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Green Tea Polyphenol Epigallocatechin Gallate Activates TRPA1 in an Intestinal Enteroendocrine Cell Line, STC-1

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Abstract

A characteristic astringent taste is elicited by polyphenols. Among the polyphenols, catechins and their polymers are the most abundant polyphenols in wine and tea. A typical green tea polyphenol is epigallocatechin gallate (EGCG). Currently, the mechanism underlying the sensation of astringent taste is not well understood. We observed by calcium imaging that the mouse intestinal endocrine cell line STC-1 responds to the astringent compound, EGCG. Among major catechins of green tea, EGCG was most effective at eliciting a response in this cell line. This cellular response was not observed in HEK293T or 3T3 cells. Further analyses demonstrated that the 67-kDa laminin receptor, a known EGCG receptor, is not directly involved. The Ca^{2+} response to EGCG in STC-1 cells was decreased by inhibitors of the transient receptor potential A1 (TRPA1) channel. HEK293T cells transfected with the mouse TRPA1 (mTRPA1) cDNA showed a Ca^{2+} response upon application of EGCG, and their response properties were similar to those observed in STC-1 cells. These results indicate that an astringent compound, EGCG, activates the mTRPA1 in intestinal STC-1 cells. TRPA1 might play an important role in the astringency taste on the tongue.

Key words: astringency, catechin, intestine, STC-1, taste

Introduction

Tastants are detected mainly by taste receptor cells (TRCs) in taste buds on the tongue. Among the 5 basic taste stimuli, sweet, umami, and bitter taste are recognized by G protein–coupled receptors (Chandrashekar et al. 2000, 2006; Nelson et al. 2001, 2002; Ishimaru 2009). As a candidate sour taste receptor, the heteromer of transient receptor potential (TRP) channels (PKD1L3 and PKD2L1) has been identified (Huang et al. 2006; Ishimaru et al. 2006). In the case of salty taste, epithelial Na^+ channels have been identified as amiloride-sensitive salty receptors and are considered to play a role at least partly (Chandrashekar et al. 2006; Ishimaru 2009). In addition to the 5 basic taste stimuli, the pungent stimulation of hot peppers is also recognized in the mouth. This pungent taste is mainly mediated by TRPV1 receptors, which can be activated by capsaicin from pepper and are expressed in TRCs and sensory neurons in the oral cavity (Ishida et al. 2002). Furthermore, in beverages, such as tea, cider, and red wine as well as in several types of fruits, nuts, and chocolate, a characteristic astringent taste is elicited primarily by compounds known as polyphenols. Of these polyphenols, catechin, epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), and their polymers are most abundant in wine and tea. A typical green tea polyphenol is EGCG (Drewnowski and Gomez-Carneros 2000; Lesschaeve and Nobel 2005). Although recent reports demonstrated that a bitter taste receptor, hTAS2R39, is an oral sensor of EGCG (Slack et al. 2010; Narukawa et al. 2011), the mechanism underlying the sensation of astringent taste is not well understood.

Green tea has been shown to have anticancer activity in many organs (Yang et al. 2006; Bettuzzi et al. 2006). Among constituents of green tea, EGCG is the major polyphenol and

exhibits the greatest cancer-preventive effects (Chung et al. 1999; Saeki et al. 2000). Recently, Tachibana et al. (2004) have found that the 67-kDa laminin receptor (67LR) functions as a cell surface EGCG receptor inducing anticancer action. 67LR is a nonintegrin-type laminin receptor and expressed on a variety of tumor cells. Furthermore, EGCG has been shown to induce the disruption of actin fibers and the dephosphorylation of the myosin II regulatory light chain through the 67LR to inhibit the growth of cancer cells (Umeda et al. 2005). Because activation of 67LR with EGCG does not influence the intracellular Ca^{2+} level (Fujimura et al. 2006), it seems that the EGCG signaling using 67LR may not induce the astringent sensation in sensory terminals in the oral cavity. Other receptor molecule for EGCG must be present as an astringent sensor on the tongue.

In addition to the gustatory system, chemosensory information perceived during the gastric and intestinal phases of digestion is important for the control of gastrointestinal (GI) function, such as the secretory activity of GI glands, the resorptive activity, motility and blood supply of the intestinal tract, and satiation (Dockray 2003). The enteroendocrine cells are specialized transducers of luminal factors. STC-1 cells were established in 1990 as a line of enteroendocrine cells (Rindi et al. 1990). A decade later, Wu et al. (2002) reported that STC-1 cells express T2R bitter taste receptors and respond to bitter taste substances. We also characterized the bitter taste responses of STC-1 cells (Masuho et al. 2005). Then, we recently investigated the cellular responses of intestinal STC-1 cells to compounds of 5 basic tastants using a calcium-imaging technique. Although this cell line was known to respond to bitter compounds, we found that compounds of 4 other basic tastants also stimulated STC-1 cells. When solutions containing glutamate, sucrose, HCl, or NaCl were applied, the intracellular Ca^{2+} concentration in STC-1 cells significantly increased. Therefore, we demonstrated that the GI system can sense all 5 of the basic taste stimuli and that it might contain a taste receptor signaling system similar to the oral taste system (Saitoh et al. 2007). The expression of T1R taste receptors in the gut cells has also been reported by Dyer et al. (2005) and Margolskee et al. (2007).

Here, we investigated whether the intestinal STC-1 can respond to the astringent compound of green tea, EGCG, by the calcium-imaging technique. Interestingly, the results clearly indicated that STC-1 cells have a novel sensor for EGCG, which has not been described. When EGCG was applied to STC-1, a significant increase in the intracellular Ca^{2+} concentration occurred. This cellular response was not observed in HEK293T or 3T3 cells, both of which express 67LR. Using some channel blockers, we focused on members of the TRP channels and found that mouse TRPA1 (mTRPA1) is utilized in the EGCG-induced Ca^{2+} response in STC-1 cells. Then, we characterized the responding properties of heterologously expressed mTRPA1 to EGCG in HEK293T cells.

Materials and methods

Materials

(–)-epigallocatechin-3-gallate (EGCG), (–)-epicatechin (EC), (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC), sodium L-glutamate (Glu-Na), menthol, capsaicin, and sodium saccharin were from Wako. Caffeine, ruthenium red (RR), and $GdCl_3$ were from Sigma-Aldrich. AP-18 and HC-030031 were from Enzo Life Sciences. Denatonium benzoate was from Fluka (via Sigma-Aldrich). Fluo8 acetoxymethyl ester (Fluo8-AM) was from AAT Bioquest, and Rhodamine-phalloidin was from Molecular Probes (via Invitrogen). Growth Factor Reduced MATRIGEL Matrix (Matrigel) was from Becton Dickinson. The STC-1 cell line was a gift from Dr D. Hanahan (University of California, San Francisco, CA). The expression vector for mTRPA1 was previously described (Nagatomo and Kubo 2008), and the vectors for rat TRPM8 and rat TRPV1 were provided by Dr D. Julius (University of California, San Francisco).

