which is formed in the course of auto-oxidation of EGCG, activates DRG sensory neurons, and that this activation is mediated by TRPV1 and TRPA1 channels, providing an important information about the mechanism for astringent taste of food and beverages and one possible molecular explanation for sensing astringency of green tea after longer incubation.

Response of TRPV1 and TRPA1 channels from chick and snake

Mammalian TRPV1 is activated by the pungent vanilloid CAP (Caterina et al. 1997). On the other hand, it has been reported that a chick homolog of TRPV1 channel is insensitive to CAP (Jordt and Julius 2002). It has been shown that mammalian TRPA1 channels are not activated by heat, but snake TRPA1 channels are heat sensitive (Gracheva et al. 2010). Also, the diversity of channel properties among vertebrate TRPV1 and TRPA1 has been known (Nagatomo and Kubo 2008; Gracheva et al. 2010; Saito et al. 2012). Here, we studied whether or not TRPV1 and TRPA1 channels from chick and snake have sensitivity to the auto-oxidized EGCG. For this study, we cloned a chick TRPA1 cDNA. Recently, Saito et al. (2014) reported cloning and functional characterization of chick TRPA1. Their sequences of amino acids were identical. They demonstrated that TRPA1 responses to methyl anthranilate varied among 5 diverse vertebrate species. Here, the expression vector for chick TRPV1, chick TRPA1, rattlesnake TRPV1, or rattlesnake TRPA1 was transfected into HEK293T cells and calcium-imaging

