Evidence that polyphenols do not inhibit the phospholipid scramblase TMEM16F

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ACCELERATED COMMUNICATION

The TMEM16 membrane protein family consists of Ca2+-activated phospholipid scramblases (CaPLSases) that mediate rapid transmembrane phospholipid flip-flop and as such play essential roles in various physiological and pathological processes such as blood coagulation, skeletal development, viral infection, cell-cell fusion, and ataxia. Pharmacological tools specifically targeting TMEM16 CaPLSases are urgently needed to understand these novel membrane transporters and their contributions to health and disease. Tannic acid (TA) and epigallocatechin gallate (EGCG) were recently reported as promising TMEM16F CaPLSase inhibitors. However, our present study shows that TA and EGCG do not inhibit the phospholipid-scrambling or ion conduction activities of the dual-functional TMEM16F. Instead, we found that TA and EGCG mainly acted as fluorescence quenchers that rapidly suppress the fluorophores conjugated to annexin V, a phosphatidylserine-binding probe commonly used to report on TMEM16 CaPLSase activity. These data demonstrate the false positive effects of TA and EGCG on inhibiting TMEM16F phospholipid scrambling and discourage the use of these polyphenols as CaPLSase inhibitors. Appropriate controls as well as a combination of both fluorescence imaging and electrophysiological validation are necessary in future endeavors to develop TMEM16 CaPLSase inhibitors.

In this study, we attempted to assess the inhibitory effects of TA and EGCG on TMEM16F CaPLSase and ion channel activities by fluorescence imaging and electrophysiology. To our surprise, TA and EGCG displayed minimal effects on inhibiting TMEM16F channel activity. This prompted us to re-examine the effects of these polyphenols on TMEM16F lipid scramblase activity. We found that TA and EGCG indeed quenched various fluorophores, including those that are tagged to the AnV probes. We further demonstrated that the previous inhibitory effects of TA and EGCG on TMEM16F were mainly due to the fluorescence-suppressing effects of the polyphenols on the fluorophores conjugated to the extracellular AnV probes and thus prevent AnV from reporting the scrambling activity. We therefore conclude that TA and EGCG do not function as TMEM16F CaPLSase inhibitors. These results also suggest that precautions need to be taken in developing inhibitors targeting TMEM16 CaPLSases.

Results

Extracellular fluorescently tagged AnV cannot report TMEM16F lipid-scrambling activity in the presence of TA and EGCG

We applied a previously established fluorescence imaging–based scrambling assay (10, 25) to characterize the effects of TA and EGCG on TMEM16F-CaPLSase. In this assay,
fluorescently tagged AnV (henceforth referred to as AnV) was included in the extracellular solution to monitor scramblase activity of HEK293 cells stably expressing murine TMEM16F. Upon its activation by ionomycin-induced Ca\(^{2+}\) influx, TMEM16F CaPLSase rapidly translocates PS from the inner to outer leaflet of the plasma membrane. Because the externalized PS recruits AnV to the cell surface, the gradual accumulation of fluorescence signal from AnV on the cell membrane is indicative of TMEM16F scrambling activity (Fig. 1, B and C). Consistent with previous reports (22, 23), there was no AnV fluorescence signal on the surface of TMEM16F-expressing HEK293 cells when TA (20 \(\mu M\)) and EGCG (20 \(\mu M\)) were present (Fig. 1, D–G). On the other hand, robust AnV signal was observed on the cell surface in the control experiment without TA or EGCG (Fig. 1, B and C). Furthermore, Ca\(^{2+}\) influx via ionomycin was not affected by the polyphenols as intracellular Ca\(^{2+}\) was instantly mobilized following Ca\(^{2+}\) ionophore stimulation regardless of the presence of TA or EGCG (Fig. 1, B–G). All of these observations imply that the polyphenols could impair TMEM16F lipid-scrambling activity. It is worth noting that 20 \(\mu M\) TA completely abolishes AnV surface signal, whereas 20 \(\mu M\) EGCG still allows weak AnV accumulation on the cell membrane, suggesting that EGCG may have a weaker inhibitory effect than TA (Fig. 1, D–G).