Culture and calcium-imaging analysis of STC-1 cells

A culture medium consisting of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 μg/mL kanamycin) was used for STC-1 and HEK293T cells. For 3T3 cells, newborn calf serum was added to the culture medium instead of FBS. For calcium-imaging analysis, cells grown on a Matrigel-coated μ-Slide 8 well (80826, ibidi, MPI für Infektionsbiologie) were washed with Hanks' balanced salts solution (HBSS; Sigma-Aldrich) and then incubated in HBSS containing 5 μM Fluo8-AM for 30 min at room temperature. Cells were then washed with HBSS and left at room temperature for an additional 30 min to allow cleavage of the AM ester. Each recording chamber was filled with 150 μL of HBSS. To achieve abrupt changes in ligand concentration, 150 μL of 200% concentrated ligand solution was applied by pipette. $[Ca^{2+}]_i$ was monitored at 470 nm Fluo8 emission excited by illumination at 525 nm using Axiovert 200 (Carl Zeiss). Fluo8 fluorescence was recorded usually every 3 s, and changes of fluorescence intensity were analyzed by Image-Pro Plus imaging software (Media Cybernetics). The mean fluorescence from at least 5 cells was obtained, and the signals were expressed as the relative change in fluorescence:

$$\Delta F/F = (F - F_0)/F_0.$$

All calcium-imaging experiments were repeated 2 or 3 times.

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 experiments for the expression

of TRPA1, cells were incubated in 3 μM RR for 24–48 h, then washed with HBSS, and used for the calcium imaging.

Reverse transcriptase–polymerase chain reaction assay

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen) and subjected to reverse transcription with random primers. The reverse-transcribed cDNA was used as a template for polymerase chain reaction (PCR). Total RNA treated under the same conditions without reverse transcriptase was used as a negative control. The primers used were as follows: for mouse 67-kDa laminin receptor, 5′-TAAACCTGAAGAGGACCTGG-3′ and 5′-GGTCCATTCACCCTGGAATT-3′; for mTRPA1, 5′-CATCTTCGTGTTGCCCTTGT-3′ and 5′-AAAAACCGTAGCATCCTGCC-3′; for human TRPA1, 5′-CATTTTTGTGCTGCCCTTGT-3′ and 5′-GGAATAACATCCCACCAGA-3′; for mouse TRPV1, 5′-TCAGCCATCGCAAGGAGTAT-3′ and 5′-CAGTTCACCTCATCCACCCT-3′; for human TRPV1, 5′-TCA GCCACCTCAAGGAGTAT-3′ and 5′-TTCACCTC GTCCACCCTGAA-3′; for mouse TRPM8, 5′-ACTG CAACCGCCTAAACATC-3′ and 5′-TCGTGGGA AAGGAGTGTCAA-3′; and for human TRPM8, 5′-ACTGCAGCCGCCTCAATATC-3′ and 5′-GGAAAAAGGAG GCGGTAAGA-3′.

PCR products were analyzed on 1% agarose gels.

Fluorescent staining for actin fibers

Cells were fixed for 20 min in 4% paraformaldehyde in PBS at room temperature, washed with PBS, and permeabilized with 0.5% TritonX-100 in PBS. Then, cells were stained with Rhodamine-phalloidin for 1 h at room temperature, washed with PBS, and mounted with Prolong Gold antifade reagent (Invitrogen). The specimens were observed and recorded with an Axiovert 200 microscope equipped with phase contrast and epifluorescence optics.

Results

Green tea polyphenol EGCG can stimulate intestinal STC-1 cells

We previously demonstrated that intestinal STC-1 cells can sense all of the 5 basic taste stimuli and that a taste receptor signaling mechanism similar to the oral taste system might be present (Saitoh et al. 2007). Here, we investigated whether this intestinal STC-1 might be able to respond to the major astringent compound of green tea, (–)-epigallocatechin-3-gallate (EGCG), by means of the calcium-imaging technique. Approximately 1.5 mM EGCG is known to be present in standard green tea (Wang et al. 1992; Wolfram 2007). Addition of 200 μM EGCG to cultures of STC-1 cells, loaded with the fluorescence Ca^{2+} indicator Flou8-AM, induced a significant but relatively slow elevation in $[Ca^{2+}]_i$. The responses to EGCG of STC-1 cells were dose dependent, and quite low activation was detected at 20 μM EGCG (Figure 1A). On the other hand, when 3T3 or HEK293T cells were stimulated by 200 μM EGCG, no significant calcium elevation was observed (Figure1C,D). EGCG is the most abundant green tea polyphenol. In addition to EGCG, EC, ECG, and EGC are also present and generally known as tea catechins. The effects of these catechins on the intracellular levels of Ca^{2+} of STC-1 cells were further examined. As shown in Figure 1E, each of these catechins induced a different level of stimulation of STC-1 cells at 200 μM. The order of potency of these catechins was EGCG > EGC > ECG > EC. The results indicated that EGCG was the most effective at inducing a Ca^{2+} response in STC-1 cells.

67LR-mediated signaling is not involved in the EGCG response of STC-1

Among the green tea constituents, EGCG is the most active constituent in inhibiting experimental carcinogenesis and related reactions. 67LR has been shown to function as a cell surface EGCG receptor to mediate the anticancer action of EGCG (Tachibana et al. 2004). Activation of 67LR by EGCG induces the disruption of stress fibers with actin cytoskeleton rearrangement and growth inhibition in cancer cells (Umeda et al. 2005, 2008). We next investigated whether 67LR-mediated signaling is involved in the EGCG response observed in intestinal STC-1 cells. First, we examined the expression of 67LR messenger RNA (mRNA) by reverse transcriptase–PCR (RT-PCR) analysis. Total RNA was isolated from STC-1, HEK293T, and 3T3 cells, and the reverse-transcribed cDNA with random primers was used as a template for PCR. As shown in Figure 2A, 67LR mRNA appeared to be expressed in all cell lines examined.

We further studied the effects of EGCG on the actin cytoskeleton. To visualize the distribution of F-actin structures, fluorescently labeled phalloidin was utilized. As a control, we first examined HEK293T cells. We observed that HEK293T cells showed no significant elevation of $[Ca^{2+}]_i$ after the treatment with EGCG and that 67LR mRNA was expressed in this cell line. When HEK293T cells were treated with 200 μM EGCG, disappearance of actin fibers in the central body of cells occurred 2 min after the addition of EGCG, and only the cell–cell junctions were weakly visible at 4 min after the EGCG addition. On the other hand, when STC-1 cells were treated with 200 μM EGCG, interestingly, actin fibers were newly formed 2 min upon treatment with EGCG and the fiber formation continued for at least 4 min. As a result, the intensity of phalloidin staining in STC-1 cells appeared to increase after the EGCG treatment (Figure 2B). This effect on actin fibers was observed from 50 μM EGCG. Thus, we found that the effects of EGCG on the actin cytoskeletal structures were completely opposite in HEK293T and STC-1 cells, suggesting that the 67LR-mediated signaling observed in many cancer cells might not be involved in the mechanism underlying the EGCG-induced response of intracellular Ca^{2+} elevation in STC-1 cells.