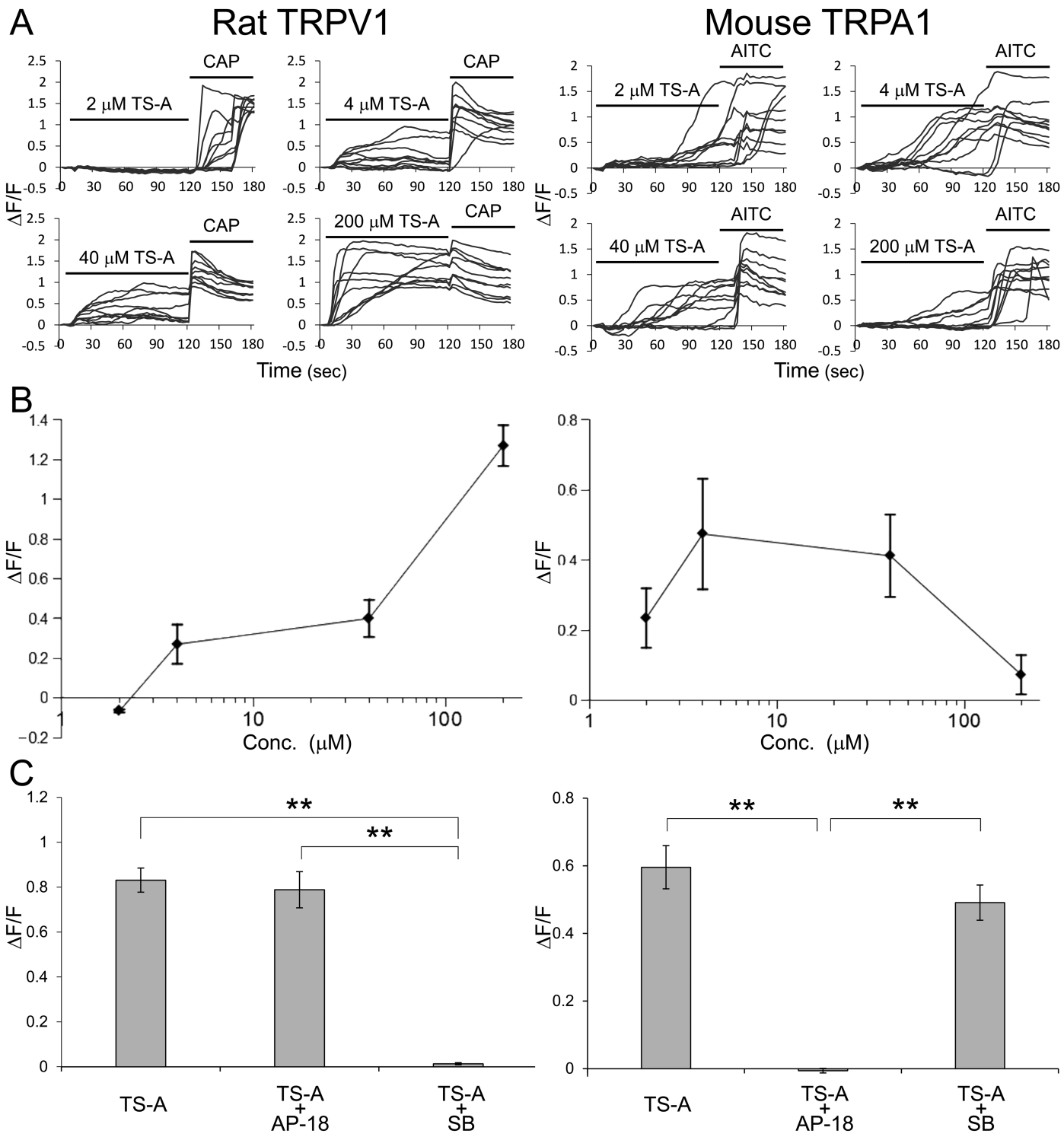

Figure 7 TS-A stimulates rat TRPV1 and mouse TRPA1 channels. (**A**) Effects of prepared TS-A on $[Ca^{2+}]_i$ in HEK293T cells expressing rat TRPV1 or mouse TRPA1 were examined. After transfection with the expression vector (rat TRPV1 or mouse TRPA1), the calcium-imaging analysis was performed and Δ*F*/*F* was determined. At 6 s, TS-A (2, 4, 4, and 200 μM) was applied. At 120 s, 10 μM CAP or 100 μM AITC was further added to the bath solution containing TS-A to confirm the channel expression. Time courses of Δ*F*/*F* of individual cell recordings were shown. (**B**) From results described A, the average Δ*F*/*F* at 90 s was obtained and plotted against the concentration of TS-A. (**C**) Effects of channel blockers on the Ca^{2+} response in rat TRPV1-expressing or mouse TRPA1-expressing cells induced by TS-A. To HEK293T cells expressing rat TRPV1, 200 μM TS-A was singly, with 100 μM AP-18 or with 0.1 μM SB applied to cells at 6 s. The average Δ*F*/*F* at 90 s was obtained and compared (left). To HEK293T cells expressing rat TRPA1, 40 μM TS-A was singly, with 100 μM AP-18 or with 0.1 μM SB applied to cells at 6 s. The average Δ*F*/*F* at 90 s was obtained and compared (right).

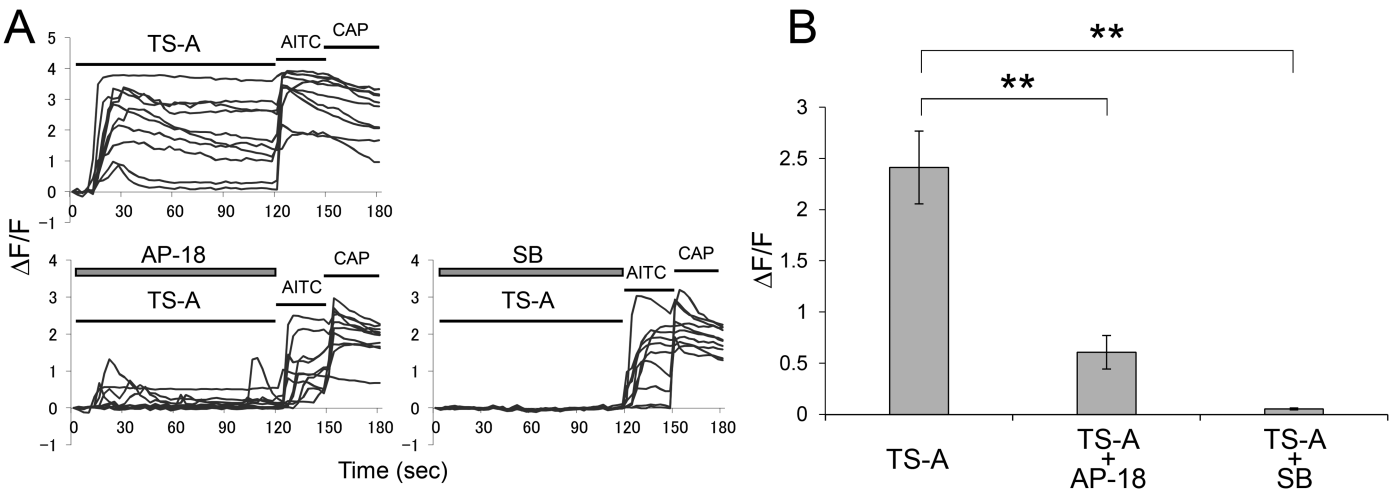

Figure 8 TS-A stimulates mouse DRG neurons. (**A**) Effects of prepared TS-A on $[Ca^{2+}]_i$ in DRG neurons were examined by the calcium-imaging analysis. At 6 s, 80 μM TS-A was singly, with 100 μM AP-18 or with 0.1 μM SB applied to cells. AITC (100 μM) was applied at 120 s, and 10 μM CAP was further added to the bath solution containing TS-A at 150 s. Time courses of Δ*F*/*F* of individual cell recordings were shown. (**B**) From results in A, the average of the highest response in individual neurons during the first 120 s stimulation was obtained and compared.

analysis was performed (Figure 9). The auto-oxidized products of major 4 catechins after 3-h incubation were used. The increase in $[Ca^{2+}]_i$ was observed only in HEK293T cells expressing chick TRPV1. Further, only the incubated EGCG induced a significant response. Results show that the sensitivity to the oxidized catechins of TRP channels is species specific, and that birds and reptilians have limited sensitivity to the astringent taste of oxidized polyphenols from plants.

