

RESEARCH PAPER

Genistein potentiates activity of the cation channel TRPC5 independently of tyrosine kinases

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Background and purpose: TRPC5 is a Ca²⁺-permeable channel with multiple modes of activation. We have explored the effects of genistein, a plant-derived isoflavone, on TRPC5 activity, and the mechanism(s) involved.

Experimental approach: Effects of genistein on TRPC5 channels were investigated in TRPC5-over-expressing human embryonic kidney 293 (HEK) cells and bovine aortic endothelial cells (BAECs) using fluorescent Ca²⁺ imaging and electrophysiological techniques.

Key results: In TRPC5-over-expressing HEK cells, genistein stimulated TRPC5-mediated Ca²⁺ influx, concentration dependently (EC₅₀ = 93 µM). Genistein and lanthanum activated TRPC5 channels synergistically. Effects of genistein on TRPC5 channels were mimicked by daidzein (100 µM), a genistein analogue inactive as a tyrosine kinase inhibitor, but not by known tyrosine kinase inhibitors herbimycin (2 µM), PP2 (20 µM) and lavendustin A (10 µM). Action of genistein on TRPC5 channels was not affected by an oestrogen receptor inhibitor ICI-182780 (50 µM) or a phospholipase C inhibitor U73122 (10 µM), suggesting genistein did not act through oestrogen receptors or phospholipase C. In BAECs, genistein (100 µM) stimulated TRPC5-mediated Ca²⁺ influx. In patch clamp studies, both genistein (50 µM) and daidzein (50 µM) augmented TRPC5-mediated whole-cell cation current in TRPC5 over-expressing HEK cells. Genistein stimulated TRPC5 channel activity in excised inside-out membrane patch, suggesting that its action was relatively direct and did not require cytosolic factors.

Conclusions and implications: The present study is the first to demonstrate stimulation of a TRP channel by isoflavones. Genistein is a lipophilic compound able to stimulate TRPC5 activity in TRPC5-over-expressing HEK cells and in native vascular endothelial cells.

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Abbreviations: AM, acetoxymethyl ester; 2-APB, 2-aminoethoxydiphenyl borate; BAEC, bovine aortic endothelial cell; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; FBS, fetal bovine serum; G-418, geneticin; HEK, human embryonic kidney; IgG, immunoglobulin G; OAG, 1-oleoyl-2-acetyl-sn-glycerol; pS, piconSiemens; TRP, transient receptor potential; TRPC5, canonical transient receptor potential isoform 5

Introduction

TRPC5 is a Ca²⁺-permeable non-selective cation channel (nomenclature follows Alexander *et al.*, 2009) that is expressed in many different tissues/cells (Beech, 2007b). Functionally, the channel plays an important role in a variety of physiological processes including neuronal growth cone extension (Greka *et al.*, 2003) and smooth muscle motility (Xu *et al.*, 2006b). Excessive expression of TRPC5 channels is

associated with cardiovascular disorders including hypertension (Liu *et al.*, 2006) and heart failure (Bush *et al.*, 2006).

There are diverse modes of activation for TRPC5 channels (Zeng *et al.*, 2004). The channel can be activated by Ca²⁺ store depletion, lanthanides, lysophosphatidylcholine, extracellular thioredoxin and vesicular trafficking (Beech, 2007a,b; Xu *et al.*, 2008). The channel activity is also stimulated following the activation of G-protein-coupled receptors and/or receptor tyrosine kinases (Schaefer *et al.*, 2000). Although TRPC5 can be activated by multiple factors, there is still insufficient knowledge on the endogenous agonist(s) that directly opens the channel. In this regard, a recent study suggests that reduced thioredoxin may directly activate TRPC5 by breaking a disulphide bridge in the predicted extracellular pore loop adjacent to the ion selectivity filter of the channel (Xu *et al.*, 2008). There is also evidence that lysophospholipids can

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activate TRPC5 through a relatively direct action, probably by causing a physical distortion of lipid bilayers (Xu *et al.*, 2006a).

Plant-derived isoflavones have received considerable attention for their potential beneficial properties in protecting against cancer, heart diseases, rheumatoid arthritis, Alzheimer's disease and other pathological conditions (Si and Liu, 2007). Genistein is one of the most widely studied isoflavones and is a phytoestrogen that can act on oestrogen receptors at nanomolar level (Escande *et al.*, 2006). At the concentration range of 10–100 μM , it also inhibits tyrosine kinases (Barnes *et al.*, 2000). In addition, genistein may exert a direct, non-catalytic blocking effect on several ion channels, including L-type Ca^{2+} channels (Chiang *et al.*, 1996; Belevych *et al.*, 2002), voltage-gated Na^{+} channels (Paillart *et al.*, 1997), voltage-gated K^{+} channels (Smirnov and Aaronson, 1995) and inwardly rectifying K^{+} channels (Chiang *et al.*, 2002), in a tyrosine kinase-independent manner.

In the present study, we used the live-cell fluorescent calcium imaging and patch clamp technique to study the effect of genistein on TRPC5 channels. We found that genistein was capable of stimulating TRPC5 activity in human embryonic kidney (HEK) cells that heterologously expressed TRPC5 channels. This stimulatory effect was independent of tyrosine kinase activity and oestrogen receptors. Genistein also increased TRPC5 channel open probability as recorded in excised membrane patches, suggesting a relatively direct action. Furthermore, genistein also potentiated the stimulating action of La^{3+} on TRPC5 activity. To our knowledge, this is the first report showing an action of genistein on a TRP cation channel.

Methods

Cell culture and stable transfection

Mouse TRPC5 channel clone was a generous gift from Dr L Birnbaumer, NIH. The full-length cDNA was subcloned into pcDNA3 and transfected into HEK cells (ATCC). Transfection was done as described elsewhere (Kwan *et al.*, 2004). Briefly, 4 μg of DNA plasmid was transfected into HEK cells using 6 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 200 μL Opti-MEM reduced serum medium (Invitrogen) in six-well plates, which contained $\sim 6 \times 10^4$ cells per well. HEK cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). Then, 1 $\text{mg}\cdot\text{mL}^{-1}$ G-418 (Invitrogen) was added during culture to establish a stable TRPC5-expressing cell line. The primary cultures of bovine aortic endothelial cells (BAECs) were prepared from bovine aorta as described previously (Cheng *et al.*, 2008). Briefly, bovine aortic segments were cut open longitudinally. The intima layer was peeled off and then digested with 0.1% collagenase in phosphate-buffered saline (PBS; in mM: 140, NaCl; 3, KCl; 25, Tris, pH 7.4) for 15 min at 37°C under vigorous shaking. Dissociated cells were centrifuged, resuspended and then grown in a culture medium RPMI-1640 (Invitrogen) supplemented with 10% FBS. Ca^{2+} imaging experiments with BAECs were carried out 2 days after seeding the cells on glass coverslips.