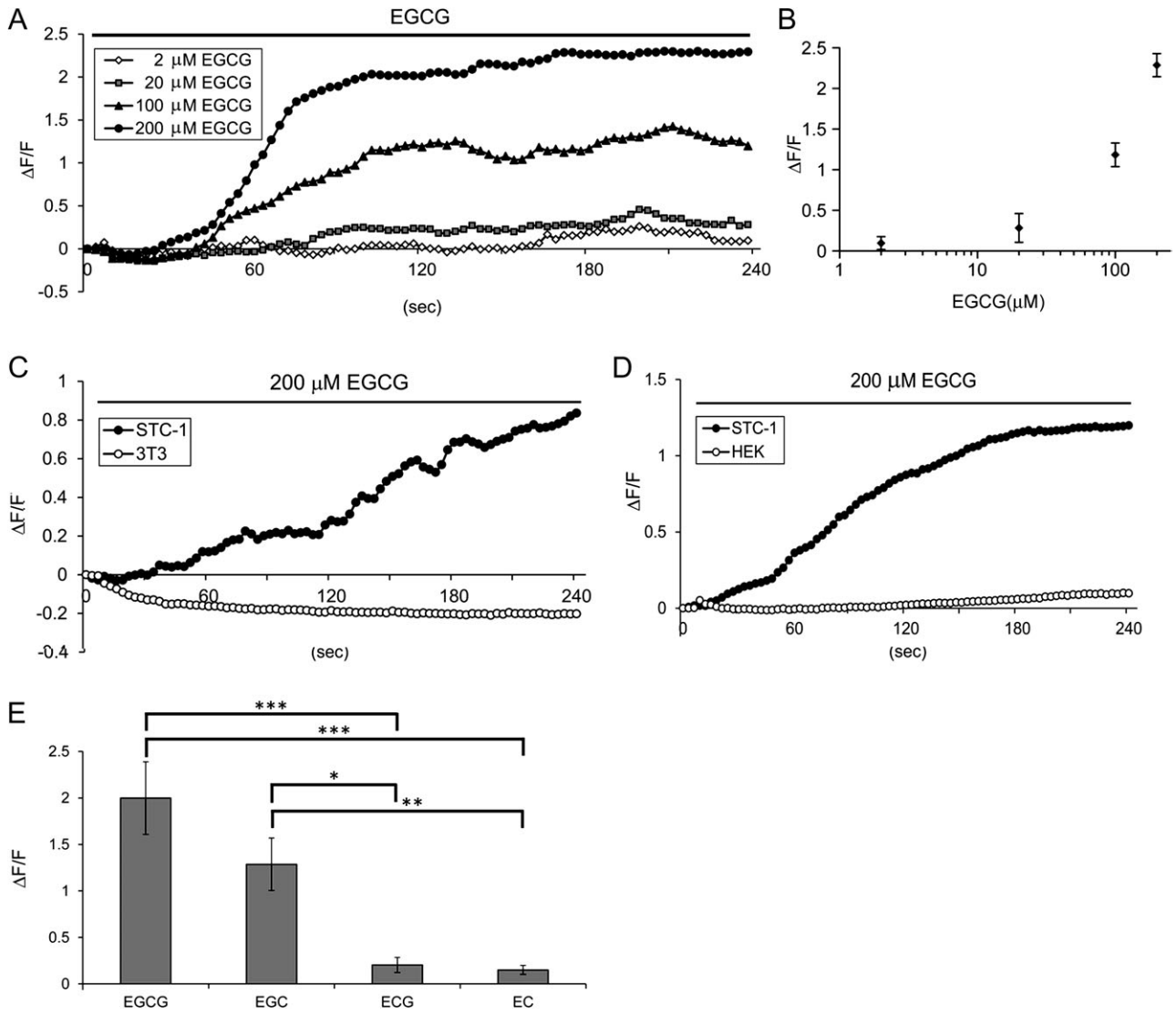

Figure 1 Green tea polyphenol EGCG can stimulate intestinal STC-1 cells. **(A)** STC-1 cells preloaded with 5 μM Fluo8-AM and were challenged with 2, 20, 100, or 200 μM EGCG. The Fluo8 fluorescence was recorded every 3 s, and the relative fluorescence change (Δ*F*/*F*) was determined as described in the Materials and Methods. At 9 s, the ligand was applied to the bath. **(B)** As shown in A, STC-1 cells were stimulated with various doses of EGCG, and the dose–response relationship was observed. The means of Δ*F*/*F* at 237 s after ligand stimulation are plotted. **(C)** STC-1 or 3T3 cells preloaded with 5 μM Fluo8-AM and were activated with 200 μM EGCG. The Fluo8 fluorescence was recorded every 3 s and Δ*F*/*F* was analyzed. At 9 s, the ligand was applied to the bath. **(D)** STC-1 or HEK293T cells preloaded with 5 μM Fluo8-AM and were stimulated with 200 μM EGCG. The Fluo8 fluorescence was recorded every 3 s and Δ*F*/*F* was analyzed. At 9 s, the ligand was applied to the bath. **(E)** STC-1 cells preloaded with 5 μM Fluo8-AM and were stimulated with 200 μM of EGCG, EC, ECG, or EGC. The Fluo8 fluorescence was recorded every 6 s and Δ*F*/*F* was analyzed. The average Δ*F*/*F* at 231 s after ligand stimulation is shown. Differences judged to be significant by the Tukey–Kramer method are marked with 1–3 asterisks as follows: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Possible involvement of the TRPA1 channel in the EGCG response of STC-1

To determine the contribution of Ca^{2+} influx through the plasma membranes from the extracellular medium on the increase in $[Ca^{2+}]_i$ induced by EGCG in STC-1 cells, we performed calcium imaging using Ca^{2+}-free HBSS as the bath solution. As shown in Figure 3A, the response was completely abolished in the absence of $Ca^{2+}{}_o$. This result indicated that $Ca^{2+}{}_o$ is responsible for a major component of the increase in $[Ca^{2+}]_i$ induced by EGCG. Furthermore, the effects of blockers for TRP channels were studied. Gd^{3+} or RR significantly inhibited the Ca^{2+} response in STC-1 cells (Figure 3B). The results suggested that TRP channels might be involved in the unique response of STC-1 cells to EGCG. Among TRP channels, the TRPA1 channel is activated by various pungent compounds, such as isothiocyanates, allicin, cinnamaldehyde, menthol, and sanchol (Bandell et al. 2004; Jordt et al. 2004; Macpherson et al. 2005; Karashima et al. 2007; Bautista et al. 2008). It is possible that TRPA1 might be involved in the