To approach the molecular mechanism for the sensitivity of TRPV1 and TRPA1 to the oxidized EGCG and TS-A, we constructed chimera channels. The whole N-terminus cytoplasmic region containing ankyrin repeats was swapped between mouse and chick TRPA1 to produce the mouse–chick TRPA1 chimera (MC TRPA1) and the chick–mouse TRPA1 (CM TRPA1). Further, between rat and rattlesnake TRPV1, the whole N-terminus cytoplasmic region was swapped to obtain the snake–rat TRPV1 chimera (SR TRPV1) and the rat–snake TRPV1 (RS TRPV1). These 4 chimeras were expressed in HEK293T cells and calcium-imaging analysis was performed to examine the sensitivity to the oxidized EGCG or TS-A (Figures 10 and 11, respectively). MC TRPA1 responded to AITC, but TS-A could not activate MC TRPA1. On the other hand, CM TRPA1 could be activated by TS-A (Figure 10). Results show that the C-terminal part containing 6 transmembrane domains of mouse TRPA1 is necessary for the sensitivity to TS-A. In case of TRPV1, only SR TRPV1 could be expressed in HEK293T cells. The oxidized EGCG could activate SR TRPV1 (Figure 11). Since we could not detect the expression of RS TRPV1 with immunofluorescent staining using the antibody which recognizes the peptide (25–75 aa) present near the N-terminus of rat TRPV1 (SC-12498, Santa Cruz Biotechnology), it seems that RS TRPV1 protein could not be correctly folded and might be degraded in cells. Results show that the region providing the sensitivity to the oxidized EGCG is also located at the C-terminal part containing 6 transmembrane domains within rat TRPV1.

The TRPV1- and TRPA1-mediated ionic current response induced by the oxidized EGCG

To examine whether the oxidized EGCG may indeed affect the channel activity of TRPV1 and TRPA1, whole-cell patch-clamp recordings were performed. Rat TRPV1 was activated with the oxidized EGCG at 20 μM (Figure 12A, upper trace and middle panel), but the increases in the currents were variable. It might be partly due to the degree of oxidation of EGCG. Further, the induced currents by CAP (0.1 μM) were blocked with the oxidized EGCG at 200 μM (Figure 12A, lower trace, 64 ± 18%, n = 4). For activation of TRPA1, Ca^{2+}-imaging analysis indicated that TS-A is more potent than the oxidized EGCG. We examined effects of TS-A on the channel activity of mouse TRPA1 instead of the EGCG. Activation of mouse TRPA1 by 4 μM TS-A was observed in 6 out of 11 caffeine responded cells (Figure 12B, upper trace and middle panel). TS-A-induced currents did not correlate well with caffeine-induced currents. Some unknown condition(s) might be required for the response to TS-A. The inhibition of caffeine-induced currents was evident with 100 μM TS-A (Figure 12B, lower trace, 59 ± 11 %, n = 2). Results clearly indicated that the channel activity of rodent TRPV1 and TRPA1 is activated with the oxidized EGCG, but that the oxidized EGCG at higher concentration has an inhibitory effect on TRPV1 and TRPA1 channels. We further examined the channel activity with 2-electrode voltage clamp methods using *Xenopus* oocytes. The activation of TRPA1 nor TRPV1 channels with TS-A was not clearly observed, but we could detect the inhibitory effect of TS-A on both TRP channels (data not shown).