Immunoblots

The procedures were as described elsewhere (Kwan *et al.*, 2004). Briefly, whole-cell lysates were extracted with detergent extraction buffer, which contained 1% (v/v) Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, with addition of protease inhibitor cocktail tablets. Extracted proteins (80 μg) were applied to each lane and separated on 7.5% sodium dodecyl sulphate/polyacrylamide gel electrophoresis. The proteins were then blotted onto a PVDF membrane. The membrane was incubated at 4°C overnight with anti-TRPC5 antibody (dilution 1:200; Alomone Laboratory, Jerusalem, Israel) in PBS buffer containing 0.1% Tween 20 and 5% non-fat dry milk. Immunodetection was accomplished with horseradish peroxidase-conjugated secondary antibodies, followed by the ECL detection system. Immunoblots with anti- β -tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to confirm that an equal amount of proteins was loaded onto each lane. The intensity of the bands was analysed by FluorChem 8000 imaging system (Alpha Innotech, San Leandro, CA, USA).

Immunodetection of TRPC5 channels in BAECs

BAECs were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100. Non-specific immunostaining was blocked by pre-incubating the cells with 5% BSA. The fixed cells were incubated with or without anti-TRPC5 (dilution 1:100, Alomone Laboratory) at 4°C overnight, followed by incubation with secondary goat anti-rabbit IgG conjugated to Alexa Fluor 546 (1:200). Immunofluorescence was detected by a FV1000 confocal system (Olympus, Tokyo, Japan). Fluorescence signals were acquired at the settings under which the signals from the control cells (without anti-TRPC5) were absent.

Preparation of a TRPC5-blocking antibody T5E3

T5E3 was raised in rabbits using the strategy developed by Xu *et al.* (2005b). Briefly, a peptide corresponding to TRPC5 channel putative pore region (CYETRAIDEPNCKG) conjugated to keyhole limpet haemocyanin (Alpha Diagnostic International, San Antonio, TX, USA) was injected subcutaneously in the back of a rabbit followed by two boost doses. T5E3 antiserum was collected 4 weeks after the second boost. IgG was purified from T5E3 antiserum and pre-immune serum using a protein G column. The specificity of T5E3 on TRPC5 channel activity has been previously demonstrated by Dr Beech's lab (Xu *et al.*, 2006a). We also tested the specificity of our batches of T5E3. The results showed that T5E3 inhibited carbachol-induced activation of TRPC5 channels in TRPC5-over-expressing HEK cells, but it had no effect on 1-oleoyl-2-acetyl-sn-glycerol (OAG)-induced rise of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in TRPC3-expressing HEK cells (data not shown), confirming the specificity of T5E3 to TRPC5.

Measurement of $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was measured as described elsewhere with slight modification (Kwan *et al.*, 2004). Briefly, cells were seeded onto poly-L-lysine-coated glass discs 1 day before $[\text{Ca}^{2+}]_i$

measurement. The cells were loaded for 1 h in dark with 5 μM Fluo-3/AM and 0.02% Pluronic F-127 in culture media, before mounting onto a microscope chamber containing the bath solution. Normal physiological saline solution (NPSS) contained in mM: 140, NaCl; 5, KCl; 2, MgCl_2 ; 1, CaCl_2 ; 10, glucose; 10, HEPES, pH 7.4. 0Ca^{2+} -PSS contained in mM: 140, NaCl; 5, KCl; 2, MgCl_2 ; 10, glucose; 0.2, EGTA; 10, HEPES, pH 7.4. When needed, cells were pretreated with genistein or LaCl_3 for 10 min. In experiments with BAECs, cells were loaded with 5 μM Fura-2/AM for ratiometric Ca^{2+} -fluorescent measurement, and the bath solution was HBS, which contained in mM: 107, NaCl; 6, KCl; 1.2, MgSO_4 ; 2, CaCl_2 ; 11.5, glucose; 20, HEPES, pH 7.4. Ca^{2+} -free ($0[\text{Ca}^{2+}]_o$) HBS was equivalent to HBS except that there was no CaCl_2 . Fluorescence $[\text{Ca}^{2+}]_i$ signals were recorded by InCyt Basic Fluorescence Imaging System (Intracellular Imaging, Cincinnati, OH). In HEK cell experiments using Fluo-3 indicator, $[\text{Ca}^{2+}]_i$ response was expressed as a ratio (F_i/F_0) of real-time fluorescence (F_i ; excitation at 490 nm) relative to the intensity at the beginning of the experiment (F_0). In experiments with BAECs using Fura-2 indicator, $[\text{Ca}^{2+}]_i$ response was expressed as ratio of the fluorescence signal from excitation at 340 nm to that at 380 nm (F_{340}/F_{380}). All experiments were performed at room temperature ($\sim 23^\circ\text{C}$).

Electrophysiology

TRPC5 channel activity was measured in whole-cell or excised inside-out patch mode as described elsewhere (Shen *et al.*, 2008). Briefly, whole-cell or single-channel activity was recorded using an EPC9 patch clamp amplifier (HEKA, Freiburg, Germany) controlled by Pulse software (HEKA). In time-series recordings of whole-cell current, the currents at +60 and -60 mV were sampled at 5 s intervals after break-in. Whole-cell current-voltage relationship (I/V) was determined from the currents recorded in response to successive voltage pulses of 500 ms duration, increasing in 20 mV increments from -80 to +80 mV. For whole-cell recording, the patch pipette contained in mM: 145, CsCl; 2, MgCl_2 ; 10, glucose; 0.2, ATP; 10, HEPES, pH 7.2 titrated with CsOH. The bath contained NPSS. Free Ca^{2+} in the deionized water was estimated to be <50 pM. Although deionized water was used to prepare all solutions, there would be some calcium contamination in the major salts used (CsCl, NaCl). For the excised inside-out patches, the pipette solution was NPSS, and the bath solution contained in mM: 135, CsCl; 2, MgCl_2 ; 1, EGTA; 20, HEPES, pH 7.2 titrated with CsOH. All currents were sampled at 50 kHz and filtered at 3 kHz, and the data were analysed with PulseFit and TAC. Electrophysiological experiments were performed at room temperature ($\sim 23^\circ\text{C}$).