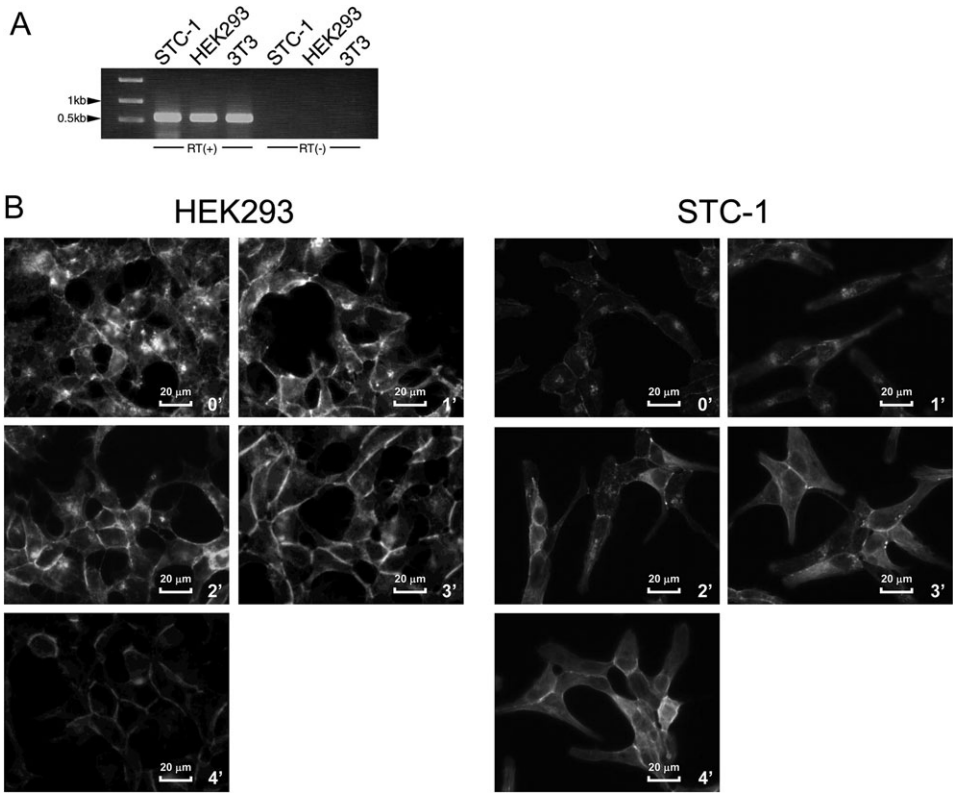

Figure 2 67LR-mediated signaling is not involved in the EGCG response of STC-1. **(A)** Expression of 67LR was examined in STC-1 cells. Total RNA was isolated from STC-1, HEK293T, and 3T3 cells, and RT-PCR analysis was performed using primers for 67LR as described in the Materials and Methods. When using the reaction mixture without reverse transcriptase (RT(−)), no PCR product was observed. **(B)** The effect of EGCG on the actin cytoskeleton organization in STC-1 cells was examined. STC-1 and HEK293T cells were cultured on Matrigel-coated coverslips, and culture medium was replaced with HBSS before addition of EGCG. At 0, 1, 2, 3, and 4 min after stimulation with 200 μM EGCG, the cells were fixed and stained for actin fibers as described in the Materials and Methods. All images were obtained with the same exposure time.

response to the astringent stimulus with EGCG. We next examined the effects of TRPA1-specific inhibitors, AP-18 and HC-030031 (McNamara et al. 2007; Petrus et al. 2007; Kerstein et al. 2009), on the Ca^{2+} response induced by EGCG in STC-1 cells. In the presence of AP-18 or HC-030031, the EGCG-induced response was completely attenuated (Figure 3C). We further investigated the expression levels of mRNAs of TRPA1, TRPV1, and TRPM8 in STC-1 cells by RT-PCR analysis. A significant expression of TRPA1 mRNA was detected, and low levels of TRPV1 mRNA were also detected (Figure 3D). Treatment with capsaicin could not activate STC-1 cells, when examined using the calcium-imaging technique (Figure 3E). The results strongly suggested that EGCG might activate TRPA1 channels to induce an increase in $[Ca^{2+}]_i$ in STC-1 cells. Although a low level of TRPV1 was also detected in HEK293T cells (Figure 3D right panel), capsaicin could not activate HEK293T cells (Figure 4C).

HEK293T cells respond to EGCG when expressing mTRPA1 channels

We next measured $[Ca^{2+}]_i$ in HEK293T cells expressing mTRPA1 channels. The expression vector for mTRPA1 cDNA was transfected into HEK293T cells, and the effect of EGCG on $[Ca^{2+}]_i$ was examined. We observed that 200 μM EGCG induced an increase in $[Ca^{2+}]_i$ in cells transfected with mTRPA1 cDNA but not in cells transfected with the empty vector (Figure 4A). TRPV1 and TRPM8 are known to have some features in common with TRPA1 (Jordt et al. 2004; Macpherson et al. 2005). HEK293T cells were transfected with the expression vectors of rat TRPV1 (rTAPV1) and rat TRPM8 (rTRPM8), and their responses to EGCG were also studied. A significant response was not observed in HEK293T cells expressing rTRPM8, but quite interestingly, the Ca^{2+}-response was also induced in HEK293T cells expressing rTRPV1 (Figure 4B,C). Because rTRPV1 does not function in STC-1 cells, it is considered that the mTRPA1 channel mainly contributes to the response to EGCG observed in STC-1 cells.

Characterization of the response to tea catechins of HEK293T cells expressing mTRPA1 channels

We next examined the responses of HEK293T cells expressing mTRPA1 to various doses of EGCG. The results are shown in Figure 5A,B. Significant activation was observed