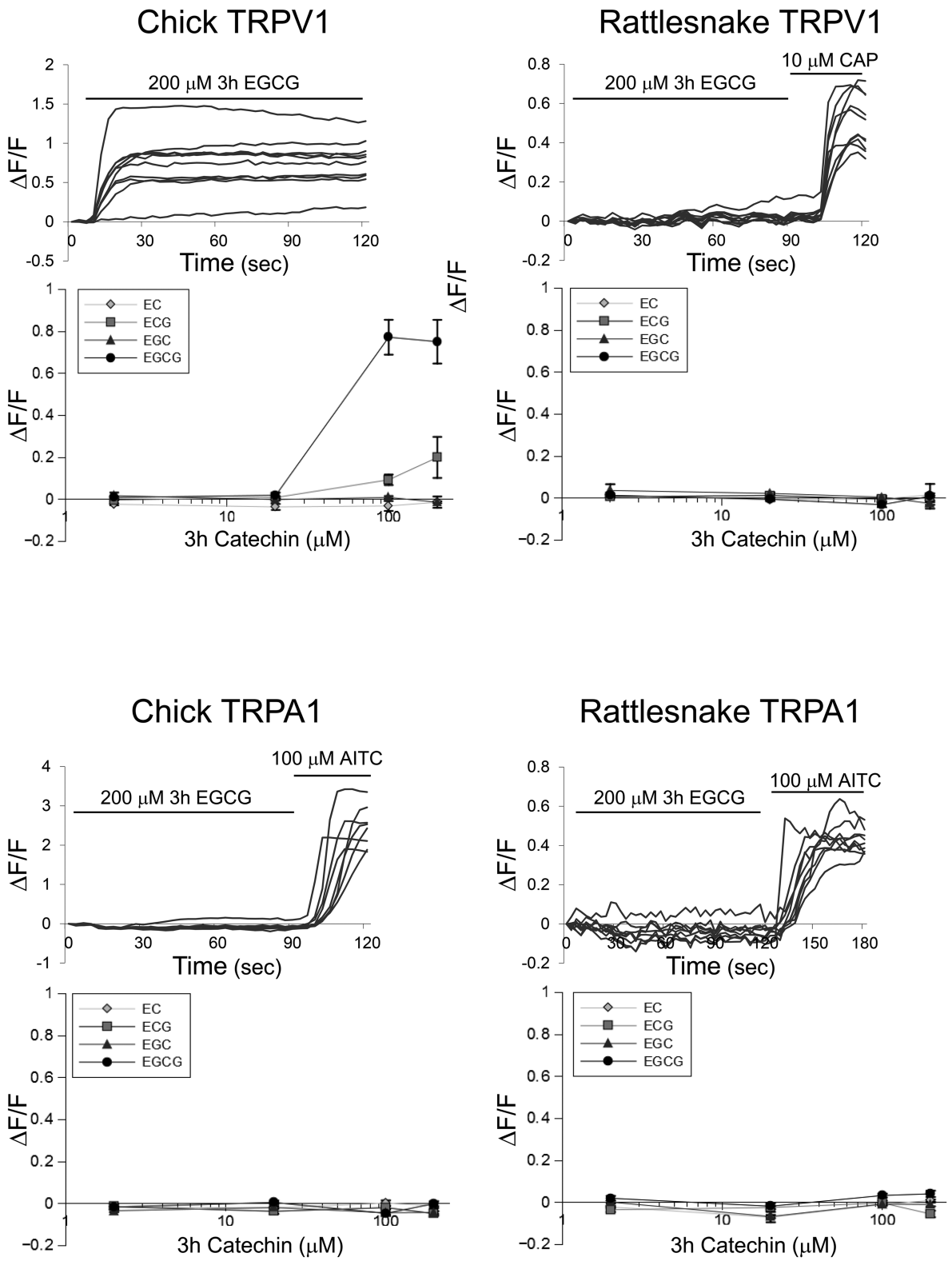

Figure 9 The incubated EGCG activates chick TRPV1 channels. Effects of incubated catechins on $[Ca^{2+}]_i$ in HEK293T cells expressing chick TRPV1, rattlesnake TRPV1, chick TRPA1, and rattlesnake TRPA1 were examined. After transfection with the expression vector, calcium-imaging analysis was performed. EC, EGC, ECG, or EGCG was prepared in HBSS and incubated at 25 °C for 3 h. They were used as a ligand solution at 6 s. Except for cells expressing chick TRPV1, 10 μM CAP or 100 μM AITC was further added to the bath solution containing the incubated catechins to confirm the channel expression. Result of calcium imaging for the incubated EGCG (200 μM) is indicated (upper). The average Δ*F*/*F* at 90 s was obtained and plotted against the catechin concentration (lower). Only chick TRPV1 channels were activated with the incubated EGCG.