Data analysis and presentation

Data points in Ca^{2+} -imaging traces represent the mean \pm SEM from cells in one experiment. Bar charts represent means \pm SEM of multiple experiments. Current-voltage curves in electrophysiology were means \pm SEM of multiple experiments with number of experiments given as n in parenthesis. Student's t -test was used for statistical comparison, with $P < 0.05$ (*) or $P < 0.01$ (**) as a significant difference. Half-maximal response of TRPC5 channels to genistein (EC_{50}) was

determined by curve-fitting with Hill's equation using Prism 3.0 (GraphPad software).

Materials

Genistein, PP2, lavaendustin A, carbachol, OAG and 2-aminoethoxydiphenyl borate (2-APB) were from Calbiochem/Merck KGaA (Darmstadt, Germany). Daidzein, herbimycin, LaCl_3 and dimethyl sulphoxide (DMSO) were from Sigma (St Louis, MO, USA). ICI-182780 was from Tocris Bioscience (Bristol, UK).

Results

Effect of genistein on TRPC5 channel current in TRPC5-over-expressing cells

TRPC5 cDNA was stably transfected into HEK cells. Compared to vector-transfected cells, these TRPC5 cDNA-transfected cells had a higher TRPC5 protein level on Western blots (Figure 1A). In whole-cell patch clamp recordings, bath application of 50 μM genistein to TRPC5-over-expressing cells induced an outward current at +60 mV and an inward current at -60 mV, peaking at around 5 min after application (Figure 1B-D). Consistent with the reported characteristics of TRPC5 channels (Jung *et al.*, 2003; Xu *et al.*, 2005a), extracellular LaCl_3 (100 μM) enhanced the currents, whereas 2-APB (75 μM) abolished them (Figure 1B and C). Current-voltage relationship of the genistein-stimulated currents displays double rectification (Figure 1F), which is characteristic of TRPC5 channels (Beech, 2007b). In control experiments, genistein (50 μM) failed to increase whole-cell current in wild-type HEK cells (Figure 1H). Because genistein stock was prepared in DMSO, a vehicle control experiment was also performed. Addition of vehicle (DMSO, 0.1%) also had no effect on the whole-cell current (Figure 1G).

Single-channel recordings were made in inside-out membrane patches excised from TRPC5-over-expressing cells. A low level of basal channel activity could be observed before any additions (Figure 2A and B). Application of genistein (50 μM) to the cytoplasmic side caused a significant increase in channel activity (Figure 2B-D), while application of vehicle (DMSO, 0.1%) did not increase the channel activity (Figure 2A). The channel activity was blocked by 75 μM 2-APB (data not shown). Single-channel current-voltage relationship was obtained (Figure 2E), and the single-channel slope conductance was estimated to be ~ 46 pS.

Effect of genistein on TRPC5 channel-mediated rise in $[\text{Ca}^{2+}]_i$

Fluorescence Ca^{2+} imaging was used to test the effect of genistein on TRPC5-mediated Ca^{2+} influx; 100 μM La^{3+} was included in the bath to block other non-selective cation channels and to select for TRPC5-mediated Ca^{2+} influx. In one protocol, cells bathed in 0Ca^{2+} -PSS were first treated with a muscarinic agonist carbachol (100 μM), which initiated a $[\text{Ca}^{2+}]_i$ rise (Figure 3A and B) by releasing Ca^{2+} from intracellular Ca^{2+} stores (Schaefer *et al.*, 2000). Subsequent addition of extracellular Ca^{2+} (1 mM) evoked another $[\text{Ca}^{2+}]_i$ rise in TRPC5-transfected (Figure 3A), but not in vector-transfected