**TA and EGCG do not specifically block TMEM16F ion channel activity**

TMEM16F is a dual-functional moonlighting protein with both CaPLSase and ion channel activities (8, 18, 25, 26). Several lines of structural and functional evidence have suggested that in TMEM16F, phospholipids and ions share the same activation gates and Ca\(^{2+}\)-dependent activation mechanism (18, 25, 27–29). As Ca\(^{2+}\)-dependent activation of scrambling activity accompanies ion channel activity (25), we tested the effects of extracellular TA and EGCG on TMEM16F ion channel function using whole-cell patch clamp. Surprisingly, high concentration of TA (20 \(\mu M\)) only inhibited about 50% of TMEM16F current, and 20 \(\mu M\) EGCG had a negligible effect on TMEM16F
inhibitory effect of 20 μM TA and EGCG could suppress the fluorescence of various fluorophores/dyes, including the Alexa Fluor–conjugated IgGs (Alexa Fluor 488, 594, and 647; Fig. 3, A–H), nucleic acid dyes ( Hoechst and Nuclear Green DCS1; Fig. 3, G and H), and CF488A-conjugated wheat germ agglutinin (WGA) (Fig. 3I).

**TA and EGCG quench the fluorescence signal of the extracellular PS probes accumulating on the cell membrane**

As the cell-free results evinced that TA and EGCG could quench the AnV probes in aqueous solution, we verified whether this was the case on our cell-based assays. First, we allowed AnV to accumulate on the surface of TMEM16F-expressing HEK293 cells after ionomycin stimulation (Fig. 4, A, C, and E, middle rows). Next, we introduced TA and EGCG to the medium after a significant amount of AnV had accumulated on the cell surface (2.5 min after ionomycin application). AnV fluorescence signal on the cell membrane was instantaneously decreased upon polyphenol applications (Fig. 4, B, D, and F). 20 μM TA completely abolished AnV signal, whereas 1 μM TA and 20 μM EGCG eliminated more than 80% of AnV signal.

To further validate the fluorescence-quenching effects of TA and EGCG on the AnV probes and verify that loss of AnV signal was not due to defects in TMEM16F functions, we tested whether the polyphenols also quenched the fluorescence signal of the AnV probes binding to apoptotic cells, which also have PS-exposed cell surface but are independent of TMEM16F-CaP.LSase (39). Staurosporine (STS) was used to induce apoptosis, which resulted in PS being exposed to the cell surface more than 80% of AnV signal.

**In vitro assay shows that TA and EGCG quench fluorescence signal of the PS probes and other dyes**

TA and EGCG are known to be able to quench various fluorophores (24, 35–38). Therefore, we suspected that the polyphenols might hinder the ability of the extracellular AnV to quench the fluorescence intensities of various fluorescence probes/dyes, including the Alexa Fluor–conjugated IgGs (Alexa Fluor 488, 594, and 647; Fig. 3, A–H), nucleic acid dyes ( Hoechst and Nuclear Green DCS1; Fig. 3, G and H), and CF488A-conjugated wheat germ agglutinin (WGA) (Fig. 3I).
fluorophores of the AnV probes. Interestingly, the intensity of the intracellular caspase dye was also instantly reduced upon TA and EGCG addition, suggesting that the compromised membrane, as a result of apoptosis, allowed TA and EGCG to leak into the cytosol and partially quench the caspase dye (Fig. S2, C–H). Based on the above observations, we conclude that TA and EGCG can rapidly quench the extracellular fluorescence signal from fluorophore-conjugated AnV.

Additionally, to further corroborate that TA and EGCG treatments have minimal effects on TMEM16F scrambling activity, we first stimulated TMEM16F scrambling and then reprobed the ionomycin-stimulated TMEM16F-stably expressed HEK293 cells with AnV-CF594. Prior to ionomycin treatment, the cells were stained with AnV-CF594 to verify that the cell surface was PS-negative (Fig. S3, left). The cells were first treated with either ionomycin alone or ionomycin in combination with TA or EGCG. After 10-min treatment and extensive washes to eliminate ionomycin, TA, and EGCG, the cells were reprobed with AnV-CF594 to observe surface-exposed PS. We found that the polyphenol-treated cells did not show obvious inhibitory effect on AnV-CF594 binding to the cell surface (Fig. S3, right). This observation is consistent with the lack of polyphenol inhibitory effect on TMEM16F current (Fig. 2). As TMEM16F is a moonlighting protein with both CaPLSase and ion channel activities (8, 18) and its activation leads to simultaneous phospholipid and ion permeation (25), our electrophysiology and fluorescence lipid scrambling results explicitly demonstrate that TA and EGCG do not inhibit TMEM16F.