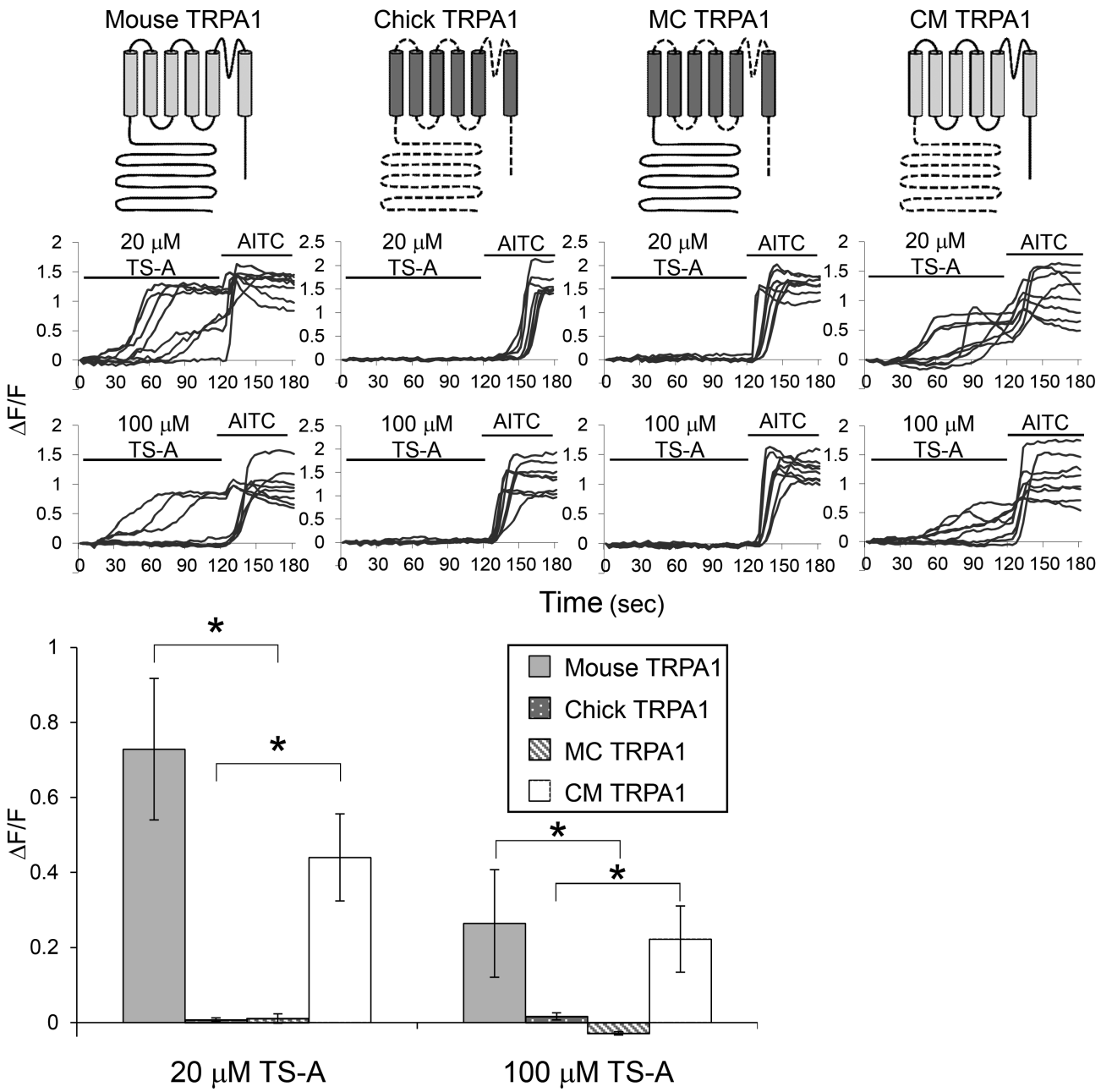

Figure 10 Necessity of the C-terminal transmembrane domains for the sensitivity to the oxidized EGCG of TRPA1 channels. The expression vectors of mouse–chick TRPA1 chimera (MC TRPA1) and chick–mouse TRPA1 chimera (CM TRPA1) were obtained as described in Materials and methods. After transfection with the expression vector (mouse TRPA1, chick TRPA1, MC TRPA1, CM TRPA1), calcium-imaging analysis was performed. TS-A was used as a ligand at 6 s. AITC (100 μM) was further added to the bath solution containing TS-A to confirm the channel expression. Results of calcium imaging for 20 μM and 100 μM TS-A are indicated upper. The average Δ*F*/*F* at 90 s of each construct is shown lower.

Discussion

In this study, we examined how TRPA1 and TRPV1 are activated by tea catechins in the course of auto-oxidation process. Neither TRPA1 nor TRPV1 could be activated by any of the freshly prepared catechins, but the incubated and auto-oxidized EGCG significantly activated both TRP channels. Furthermore, we observed that DRG neurons are activated not by nonincubated EGCG, but by the incubated EGCG through TRPV1 and TRPA1 channels.

In our previous report, we treated the cells expressing TRPA1 or TRPV1 with the EGCG solution and we observed the cellular activation using calcium-imaging technique. We interpreted that EGCG itself activated TRPA1 or TRPV1 channels. In these experiments, however, the ligands were dissolved in HBSS before loading Fluo8 to cells and the