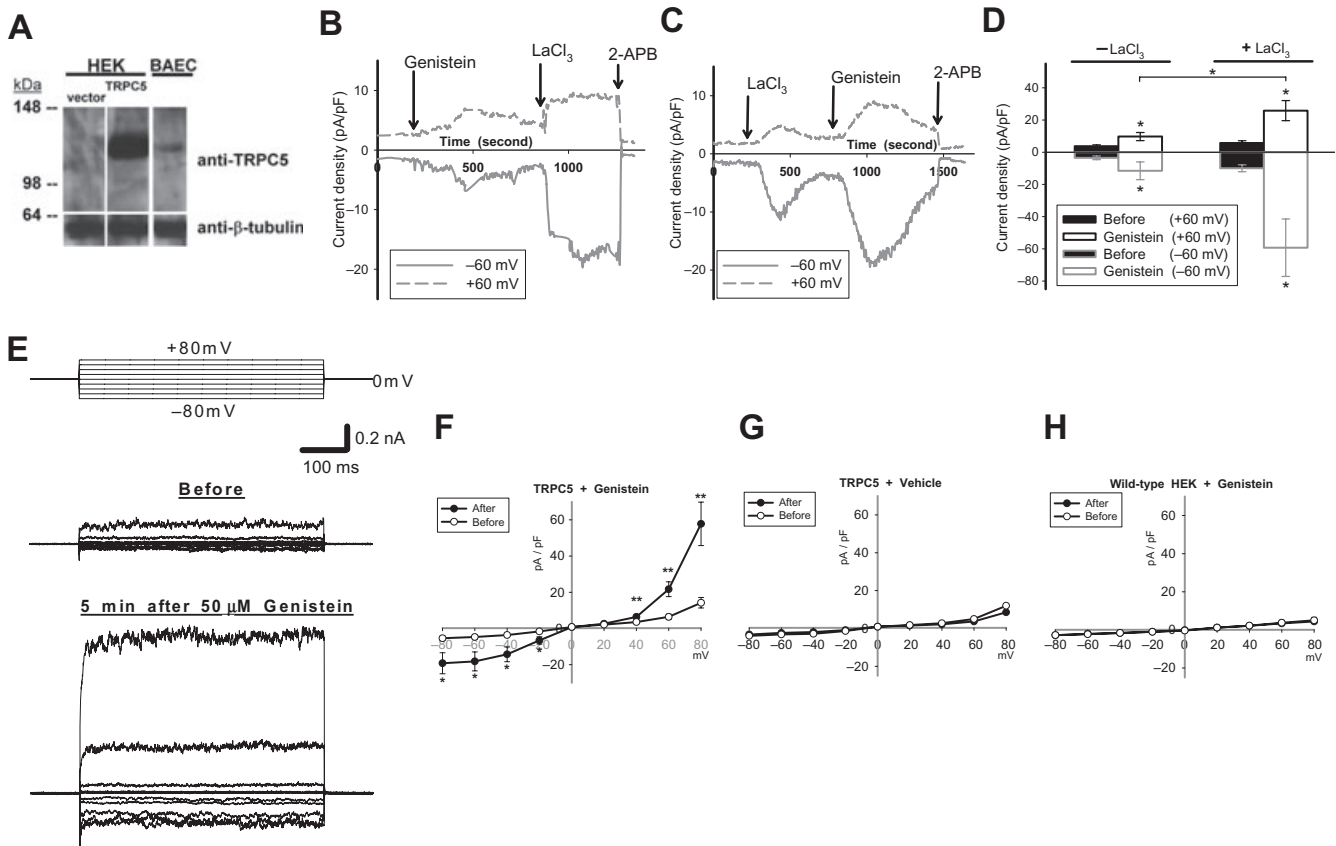


Figure 1 Effect of genistein on TRPC5 channel activity in HEK cells. (A) Representative immunoblot probed with anti-TRPC5 and anti- β -tubulin ($n = 3$). Left panel, pcDNA3-transfected HEK cells (vector); middle panel, TRPC5-transfected HEK cells; right panel, BAECs. (B and C) Representative time-course ($n = 5-6$) of whole-cell currents in TRPC5-over-expressing HEK cells. Currents were sampled every 5 s at +60 and -60 mV. Genistein (50 μ M), LaCl_3 (100 μ M) and 2-APB (75 μ M) were applied to the bath at the time-points indicated by arrows. (D) Summary of maximal whole-cell currents before and after genistein addition at +60 and -60 mV in TRPC5-over-expressing cells. + LaCl_3 , with 100 μ M LaCl_3 ; - LaCl_3 , without LaCl_3 . Mean \pm SEM ($n = 5-6$). (E) Representative traces showing voltage protocol (top) and corresponding whole-cell currents immediately before (middle) and 5 min after (bottom) bath application of 50 μ M genistein in TRPC5-over-expressing HEK cells. (F) Current-voltage relationships as in (E). (G and H) Current-voltage relationships in TRPC5-over-expressing (G) or wild-type HEK cells (H) immediately before and 5 min after bath application of 50 μ M genistein (H) or 0.1% DMSO (G). Mean \pm SEM ($n = 3-8$). * $P < 0.05$; ** $P < 0.01$ compared to 'before'.

HEK cells (Figure 3B). These results are consistent with a previous report which showed a stimulating action of carbachol on TRPC5 channels via a receptor-operated mechanism (Schaefer *et al.*, 2000). Interestingly, addition of genistein (50 μ M) into the bath caused a further rise in $[\text{Ca}^{2+}]_i$ (Figure 3A). In another protocol, cells bathed in 0Ca^{2+} -PSS were pretreated with genistein (50 μ M) for 10 min, followed by the addition of extracellular Ca^{2+} , which elicited a marked rise in $[\text{Ca}^{2+}]_i$ in TRPC5-over-expressing cells (Figure 3C), but not in cells that were transfected with vector plasmid (Figure 3D). Vehicle control experiments were performed, in which the cells were pretreated with 0.1% DMSO (vehicle) (Figure 3C and D). As expected, the $[\text{Ca}^{2+}]_i$ rise in response to extracellular Ca^{2+} addition was much smaller in vehicle-pretreated cells than in genistein-pretreated cells (Figure 3C).

Synergistic action of genistein and La^{3+} on TRPC5 channels

We further tested a range of genistein concentrations (10–800 μ M) on TRPC5 channel activity. Bath application of genistein elicited a $[\text{Ca}^{2+}]_i$ rise on TRPC5-over-expressing cells

in a concentration-dependent manner (Figure 4A–C). Analysis of the concentration–response curve yielded a half-maximal response (EC_{50}) at ~ 93 μ M (Figure 4C). Interestingly, when bath solution contained 100 μ M LaCl_3 , application of genistein induced a larger $[\text{Ca}^{2+}]_i$ rise (Figure 4B and C) and a larger whole-cell current (Figure 1D). Furthermore, La^{3+} shifted the dose–response curve of genistein to the left, resulting in a lower EC_{50} at ~ 51 μ M (Figure 4C). Conversely, the presence of genistein (50 and 100 μ M) in bath solution also potentiated the $[\text{Ca}^{2+}]_i$ response to extracellular La^{3+} (Figure 4D and E). These experiments indicate that genistein and La^{3+} act synergistically to stimulate TRPC5 channels.

Effects of genistein were independent of oestrogen receptors and phospholipase C activity

An oestrogen receptor antagonist ICI-182780 was used to determine if the genistein effect is mediated through the classic oestrogen receptor α or β . As shown in Figure 5, pre-incubation of cells with ICI-182780 (50 μ M) had no effect on genistein (100 μ M)-induced Ca^{2+} influx in