Discussion

In this study, we carefully examined the potential inhibitory effects of TA and EGCG on TMEM16F. Through a series of experiments, including patch clamp, cell-free, and cell-based fluorescence assays, we failed to find evidence to support that the polyphenols would be specific inhibitors for TMEM16F. Instead, these polyphenols quench the fluorophores of the AnV probes as well as other fluorescence dyes/probes. The quenching artifacts prevent the AnV probes from reporting TMEM16F-mediated PS exposure from fluorophore-conjugated AnV.
to study and target TMEM16 CaPLSases. Furthermore, precautions need to be taken when developing inhibitors for TMEM16 CaPLSases using fluorescence-based assays. Given the dual functionality of TMEM16F as both phospholipid scramblase and ion channel, we recommend combining fluorescence imaging–based scrambling assays with electrophysiology when trying to identify bona fide inhibitors for TMEM16 scramblases.

It is worth noting that higher concentration of TA can exert nonspecific effects on TMEM16F as evidenced by the partial inhibitory effect on TMEM16F current (Fig. 2I) and stimulating effect on different types of ion channels, such as the BK channel shown in this study (Fig. S1) or TRPM4, TRPC4, and TRPC5 channels in a previous report (33). Therefore, the nonspecific nature of TA prevents it from serving as an ideal pharmacological tool for mechanistic studies.

Experimental procedures

Cell lines and cell culture

HEK293T was purchased from the Duke Cell Culture Facility. C-terminally enhanced GFP–tagged murine (m) TMEM16F-stably expressed HEK293 cells (mTMEM16F-stable HEK293) were a generous gift from Dr. Min Li. All cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco, catalog no. 11995-065) containing 1% penicillin-streptomycin and 10% fetal bovine serum. To reselect and maintain the homogeneous population of mTMEM16F-stable HEK293 cells, 100\( \mu \)g ml\(^{-1} \) of hygromycin was occasionally added to the culture medium. All cells were cultured in a temperature-controlled (37 °C) incubator supplemented with 5% CO\(_2\).

Transfection

Plasmid carrying the cDNAs of mouse BK channel α subunit (Addgene no. 113566) was used in this study. After cells were seeded on PLL-coated coverslips for at least 24 h, X-tremeGENE9 transfection reagent (Millipore-Sigma) was used to transiently transfect the plasmid into the cells. After 5 h of transfection, culture supernatant was changed to fresh medium. Experiments were performed 24–48 h post-transfection.

Fluorescence microscopy

mTMEM16F-stable HEK293 cells were seeded on PLL-coated #0 coverslips overnight prior to the experiments. Extracellular solution in all imaging assays (imaging solution) contained 0.5 mmol l\(^{-1} \) fluorescently tagged AnV, 10 mmol l\(^{-1} \) HEPES, 140 mmol l\(^{-1} \) NaCl, 2.5 mmol l\(^{-1} \) CaCl\(_2\), pH 7.4. Stock solution (10 mmol) of TA and EGCG were prepared using ultrapure water and stored at 4 °C. Working concentrations of TA and EGCG were established by further diluting the stock TA and EGCG solutions in the imaging solution. All experiments were independently repeated at least three times.

Ca\(^{2+}\) imaging and scrambling assay

2 mmol l\(^{-1} \) stock solution of calcium indicator Calbryte\textsuperscript{TM} 590 AM (AAT Bioquest, catalog no. 20701) was prepared by using DMSO and stored at −20 °C. The stock solution was diluted to

![Image of the figure 4](attachment://figure_4.png)