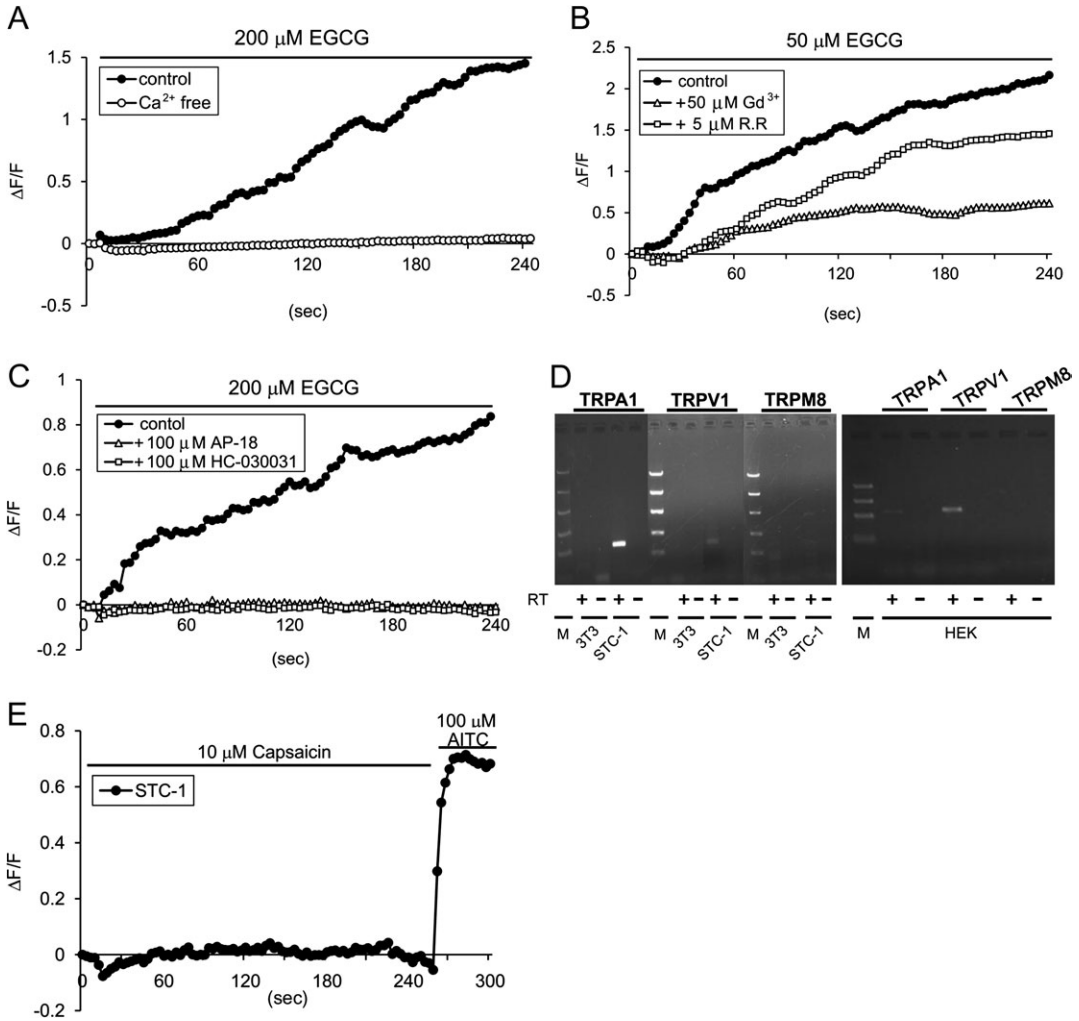

Figure 3 Possible involvement of TRP channels in the EGCG response of STC-1. **(A)** Effects of EGCG on $[Ca^{2+}]_i$ in the absence of $Ca^{2+}{}_o$ were examined. $[Ca^{2+}]_i$ was monitored in STC-1 cells loaded with 5 μM Fluo8-AM in the presence or absence of $Ca^{2+}{}_o$. The Fluo8 fluorescence was recorded every 6 s and Δ*F*/*F* was analyzed. At 6 s, 200 μM EGCG was applied to the bath. **(B)** $[Ca^{2+}]_i$ was monitored in STC-1 cells loaded with 5 μM Fluo8-AM in the absence or the presence of 50 μM Gd^{3+} or 5 μM RR. The Fluo8 fluorescence was recorded every 3 s and Δ*F*/*F* was analyzed. At 9 s, 50 μM EGCG was applied to the bath. **(C)** $[Ca^{2+}]_i$ was monitored in STC-1 cells loaded with 5 μM Fluo8-AM in the absence or the presence of 100 μM AP-18 or 100 μM HC-030031. The Fluo8 fluorescence was recorded every 3 s and Δ*F*/*F* was analyzed. At 9 s, 100 μM EGCG was applied to the bath. **(D)** Total RNA was isolated from STC-1, 3T3, and HEK293T cells. RT-PCR analysis was performed using primers for TRPA1, TRPM8, and TRPV1 as described in the Materials and Methods. When using the reaction mixture without reverse-transcriptase (RT(−)), no PCR product was observed. For mouse cells (STC-1 and 3T3), the estimated sizes of PCR products were 608 bp for TRPA1, 654 bp for TRPV1, and 617 bp for TRPM8. For human cells (HEK293T), the estimated sizes of PCR products were 605 bp for TRPA1, 655 bp for TRPV1, and 618 bp for TRPM8. The lanes labeled M contain DNA size markers (left panel: 10, 4, 2, 1, 0.5, and 0.1 Kbp; right panel: 2, 1, 0.5, and 0.1 Kbp). **(E)** $[Ca^{2+}]_i$ was monitored in STC-1 cells loaded with 5 μM Fluo8-AM. The Fluo8 fluorescence was recorded every 3 s and Δ*F*/*F* was analyzed. At 9 s, 10 μM capsaicin was applied to the bath, and 100 μM allyl isothiocyanate (AITC) was further applied at 249 s.

with 100 and 200 μM EGCG. Next, we examined the effects of 200 μM of other tea catechins on $[Ca^{2+}]_i$ in HEK293T cells expressing mTRPA1 (Figure 5C). The order of potency of these catechins was EGCG > EGC > ECG >> EC. The results indicated that EGCG was the most effective activator among the green tea catechins and demonstrated that the responses of HEK293T cells expressing mTRPA1 channels to catechins were very close to those observed with STC-1 cells. To assess the role of mTRPA1 in the response to EGCG, we investigated the effects of TRP channel blockers. First, to determine the contribution of Ca^{2+} influx to the response of $[Ca^{2+}]_i$ induced by EGCG observed in HEK293T cells expressing mTRPA1, we performed calcium imaging using Ca^{2+}-free HBSS as the bath solution. The response was completely abolished in the absence of $Ca^{2+}{}_o$ (Figure 5D). When a general blocker for TRP channels, Gd^{3+} or RR, was present in the bath solution of HEK293T cells expressing mTRPA1, the increase in $[Ca^{2+}]_i$ induced by EGCG appeared to be inhibited (Figure 5E). It was previously reported that the response of HEK293T cells expressing mTRPA1 to caffeine was almost