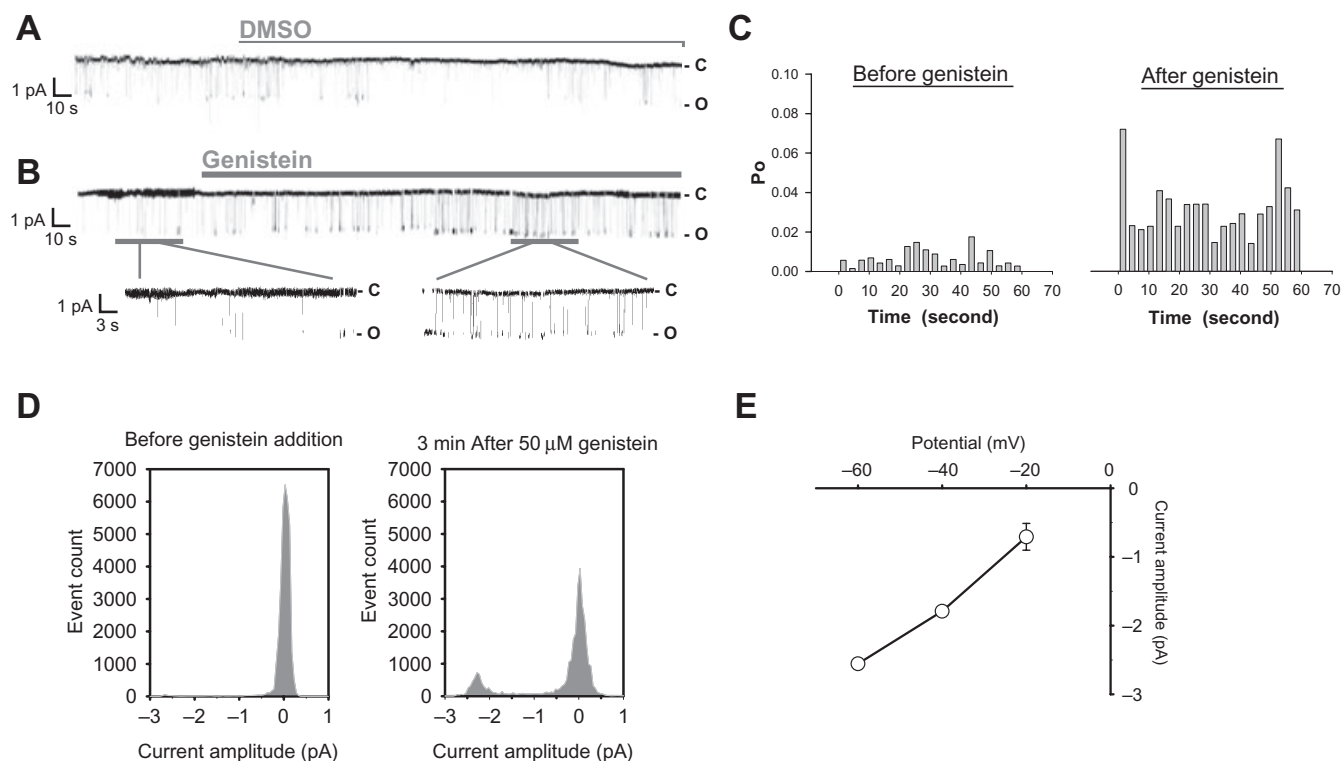


Figure 2 Single-channel recording of genistein-modulated channels in excised inside-out patches from TRPC5-over-expressing HEK cells. (A and B) Single-channel traces of membrane patches excised from TRPC5-over-expressing HEK cells; 0.1% DMSO (A) or 50 μM genistein (B) was applied as indicated by the horizontal bar. The patch potential was held at -60 mV. C, close state; O, open state. (C) Channel open probability (P_o) values of the patch (as in A and B) in 3 s intervals. (D) Event histogram of a representative experiment before and after 50 μM genistein application at -60 mV. (E) Single-channel current–voltage relationship of the genistein-modulated channel. Mean \pm SEM ($n = 3$ –5).

TRPC5-over-expressing HEK cells (Figure 5A). Other earlier reports have shown that receptor activation of TRPC5 channels relies on phospholipase C activity (Schaefer *et al.*, 2000; Beech, 2007b). However, pretreatment of cells with a phospholipase C inhibitor U73122 (10 μM) did not affect the $[\text{Ca}^{2+}]_i$ response to genistein (Figure 5B). U73343 (10 μM), an inactive analogue of U73122, also had no effect (Figure 5B). Control experiments were performed to verify the inhibitory effect of U73122 on phospholipase C activity. The results showed that a 30 min pretreatment with U73122 (10 μM) was able to inhibit carbachol-induced $[\text{Ca}^{2+}]_i$ rise in wild-type HEK cells (F_1/F_0 of 1.53 ± 0.24 ($n = 4$) in U73122-treated cells vs. 5.96 ± 0.47 ($n = 4$) in U73343-treated cells), confirming the effectiveness of U73122 towards phospholipase C. Taken together, these data suggest that the effect of genistein on TRPC5 channels was not mediated through oestrogen receptors or phospholipase C.

Effect of tyrosine kinase inhibitors and daidzein on TRPC5-mediated rise in $[\text{Ca}^{2+}]_i$

To determine if the effect of genistein was related to its inhibitory action on tyrosine kinases, a panel of other tyrosine kinase inhibitors was tested. These include a wide-spectrum tyrosine kinase inhibitor (herbimycin), an src family tyrosine kinase inhibitor (PP2) and an inhibitor for epidermal growth factor receptor tyrosine kinase (lavendustin A). Unlike genistein, herbimycin (2 μM), PP2 (20 μM) and lavendustin A

(10 μM) all failed to stimulate TRPC5 channels (Figure 5D and E). In contrast, daidzein, which is a genistein analogue not active as an inhibitor of tyrosine kinases, was capable of stimulating TRPC5 channels (Figure 5C and E).

Effect of daidzein on TRPC5 channel whole-cell currents

In agreement with the $[\text{Ca}^{2+}]_i$ measurement experiments, treatment of TRPC5-over-expressing HEK cells with daidzein (50 μM) increased the whole-cell currents at $+60$ and -60 mV (Figure 6A–C). Similar to the genistein-stimulated currents (Figure 1B), daidzein-stimulated currents also peaked at around 5 min (Figure 6A). Current–voltage relationship of the daidzein-stimulated currents displays double rectification (Figure 6C), a characteristic of TRPC5 channels. These results are consistent with the notion that genistein potentiates TRPC5 channel activity, independently of tyrosine kinases.