**Figure 4.** TA and EGCG suppress fluorescence signal of fluorescently tagged AnV-CF594 instead of inhibiting TMEM16F-mediated phospholipid-scrambling activity. A, C, and E, representative images showing 20 \( \mu \)M TA (A), 1 \( \mu \)M TA (C), and 20 \( \mu \)M EGCG (E) immediately deplete fluorescence signal of AnV-CF594 (magenta) of scrambling cells (HEK293 cells stably expressing mouse TMEM16F; green). Scale bars, 25 \( \mu \)m. B, D, and F, mean fluorescence intensity change of AnV fluorescence over 10 min of ionomycin treatment for each cell in A, C, and E, respectively. Applications of ionomycin, TA, and EGCG are marked by a downward arrow and chemical abbreviations. \( n = 3–8 \) cells. Error bars, S.E. All images are representative of at least three independent replicates. a.u., arbitrary units.
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a working concentration of 1 μM by using Hanks’ balanced salt solution (HBSS; Gibco, catalog no. 14025-092). The cells were incubated with 1 μM Calbryte\textsuperscript{TM} 590 AM for 20–30 min in the incubator supplied with 5% CO\textsubscript{2} at 37 °C. After washing the cells with HBSS, the coverslips were mounted to our customized imaging chamber containing the imaging solution that has CF 640R–tagged annexin V (AnV-CF640R) (Biotium, catalog no. 29014), and we proceeded with our scrambling assay as described previously (10, 27). In brief, a final concentration of 5 μM ionomycin was added to the imaging chamber to induce Ca\textsuperscript{2+} influx and subsequent scrambling activity of the cells. In TA or EGCG treatment, a 20 μM concentration of either TA or EGCG was added together with ionomycin into the imaging chamber. Changes in fluorescence intensity of AnV-CF594 and AnV-CF640R were recorded using a ×63/1.4 NA Oil Plan-Apochromat DIC in a Zeiss 780 inverted confocal microscope. This experiment was executed using time-lapse imaging with 5-s intervals.

**Cell-based AnV quenching assay**

mTMEM16F stable HEK293 cells were stimulated with 5 μM ionomycin, and the scrambling activity was monitored using CF594–tagged AnV (AnV-CF594) for 2–3 min. After that, either 1 μM TA, 20 μM TA, or 20 μM EGCG was added into the imaging chamber. Changes in the fluorescence intensity of AnV-CF594 were recorded using time-lapse imaging with 5-s intervals. This experiment was executed using a ×63/1.4 NA Oil Plan-Apochromat DIC in a Zeiss 780 inverted confocal microscope.

**Reprobing of the scrambling cells with AnV after TA/EGCG treatment**

mTMEM16F-stable HEK293 cells were seeded on PLL-coated #0 coverslips for 48 h. The cells were first stained and imaged with 0.5 μg ml\textsuperscript{-1} AnV-CF594 prior to any treatments. The coverslips were then transferred to a chamber containing either 5 μM ionomycin alone, 5 μM ionomycin with 1 μM TA, or 5 μM ionomycin with 20 μM EGCG. After a 10-min stimulation, the ionomycin solutions with or without TA or EGCG in the chamber were removed, and the cells were thoroughly washed twice with a large volume of HBSS. The cells were then briefly incubated with AnV-CF594 solution for 3–5 min, and AnV-CF594 signal was captured by a Prime 95B Scientific camera (Photometrics). The same set of fluorophore emissions of the fluorophores were measured using the spectroscopy mode of a Spectramax M5 plate reader (Molecular Devices) with a 5-nm increment. The same set of fluorophore solutions without TA and EGCG was also examined as a control. Each sample was done in three replicates.

**STS-induced apoptosis and active caspase-3/7 staining**

STS was diluted in culture medium and applied to the cells at a final concentration of 10 μM. The cells were incubated with STS for 4 h. Next, medium containing STS was removed and replaced with fresh medium containing TF3-DEVD-FMK (caspase-3/7 dye) from the Live Cell Caspase 3/7 binding assay kit (AAT Bioquest, catalog no. 20101). The detailed staining procedure was described previously (27). Only cells that were positive with TF3-DEVD-FMK and AnV-CF640R were focused for imaging. The control, TA, or EGCG solutions at appropriate concentrations were added to the apoptotic cells. Fluorescence changes of AnV-CF640R and caspase dye were captured with confocal time-lapse imaging at 1-s intervals. This experiment was executed using a ×63/1.4 NA Oil Plan-Apochromat DIC in a Zeiss 780 inverted confocal microscope.