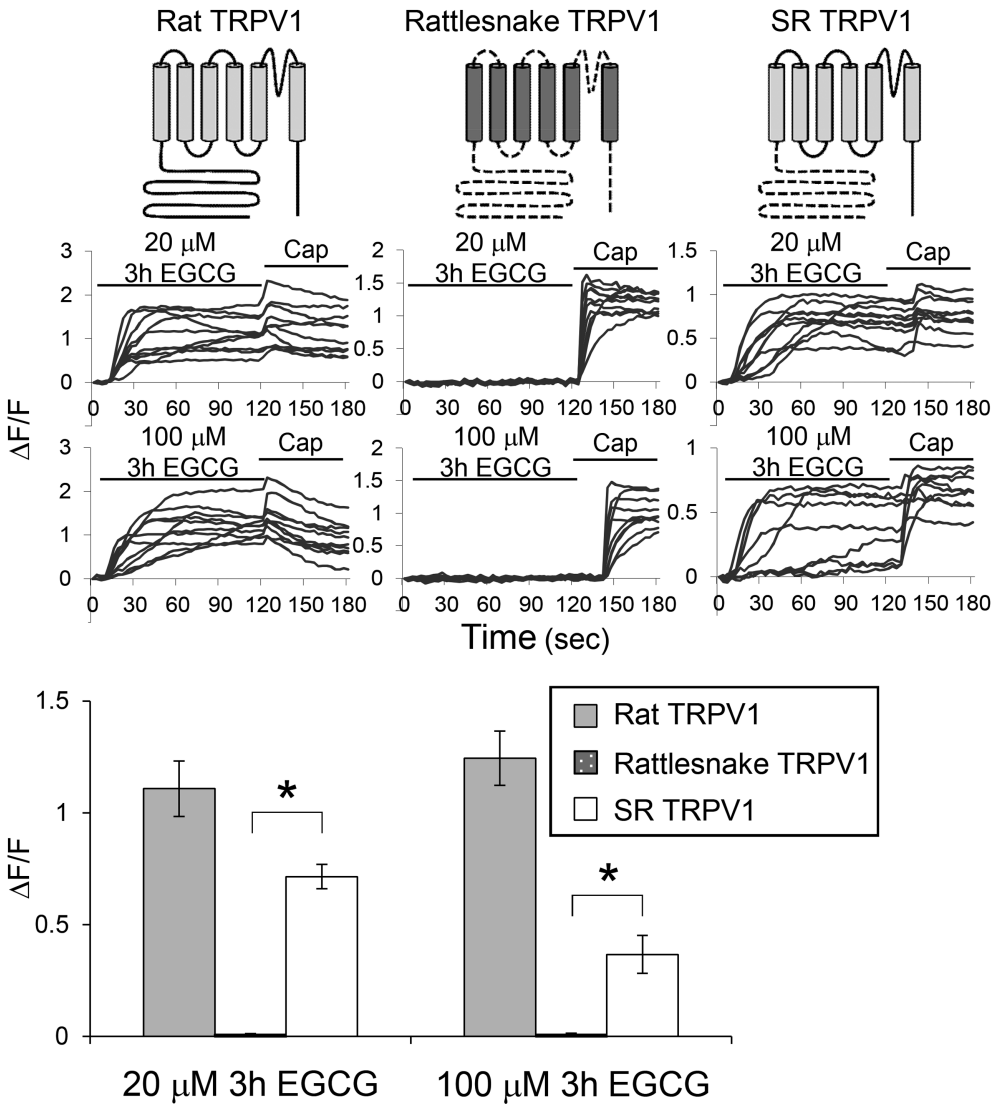

Figure 11 The C-terminal transmembrane domains of TRPV1 channels contribute to the sensitivity to the oxidized EGCG. Data of chimera TRPV1. The expression vectors (rat TRPV1, rattlesnake TRPV1, SR TRPV1, RS TRPV1) were used. The experiments and presentations are almost same as those of chimeras of TRPA1 in Figure 10. The 3-h-incubated EGCG was used as a ligand. CAP (10 μM) was used instead of 100 μM AITC to confirm the channel expression. RS TRPV1 could not be expressed.

solutions were kept for about 30 min before assay. From the present study, it is considered that EGCG started to be auto-oxidized during this preincubation, and that the solution containing auto-oxidized EGCG might be used. This may be the reason why the cellular activation was observed by this EGCG solution. In the present study, we observed that an antioxidant, AA, could completely cancel the incubation effect on EGCG, and that EGCG itself could not stimulate TRPA1 nor TRPV1 channels (Figure 1).

When examined using the heterologous expression system of HEK293T cells, the response of TRPV1 and TRPA1 induced with the incubated EGCG was apparently slow and gradually reached the maximum level. On the other hand, the responses of individual DRG neurons were sharply induced but not synchronized. The time points of the response of DRG cells were sometimes variable. Firing of DRG neurons are triggered by voltage-activated channels and are detected by Ca^{2+} imaging. Since the response of TRP channels to the incubated EGCG is very slow, the time to reach the threshold for firing is considered to differ in individual DRG neurons. Therefore, it appears that DRG neurons respond to the incubated EGCG at different time points.

We analyzed the contents in the incubated auto-oxidized EGCG by LC-MS and detected the presence of EGCG dimers, theasinensins. TS-A was purified and the activity to stimulate TRP channels was studied. TS-A, which is one of the auto-oxidized products of EGCG, was shown to activate TRPA1 and TRPV1. Further, TS-A activated DRG neurons by mediating through TRPV1 and TRPA1. TRPV1 is expressed in the nerves innervating the tongue (Ishida et al.