Effect of genistein on TRPC5 channels in native endothelial cells

TRPC5 is known to be expressed in primary cultures of BAECs (Yao and Garland, 2005). This was confirmed in the present study by Western blot and immunochemical staining of cultured BAECs using a TRPC5-specific antibody (Alomone Laboratory, 1:100) (Figures 1A and 7A). In functional studies, bath application of vehicle (DMSO, 0.1%) and 1 μM genistein did not increase $[\text{Ca}^{2+}]_i$ (Figure 7B and C), but, at higher concentrations (25–200 μM), genistein did elevate $[\text{Ca}^{2+}]_i$ in these

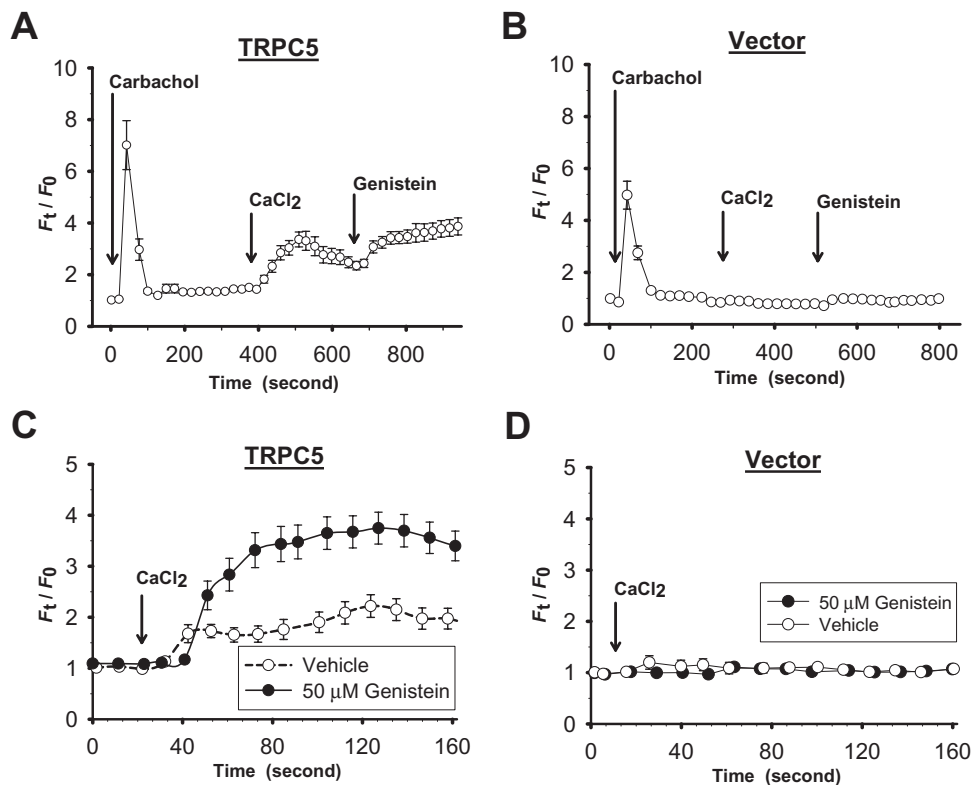


Figure 3 Effect of genistein on TRPC5-mediated $[Ca^{2+}]_i$ rise. (A–D) Representative time-course of $[Ca^{2+}]_i$ change in vector-transfected (B, D) and TRPC5-transfected HEK cells (A, C). (C and D) Cells were pretreated with genistein (50 μ M) or vehicle (0.1% DMSO) for 10 min in $0Ca^{2+}$ -PSS. In (A–D), 100 μ M La^{3+} was included in the bath to block other non-selective cation channels and to select for TRPC5-mediated Ca^{2+} influx. Each trace represents mean \pm SEM of at least 20 cells in one representative experiment ($n = 4$ –7 experiments).

cells (Figure 7B and C). This $[Ca^{2+}]_i$ rise was mostly due to Ca^{2+} influx because it was almost absent in the cells that were bathed in a Ca^{2+} -free medium (Figure 7E). As the $[Ca^{2+}]_i$ response was not further enhanced when the genistein concentration was increased from 100 to 200 μ M (Figure 7C), 100 μ M was chosen as the standard concentration for further study of the effects of genistein on TRPC5 channels in BAECs (Figure 7D and E). As shown, 100 μ M genistein induced a $[Ca^{2+}]_i$ rise in BAECs pre-incubated with the pre-immune IgG (Figure 7D and E). In contrast, this genistein-induced $[Ca^{2+}]_i$ rise was diminished in cells that had been pre-incubated with a TRPC5-specific blocking antibody T5E3 (Figure 7D and E), suggesting an involvement of TRPC5 channels. However, note that the $[Ca^{2+}]_i$ response to genistein was rather small, it is probably due to a relatively lower expression of TRPC5 channels in BAECs compared to that in TRPC5-over-expressing HEK cells.

Discussion and conclusions

In the present study, we found that genistein augmented TRPC5-mediated Ca^{2+} influx in TRPC5-over-expressing HEK cells and primary cultures of BAECs. Genistein also enhanced TRPC5-mediated cation current. The stimulating effect of genistein synergized with La^{3+} , which is a well-documented potentiator for TRPC5 channels. The effect of genistein could not be mimicked by several other tyrosine kinase inhibitors,

but it could be mimicked by daidzein, which is inactive as a tyrosine kinase inhibitor. This action of genistein was not mediated through oestrogen receptors or phospholipase C, because selective inhibitors against these targets had no effect. Importantly, the stimulating action of genistein on TRPC5 channels could be demonstrated in excised inside-out patches. Taken together, these data suggest that the action of genistein on TRPC5 channels is relatively direct and may not require a cytosolic factor.

Several different protocols were used to determine whether genistein could indeed enhance TRPC5 activity in HEK cells that heterologously expressed TRPC5 channels. In the first protocol, the TRPC5-over-expressing cells were treated with carbachol in a Ca^{2+} -free medium, followed by extracellular Ca^{2+} addition, which initiated a Ca^{2+} influx. Genistein was then added. It was found that the genistein application elicited a further Ca^{2+} rise. In the second protocol, cells were pretreated with genistein in a Ca^{2+} -free medium, followed by extracellular Ca^{2+} addition, which elicited a $[Ca^{2+}]_i$ rise. It was found that the magnitude of this $[Ca^{2+}]_i$ rise was much larger in genistein-pretreated cells than those without genistein pretreatment. In the third protocol, direct addition of genistein also caused a $[Ca^{2+}]_i$ rise in cells bathed in a Ca^{2+} -containing physiological solution. Furthermore, in patch clamp studies, genistein stimulated TRPC5-mediated cation current in whole-cell patches and in excised inside-out patches. These data confirmed a stimulating action of genistein on TRPC5 channel activity. Interestingly, we observed that La^{3+} and