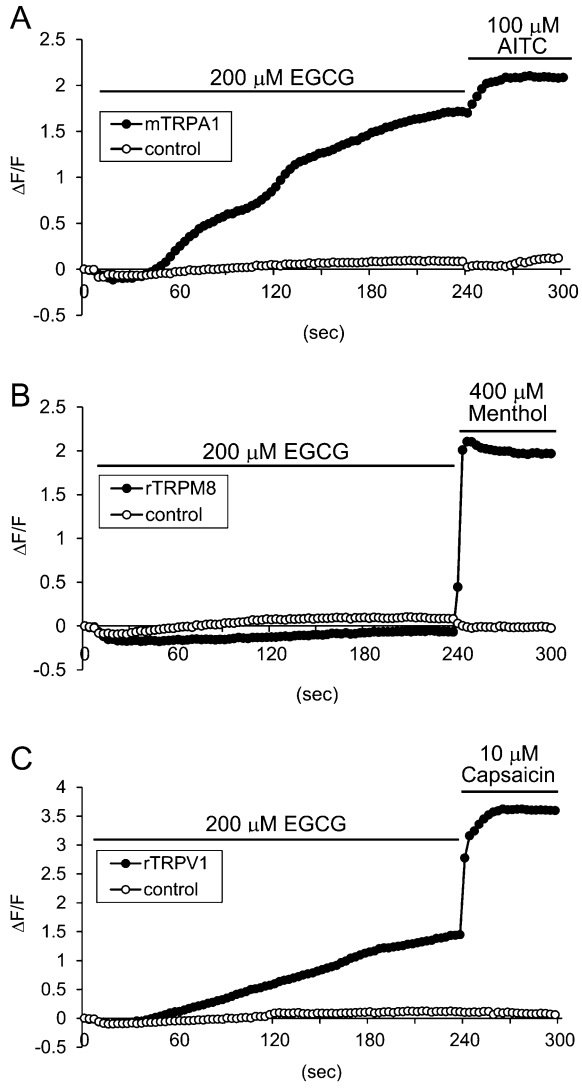

Figure 4 EGCG can stimulate HEK293T cells expressing mTRPA1 channels. **(A)** The effect of EGCG (200 μM) on $[Ca^{2+}]_i$ in HEK293T cells expressing mTRPA1 was examined. After transfection with the expression vector of mTRPA1, cells were loaded with 5 μM Fluo8-AM. The Fluo8 fluorescence was recorded and Δ*F*/*F* was analyzed. At 9 s, 200 μM EGCG was applied to the bath, and at 240 s, 100 μM allyl isothiocyanate (AITC) was further applied. **(B)** The effect of EGCG (200 μM) on $[Ca^{2+}]_i$ in HEK293T cells expressing rat TRPM8 (rTRPM8) was examined. After transfection with the expression vector of rTRPM8, cells were loaded with 5 μM Fluo8-AM. The Fluo8 fluorescence was recorded and Δ*F*/*F* was analyzed. At 9 s, 200 μM EGCG was applied to the bath, and at 240 s, 400 μM menthol was further applied. **(C)** Effect of EGCG (200 μM) on $[Ca^{2+}]_i$ in HEK293T cells expressing rat TRPV1 (rTRPV1) was examined. After transfection with the expression vector of rTRPV1, cells were loaded with 5 μM Fluo8-AM. The Fluo8 fluorescence was recorded and Δ*F*/*F* was analyzed. At 9 s, 200 μM EGCG was applied to the bath, and at 240 s, 10 μM capsaicin was further applied.

completely blocked by Gd^{3+} or RR (Nagatomo and Kubo 2008), but the EGCG-induced response here was only partially inhibited by Gd^{3+} or RR. The variation might be due to, for example, the difference of the type and concentration of ligands, as seen in previous reports (Chen et al. 2007; McNamara et al. 2007; Maher et al. 2008). We next investigated whether TRPA1-specific inhibitors, AP-18 and HC-030031, could block the EGCG-induced response. In the presence of AP-18 or HC-030031, EGCG could not induce a significant response in cells expressing mTRPA1 (Figure 5F).

Activation of mTRPA1 with EGCG induces the formation of actin fibers

In HEK293T cells, actin fibers were disassembled after addition of EGCG, but in STC-1 cells, treatment with EGCG induced the new formation of actin fibers. We next studied whether a TRPA1-specific inhibitor could block the EGCG-induced formation of actin fibers in STC-1 cells. Again, to examine the distribution of F-actin structures, fluorescently labeled phalloidin was used. As shown in Figure 6, 200 μM EGCG enhanced the formation of actin fibers within 4 min. However, in the presence of AP-18, a TRPA1-specific inhibitor, formation of the actin cytoskeleton was not enhanced. The results suggested that the EGCG treatment newly forms filamentous structures of actin through the activation of mTRPA1 in STC-1 cells. From these several lines of evidence, it was demonstrated that EGCG induces the activation of mTRPA1 in intestinal STC-1 cells, and it was suggested that the mTRPA1 channel may function as an EGCG sensor in STC-1 cells.

Discussion

EGCG response in STC-1 cells

In this study, we investigated whether the mouse intestinal cell line, STC-1, can respond to the astringent compound of green tea, EGCG. By using a calcium-imaging technique, we found that the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ of STC-1 increases in response to EGCG. We previously showed that all 5 of the basic taste stimuli induced an elevation of $[Ca^{2+}]_i$ in intestinal STC-1 cells (Saitoh et al. 2007). When the time courses of the elevations of $[Ca^{2+}]_i$ were compared, the response to EGCG, interestingly, appeared to be slower than that to any of the 5 basic taste stimuli. All of the responses to the 5 basic taste stimuli reached the maximum level within 30–60 s in STC-1 cells. On the other hand, incubation for more than 120 s was required to reach the maximum level for the EGCG-induced increase in $[Ca^{2+}]_i$. It was considered that the EGCG treatment indeed triggers a distinct mechanism in STC-1 cells. This cellular response was not observed in HEK293T or 3T3 cells, both of which express 67LR functioning as a cell surface EGCG receptor inducing anticancer action (Tachibana et al. 2004).

We further studied the effects of EGCG on the actin cytoskeleton of STC-1 cells and observed that actin stress fibers were newly formed upon treatment with EGCG. In HEK293T cells, however, actin fibers disappeared after the addition of EGCG, as previously reported (Umeda et al. 2005). It has also been reported that EGCG reduces phosphorylation of the