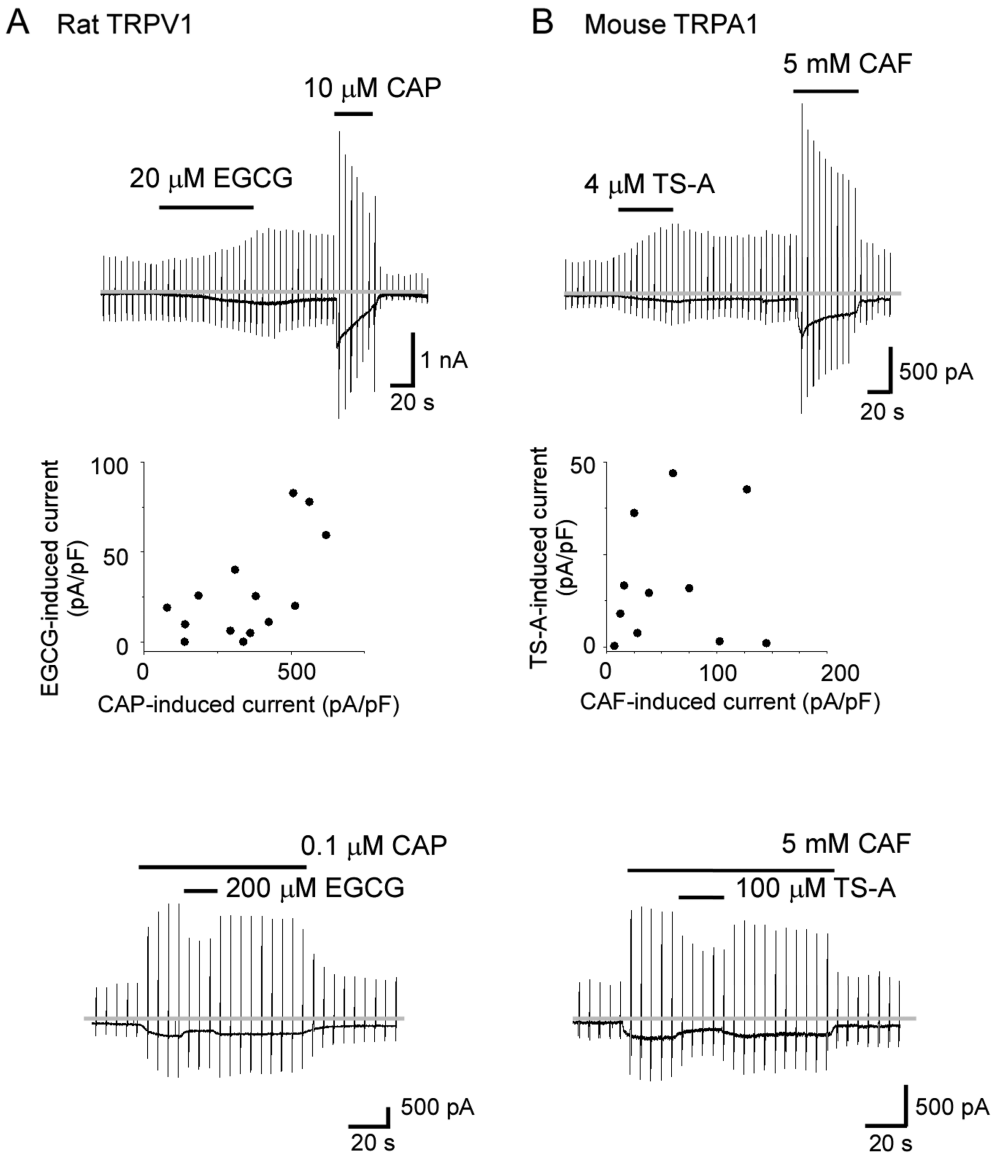

Figure 12 Effects of the oxidized EGCG on TRPV1 channels and effects of purified TS-A on TRPA1 channels. (**A**) Effects of oxidized EGCG on the rat TRPV1 channels were examined. The upper and lower current traces were recorded from cells expressing CAP-sensitive TRPV1 channels. EGCG was incubated in HBSS at 25 °C for more than 2 h. Application of the low concentration of the oxidized EGCG (20 μM) induced gradual increase in the outward current which was decreased upon the removal of the EGCG (upper trace). The EGCG-induced current density in each cell is plotted against the CAP-induced one (middle panel). High concentration of EGCG (200 μM) reversibly inhibited the TPRV1 channel activated by 0.1 μM CAP (lower trace). The zero current level is represented as the gray straight line. (**B**) Analyses of mouse TRPA1. The experiments and analyses are basically same as those of rat TRPV1 in (A). TS-A (4 μM) was applied instead of 20 μM EGCG, and 5 mM Caffeine was applied instead of 0.1 μM CAP.

2002), and TRPA1 is also suggested to be expressed in nerves on the tongue (Nagatomo and Kubo 2008). The scattered firing of sensory neuron induced by slow activation of TRPV1 and TRPA1 channels by the oxidized EGCG might be involved in the astringent sensations. Recently, Schöbel et al. reported that the activation of neurons of TG by EGCG itself is not dependent on TRP channels by using unspecific TRP blocker RR. Instead, they demonstrated that the EGCG-induced TG neuron activation requires a G protein-coupled signaling (Schöbel et al. 2014). Our present study indicated that EGCG itself could not stimulate TRPA1 nor TRPV1 channels and our results go with their report.

Auto-oxidized products of EGCG other than TS-A

When the incubated EGCG was applied to HEK293T cells expressing TRPA1, a significant increase in $[Ca^{2+}]_i$ was observed starting at 100 μM (Figure 3B). When purified TS-A was used, the $[Ca^{2+}]_i$ response was first detected at 2 μM and significantly induced at 4 μM (Figure 7B). These results indicated that TS-A is one of the oxidized products which are formed during the 3-h incubation and selectively activates TRPA1 channels. At higher concentration of TS-A, the $[Ca^{2+}]_i$ response of TRPA1 was reduced. This observation was considered to correlate with the inhibition