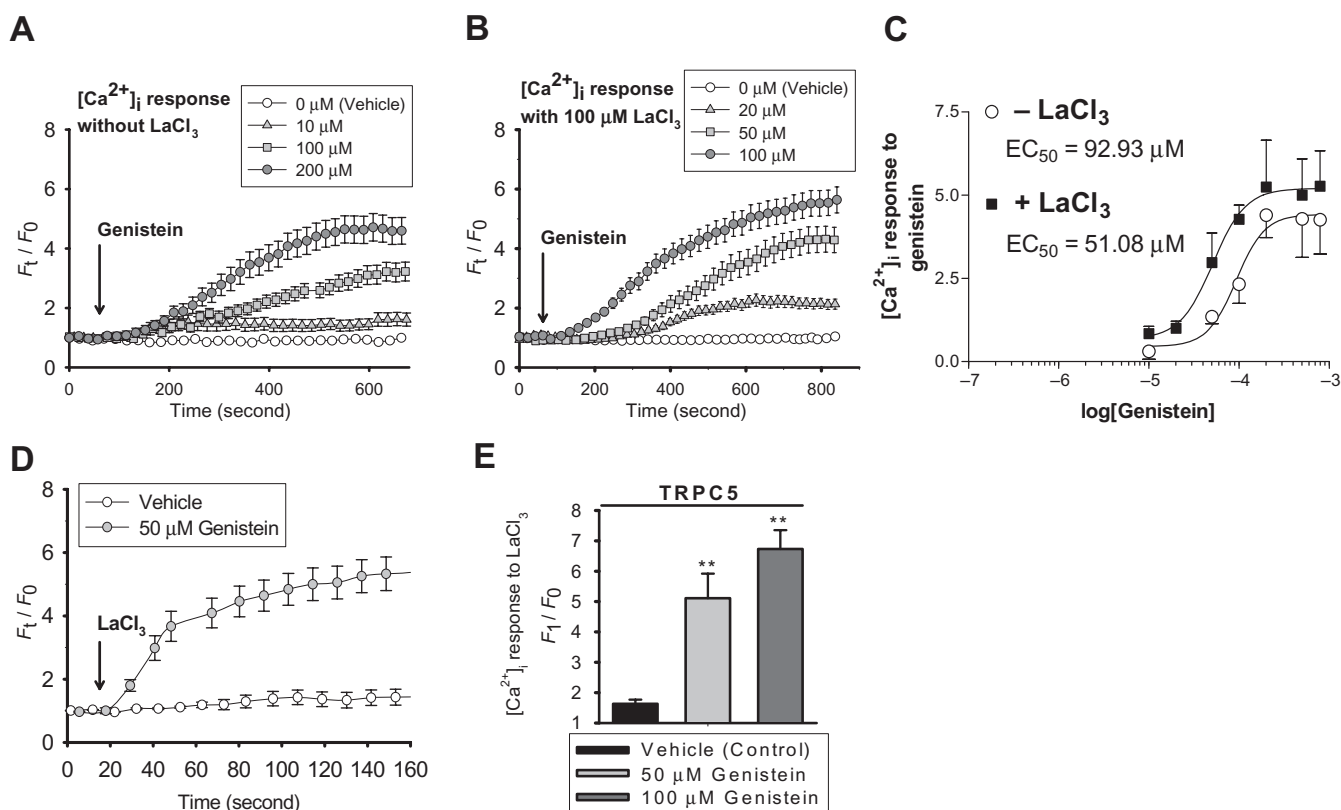


Figure 4 Concentration-dependent stimulation of TRPC5 channels by genistein and synergy between genistein and La^{3+} . (A and B) Representative time-course of $[\text{Ca}^{2+}]_i$ change in TRPC5-transfected HEK cells in response to different concentrations of genistein. Cells were bathed in NPSS (A) or NPSS + 100 μM LaCl_3 (B). (C) Plot of concentration–response with curve fitting to determine the genistein concentration for half-maximal response (EC_{50}). $[\text{Ca}^{2+}]_i$ response at each concentration was subtracted by the background given by vehicle control; means \pm SEM ($n = 4\text{--}12$). (D) Representative time-course of La^{3+} (100 μM)-induced $[\text{Ca}^{2+}]_i$ change in TRPC5-expressing HEK cells in the absence or presence of 50 μM genistein. (E) Summary of data showing the maximal $[\text{Ca}^{2+}]_i$ rise in response to La^{3+} . For A, B and D, each trace represents means \pm SEM of at least 20 cells from one representative experiment ($n = 4\text{--}10$ experiments). Vehicle contained 0.1% DMSO. $**P < 0.01$, compared to vehicle.

genistein act synergistically on TRPC5 channels. La^{3+} ions are known to interact with the negatively charged amino acid residues near the extracellular pore region of TRPC5 proteins, thereby potentiating the channel opening (Jung *et al.*, 2003). In the present study, La^{3+} was able to potentiate the stimulating action of genistein on TRPC5 channels, and genistein was capable of potentiating the action of La^{3+} on TRPC5. The exact mechanism for the observed synergy is not clear. However, one possibility could be that La^{3+} (or genistein) elevates $[\text{Ca}^{2+}]_i$, which may in turn sensitize TRPC5, resulting in an enhanced response to genistein (or La^{3+}). This interpretation is supported by a recent study which showed a potentiation by high $[\text{Ca}^{2+}]_i$ on agonist-induced activation of TRPC5 (Blair *et al.*, 2009).

Genistein is a phytoestrogen that can act on oestrogen receptors at nanomolar level (Escande *et al.*, 2006). Activity of oestrogen receptor can alter ion homeostasis in a variety of tissues and cells including the CNS, vascular system and pancreatic endocrine cells (Kelly and Levin, 2001; Nadal *et al.*, 2004; Raz *et al.*, 2008). In the present study, an oestrogen receptor antagonist ICI-182780 had no effect on the stimulating action of genistein on TRPC5, suggesting that the action is independent of classical oestrogen receptors. Genistein is also commonly used as a tyrosine kinase inhibitor,

over the concentration range of 10–100 μM (Barnes *et al.*, 2000). Indeed, genistein was found to inhibit ATP-sensitive K^+ channels, N-type Ca^{2+} channels, T-type channels, TRPC3 and TRPM2 channels, via a tyrosine kinase-dependent mechanism (Ogata *et al.*, 1997; Morikawa *et al.*, 1998; Kawasaki *et al.*, 2006; Zhang *et al.*, 2007). In the present study, we found that the genistein action could not be mimicked by other tyrosine kinase inhibitors including herbimycin, PP2 and lavendustin A. In addition, the action of genistein could be mimicked by daidzein, which is inactive as a tyrosine kinase inhibitor. Therefore, the genistein effect on TRPC5 channels was independent of tyrosine kinases. It has been reported that the membrane-anchored phospholipase C is critical to the receptor-operated TRPC5 activation (Schaefer *et al.*, 2000). However, in the present study, inhibition of phospholipase C by U73122 had no effect on the genistein action on TRPC5, suggesting that the genistein action on TRPC5 is not mediated through phospholipase C.