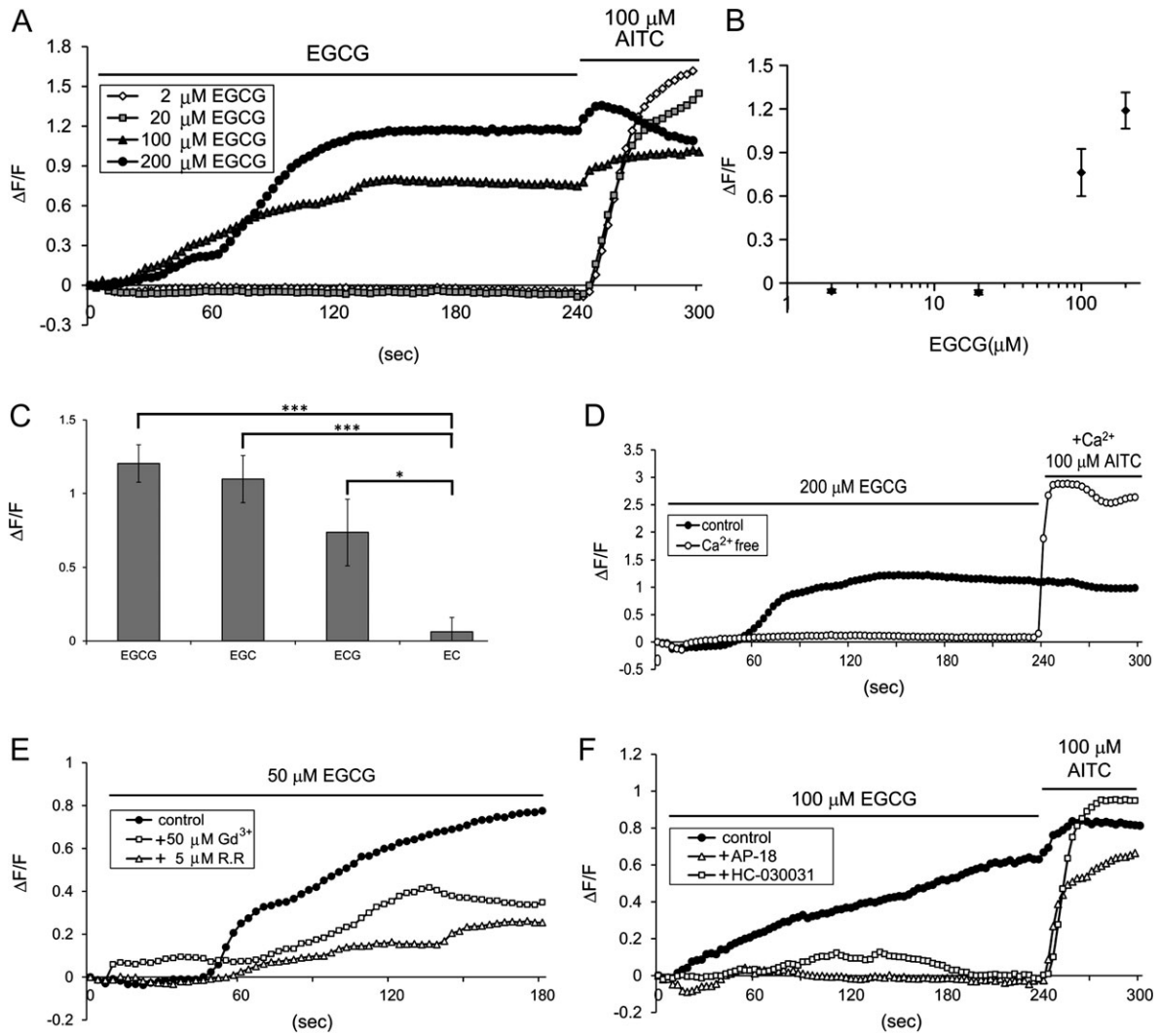

Figure 5 Characterization of the response to tea catechins of HEK293T cells expressing mTRPA1. **(A)** The effect of EGCG (2, 20, 100, and 200 μM) on $[Ca^{2+}]_i$ in HEK293T cells expressing mTRPA1 was examined. After transfection with the expression vector of mTRPA1, cells were loaded with 5 μM Fluo8-AM. The Fluo8 fluorescence was recorded and Δ*F*/*F* was analyzed. At 9 s, EGCG was applied to the bath, and at 240 s, 100 μM allyl isothiocyanate (AITC) was further applied. **(B)** As shown in panel A, HEK293T cells expressing mTRPA1 were stimulated with various doses of EGCG, and the dose–response relationship was observed. The means of Δ*F*/*F* at 237 s after ligand stimulation are plotted. **(C)** HEK293T cells expressing mTRPA1 were preloaded with 5 μM Fluo8-AM and were stimulated with 200 μM of EGCG, EC, ECG, or EGC. The Fluo8 fluorescence was recorded every 6 s and Δ*F*/*F* was analyzed. The average Δ*F*/*F* at 231 s after ligand stimulation is shown. Differences were judged to be significant by the Tukey–Kramer method (* $P < 0.05$, *** $P < 0.001$). **(D)** Effects of EGCG on $[Ca^{2+}]_i$ in the absence of $Ca^{2+}{}_o$ were examined. HEK293T cells expressing mTRPA1 were preloaded with 5 μM Fluo8-AM, and $[Ca^{2+}]_i$ was monitored in the presence or absence of $Ca^{2+}{}_o$after addition of EGCG. The Fluo8 fluorescence was recorded every 3 s and Δ*F*/*F* was analyzed. At 9 s, 200 μM EGCG was applied to the bath, and at 240 s, 100 μM AITC in Ca^{2+}-containing HBSS was further applied. **(E)** After transfection with the expression vector of mTRPA1, $[Ca^{2+}]_i$ was monitored in cells preloaded with 5 μM Fluo8-AM in the absence or the presence of 50 μM Gd^{3+} or 5 μM RR. The Fluo8 fluorescence was recorded every 3 s and Δ*F*/*F* was analyzed. At 9 s, 50 μM EGCG was applied to the bath. **(F)** $[Ca^{2+}]_i$ was monitored in HEK293T cells expressing mTRPA1 in the absence or the presence of 100 μM AP-18 or 100 μM HC-030031. The Fluo8 fluorescence was recorded every 3 s and Δ*F*/*F* was analyzed. At 9 s, 100 μM EGCG was applied to the bath, and at 240 s, 100 μM AITC was further applied.

myosin regulatory light chain (MRLC) of myosin II through 67LR and eukaryotic translation elongation factor 1A (eEF1A) to induce rearrangement of the actin cytoskeleton in cancer cells (Umeda et al. 2008). It is possible that the increase in $[Ca^{2+}]_i$ might elevate the phosphorylation of MRLC by Ca^{2+}/calmodulin-dependent myosin light-chain kinase in STC-1 cells (Citi and Kendric-Jones 1987). The phosphorylation of MRLC might lead to a conformational change in myosin II, enabling it to assemble into filaments, and might promote the formation of stress fibers (Chrzanowska-Wodnicka and Burridge 1996). Such actin fiber formations have been reported to occur within 5 min (Ridley and Hall 1992; Chrzanowska-Wodnicka and Burridge 1996). It is known that compounds present in the