of caffeine-induced currents by 100 μM TS-A, detected by electrophysiological analysis (Figure 12B). In the case of TRPV1, 20 μM of the oxidized EGCG induced an apparent increase in $[Ca^{2+}]_i$ and the decreased response was observed with 200 μM of the EGCG in TRPV1-expressing HEK293T cells (Figure 2B). On the other hand, purified TS-A induced the $[Ca^{2+}]_i$ response in TRPV1-expressing HEK293T cells, but the high level response was induced only by 200 μM TS-A (Figure 7B). Thus, the sensitivity of TRPV1 to TS-A was not so high. Further, since the TS-A dose dependence of TRPV1 looked nonlinear, it is possible that multiple TS-A-recognizing sites (high and low affinity) might be present in rat TRPV1. From the limited activation effect, we think that TS-A is not the only oxidized product which is formed from EGCG during the 3-h incubation and efficiently activates TRPV1 channels. Some oxidized and effective product(s) other than TS-A could be present in the 3-h EGCG solution. Although further investigation is required, TS-D, another dimer of EGCG, might be a candidate.

Cross-talk between TRPV1 and TRPA1

AP-18 specifically and completely inhibited $[Ca^{2+}]_i$ increase in HEK293T cells expressing TRPA1, and SB specifically and completely inhibited $[Ca^{2+}]_i$ increase in HEK293T cells expressing TRPV1. In DRG neurons, it is reported there are TRPV1- and TRPA1-expressing neurons, TRPV1- and TRPM8-expressing neurons, and TRPA1- and TRPM8-expressing neurons (Kobayashi et al. 2005). Even in the presence of a TRPA1 channel-specific blocker, it was expected that DRG neurons expressing TRPV1 can respond to the auto-oxidized EGCG or TS-A. However, when DRG neurons were treated with a TRPA1 channel-specific blocker, AP-18, cellular response induced by the auto-oxidized EGCG or TS-A was greatly inhibited (Figures 4 and 8). It has been demonstrated that TRPV1 and TRPA1 channels assemble into a complex on the plasma membrane, and that they mutually control the transduction of inflammation-induced noxious stimuli in sensory neuron (Akopian et al. 2007, 2008; Staruschenko et al. 2010). Therefore, by interaction between TRPA1 and TRPV1 channels, it seems that the treatment with AP-18 might indirectly attenuate TRPV1 channels in DRG neurons coexpressing TRPA1, and that a TRPV1 blocker, SB might function similarly on TRPV1 and TRPA1 channels.

The activation mechanisms of TRPV1 and TRPA1 by auto-oxidized EGCG

It has been known that several agonists activate TRPA1 channels through differential modification of cysteine residues in the cytoplasmic N-terminal region (Hinman et al. 2006; Takahashi et al. 2008; Takahashi and Mori 2011). Further, it has been shown that several regulatory factors activate TRPV1 channels by modification of cysteine residues located between the fifth and sixth transmembrane domains (Yoshida et al. 2006; Takahashi and Mori 2011). It is possible that the oxidized EGCG products might modify or bind to specific residues to activate TRPV1 or TRPA1 channels. Where is the region containing such critical residues recognizing the oxidized EGCG on TRPA1 and TRPV1 channels? Our results indicated that chicken TRPV1 can be activated with the incubated EGCG. Chick TRPV1 is known to be insensitive to CAP and the sensitivity to CAP is reported to be ascribed to 8 amino acids in the vicinity of transmembrane 3 that differ between chick and mammalian TRPV1s (Jordt and Julius 2002). The recognition mechanism of CAP using these amino acids may not be utilized for the sensitivity to auto-oxidized EGCG. To approach the molecular mechanism to activate these TRP channels by the oxidized EGCG and TS-A, we performed studies using chimeras between the oxidized EGCG-sensitive and insensitive TRP channels. Results showed that the region providing the sensitivity to the oxidized EGCG is located at the C-terminal part containing 6 transmembrane domains within TRPA1 and TRPV1. We observed that the N-terminal cytoplasmic region containing ankyrin repeats of TRP channels could not provide the channel with the sensitivity to the oxidized EGCG by itself. Results did not deny the possibility that the other domains than the transmembrane domains might have some supportive function in the sensitivity to the oxidized EGCG. To understand the molecular mechanism for the recognition of the oxidized EGCG and TS-A, further detailed studies using mutants of TRPV1 and TRPA1 channels are required. On the other hand, by electrophysiological analysis, we found that the oxidized EGCG at high concentration has an inhibitory effect on TRPV1 and TRPA1 channels in addition to channel activation by the oxidized EGCG. It has been reported that similar bimodal sensitivity of mouse TRPA1 to menthol (Karashima et al. 2007), and it has been further demonstrated that the transmembrane domain 5 determines menthol sensitivity of TRPA1 (Xiao et al. 2008). Similar mechanism might be used for the sensitivity to oxidized EGCG of TRPA1 and TRPV1 channels.

In conclusion, this study shows that the oxidized EGCG, a compound accumulated in the incubated green tea inducing astringent taste, especially TS-A, activates mammalian TRPV1 and TRPA1 channels expressed in sensory neurons. Our findings may help to understand the neural correlates and cellular mechanisms of astringency perception.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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