**Electrophysiology**

Voltage-clamp recordings were low-pass filtered at 5 kHz (Axopatch 200B) and digitally sampled at 10 kHz (Axon Digidata 1550A) and digitized by Clampex 10 (Molecular Devices). Electrodes were pulled from borosilicate capillaries (Sutter Instruments) and had initial resistances of 2–5 megaohms. Pipette electrodes were made from borosilicate capillaries (Sutter Instruments) and fire-polished with a microforge (Narishige). All experiments were performed at room temperature.

TA and EGCG were diluted into bath buffer at desired concentrations using aqueous stock solutions of 20 and 10 mM, respectively. External application of TA or EGCG was performed via local focal perfusion using a pressurized perfusion apparatus (ALA-VM8, ALA Scientific Instruments). For whole-cell recording, the perfusion outlet was positioned close to the patched cell before forming the whole-cell configuration for recordings. For outside-out recording, the patches were moved next to the perfusion outlet before recording.

Whole-cell TMEM16F channel recordings were performed on the mTMEM16F-stable HEK293 cells. The cells were trypsinized and plated on PLL-coated coverglass (Assistent) 1–2 h before electrophysiology. Bath solution contained 140 mM NaCl, 10 mM HEPES, 2 mM MgCl\textsubscript{2}, pH 7.3 (adjusted with NaOH). Pipette solution contained 140 mM CsCl, 10 mM HEPES, 1 mM CaCl\textsubscript{2}, pH 7.3 (adjusted with NaOH). Upon formation of whole-cell configuration, TMEM16F channels were activated by a current-voltage (I-V) protocol in which the membrane was held at −60 mV and test voltage steps from −100 to +140 mV. Typically, with the presence of 1 mM intracellular Ca\textsuperscript{2+}, considerable TMEM16F currents were observed within 30–60 s after breaking in. Following acquisition of a control I-V
recording. TA or EGCG at the desired concentrations was focally perfused to the cell for 2–3 s before recording acquisition. Next, the cell was followed by focal perfusion of a control bath solution to wash off TA or EGCG for 3–4 s before another recording acquisition.

To test TA and EGCG effects on BK channels, an outside-out configuration was used. The pipette solution contained 140 mM KCl, 10 mM HEPES, 1 mM MgCl2, 0.1 mM free Ca2+, pH 7.3 with KOH. Extracellular solution contained 140 mM KCl, 10 mM HEPES, 1 mM MgCl2, pH 7.3 with KOH. The smooth curves represent Boltzmann fits, $G/G_{\text{max}} = 1/(1 + \exp(-ze(V - V_{1/2})/kT))$, where $G_{\text{max}}$ represents tail current amplitude elicited by depolarization of +200 mV, $z$ is the number of equivalent gating charges, $V$ is the membrane voltage, $V_{1/2}$ is the half-activation voltage, $F$ is Faraday’s constant, $R$ is the gas constant, and $T$ is the absolute temperature.

**Data analysis**

All fluorescence imaging data analysis was performed in Zeiss, MetaFluor, ImageJ, Matlab, Microsoft Excel, Origin, and GraphPad Prism. All electrophysiology data analysis was performed in Clampfit, Microsoft Excel, and GraphPad Prism. Structures of TA and EGCG were depicted using ChemDraw 18.2 (PerkinElmer Life Sciences).

**Data availability**

All data are contained within the article.

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**Author contributions**—T. L., S. C. L., Y. Z., and P. L. formal analysis; T. L., S. C. L., Y. Z., and P. L. validation; T. L., S. C. L., Y. Z., and P. L. investigation; T. L. and Y. Z. methodology; T. L. and H. Y. writing—original draft; T. L. and H. Y. writing—review and editing; Y. Z. software; Y. Z. visualization; H. Y. conceptualization; H. Y. resources; H. Y. supervision; H. Y. project administration.

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**Abbreviations**—The abbreviations used are: CaPLSase, Ca2+-activated phospholipid scramblase; PLL, poly-l-lysine; PS, phosphatidylserine; TA, tannic acid; EGCG, epigallocatechin gallate; AnV, annexin V; WGA, wheat germ agglutinin; STS, staurosporine; HBSS, Hanks’ balanced salt solution; NA, numerical aperture; DIC, differential interference contrast.

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