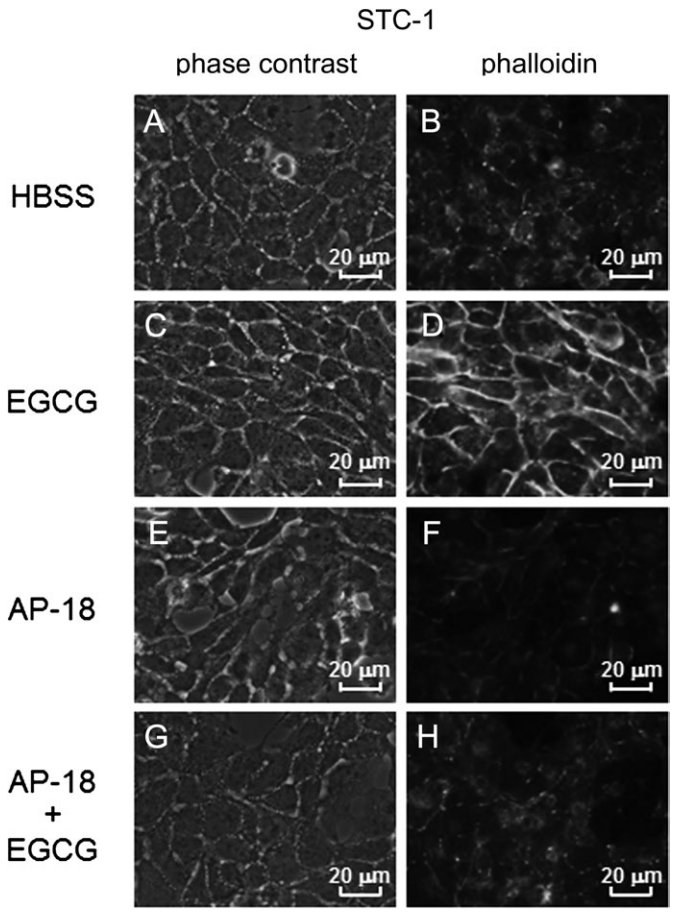

Figure 6 Activation of mTRPA1 with EGCG induces the formation of actin fibers. STC-1 cells were cultured on Matrigel-coated coverslips, and culture medium was replaced with HBSS before addition of ligands. At 4 min after stimulation with HBSS, 200 μM EGCG, 100 μM AP-18, or 200 μM EGCG and 100 μM AP-18, the cells were fixed and stained for actin fibers as described in the Materials and Methods. All images were obtained with the same exposure time. Phase contrast **(A**, **C**, **E**, and **G)** and fluorescent **(B**, **D**, **F**, and **H)** images are shown.

GI tract activate the secretion of GI hormones, such as cholecystokinin from STC-1 cells (Chen et al. 2006). Therefore, the formation of stress fibers by EGCG may also contribute to the secretion of GI hormones. To examine this point, further detailed experiments are required.

The present results strongly suggested that a novel sensor molecule is present on the surface of STC-1 cells. We showed that TRPA1-specific inhibitors, AP-18 and HC-030031, attenuated the Ca^{2+} response and the increase in actin fibers in EGCG-treated STC-1 cells. Because the inhibition with either blocker was almost complete, it was considered that mTRPA1 might be a main contributor and that other channels or receptors may not contribute to the EGCG activation of STC-1 cells. It was reported recently that the human bitter taste receptor hTAS2R39 responds to tea catechins (Narukawa et al. 2011). In that report, the authors found that the strongest response was observed with ECG, followed in order by EGCG, EC, and EGC. On the other hand, in the present study, we demonstrated that the order of potency of tea catechins to activate STC-1 cells or mTRPA1 channels was EGCG > EGC > ECG > EC. Therefore, it is considered that the mouse homologue of hTAS2R39 may not mediate the response of STC-1 to tea catechins.

We observed a high-level expression of TRPA1 mRNA in STC-1 cells. In addition, we detected a low-level expression of TRPV1 mRNA. However, an agonist of TRPV1, capsaicin, could not induce any Ca^{2+} response in STC-1 cells. The expression level of TRPV1 may be quite low, or the channel activity of TRPV1 might be blocked by an unknown mechanism in STC-1 cells. At least, it seems that TRPV1 does not contribute to the EGCG-induced response observed in STC-1 cells.

Expression of mTRPA1 channels converts cells to respond to the astringent stimulus with EGCG

mTRPA1 channel was expressed in the HEK293T cells, which normally cannot respond to EGCG, as a candidate sensor for EGCG. The transfected cells could be activated by EGCG with the characteristic slow time course observed in STC-1 cells and in a dose-dependent manner similarly to STC-1 cells. We also found that the order of potency of the 4 green tea catechins (EGCG, EGC, ECG, and EC) to activate mTRPA1 was very close to the order observed using STC-1 cells. Finally, the activation with EGCG of HEK293T cells expressing mTRPA1 was abolished in the presence of AP-18 or HC-030031. These results clearly demonstrated that the mTRPA1 channel is required for and functions in the EGCG-induced response in intestinal STC-1 cells. Furthermore, HEK293T cells expressing rTRPV1 also could be activated with EGCG. Compared with the rapid response to capsicin, the activation time course for the EGCG response of rTRPV1 appeared to be as slow as that for mTRPA1. The mechanism for the EGCG-induced slow response is currently unknown. Although further investigation is required, it is possible that some signaling process might be activated to induce gradual elevation of $[Ca^{2+}]_i$ after initial activation of TRP channels with EGCG.

The EGCG treatment activates 67 LR but cannot induce the elevation of $[Ca^{2+}]_i$ in cells other than STC-1 cells. However, it is possible that the presence of 67LR might be required for the EGCG-induced elevation of $[Ca^{2+}]_i$ in the cells expressing mTRPA1 or rTRPV1. We could not exclude this possibility. To examine this point, cell lines specifically lacking the expression of 67LR or 67LR-KO cells are required. Furthermore, we examined the response to EGCG of *Xenopus* oocytes expressing mTRPA1 under 2-electrode voltage clamp. We tried to record the current through mTRPA1 channels itself in the absence of extracellular Ca^{2+}, and also the $Ca^{2+}{}_i$-Cl^- current activated by Ca^{2+} influx through TRPA1 channels in the presence of extracellular

Ca^{2+}. We could not detect any clear changes of the membrane current in either case (data not shown), although positive control responses to allyl isothiocyanate (AITC) were clearly observed. The results suggest that additional protein factors endogenously present in HEK293T cells might be required for mTRPA1 to constitute a functional sensing system of EGCG or that posttranslational modifications that occur only in mammalian cells might be essential for mTRPA1 to recognize EGCG.

A characteristic oral astringent taste is elicited primarily by polyphenols. Of these polyphenols, EC, EGC, ECG, and EGCG are present in wine and tea. A typical green tea polyphenol is EGCG (Drewnowski and Gomez-Carneros 2000; Lesschaeve and Nobel 2005). The mechanism underlying the sensation of the astringent taste, however, is not well known. Here, we first demonstrated that the intestinal STC-1 cells can be activated with EGCG and that EGCG can stimulate HEK293T cells expressing mTRPA1 channels. It is known that typical green tea contains –1.5 mM EGCG (Wang et al. 1992; Wolfram 2007). Therefore, our results clearly indicate that the EGCG present in green tea can activate mTRPA1 on the cell surface. TRPA1 is a member of the TRP family of ion channels and expressed in a subset of nociceptive neurons. Recently, it has been reported that this TRP channel protein is expressed in the nerve fibers in the mouse tongue (Nagatomo and Kubo 2008). Therefore, although further investigation is required, it is possible that mTRPA1 plays an important role in the sensation of astringency taste on the tongue.

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