Evidence shows that genistein may inhibit multiple ion channels via a relatively direct mechanism. These include L-type Ca^{2+} channels (Chiang *et al.*, 1996; Belevych *et al.*, 2002), voltage-gated Na^+ channels (Paillart *et al.*, 1997), voltage-gated K^+ channels (Smirnov and Aaronson, 1995) and inwardly rectifying K^+ channels (Chiang *et al.*, 2002). Unlike

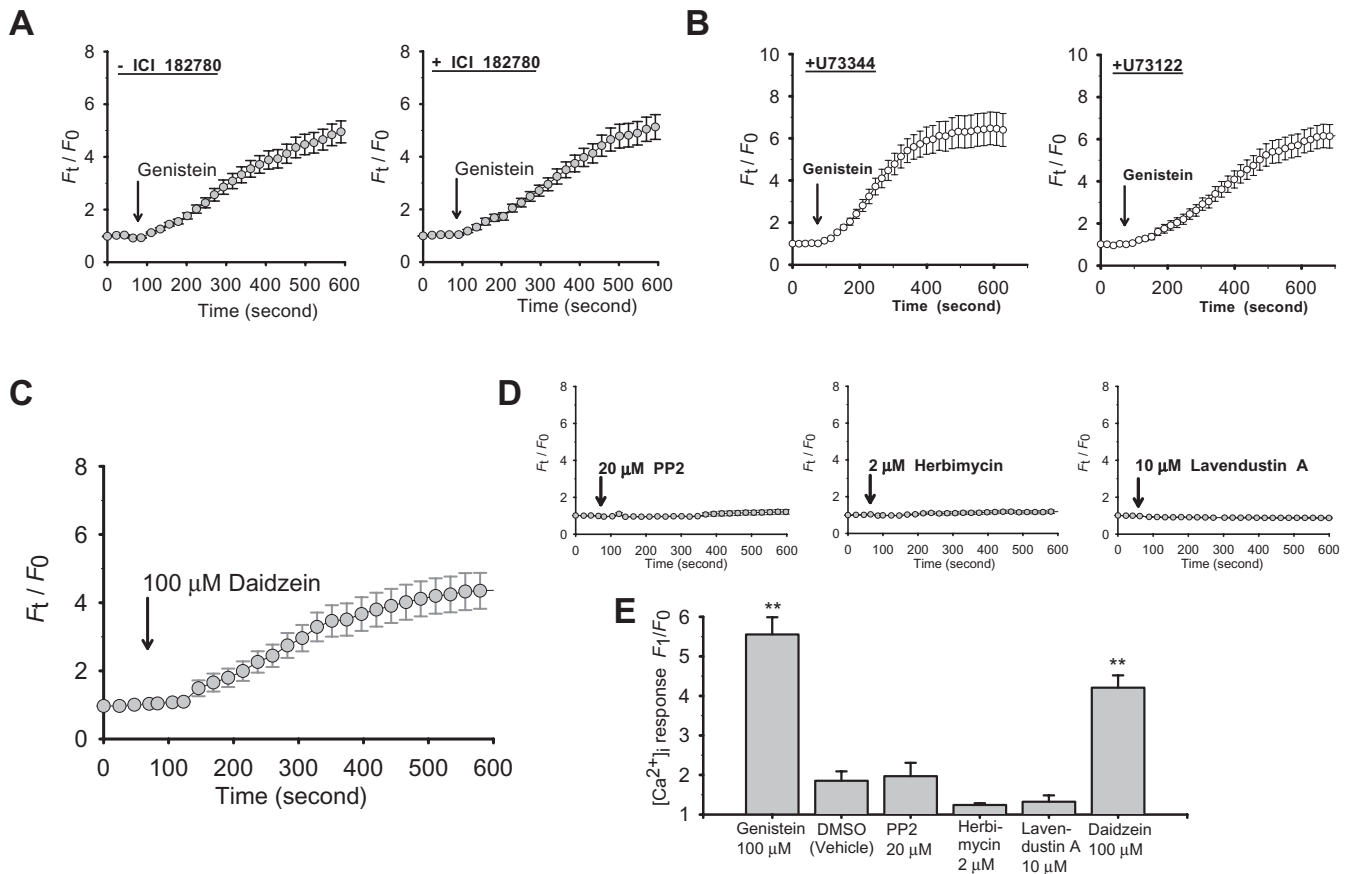


Figure 5 Effect of ICI-182780, U73122, a panel of tyrosine kinase inhibitors and an inactive genistein analogue, daidzein, on TRPC5-mediated $[Ca^{2+}]_i$ rise. (A and B) Representative time-course of $[Ca^{2+}]_i$ change in response to 100 μ M genistein in TRPC5-over-expressing HEK cells in the absence (A, left) or the presence (A, right) of 50 μ M ICI-182780, or in the presence of 10 μ M U73343 (B, left) or 10 μ M U73122 (B, right). Cells were pretreated with ICI-182780 for 5 min, or with U73122 or U73343 for 30 min. (C and D) Representative time-course of $[Ca^{2+}]_i$ change in response to 100 μ M daidzein (C), 20 μ M PP2 (D, left), 2 μ M herbimycin (D, middle) or 10 μ M lavendustin A (D, right) in TRPC5-over-expressing HEK cells. Each trace in A–D represents mean \pm SEM of at least 20 cells from one representative experiment ($n = 3–8$). Cells were bathed in NPSS in the presence of 100 μ M $LaCl_3$. (E) Summary of data showing the maximal $[Ca^{2+}]_i$ rise as in A–D. Mean \pm SEM ($n = 3–8$). ** $P < 0.01$, compared to vehicle.

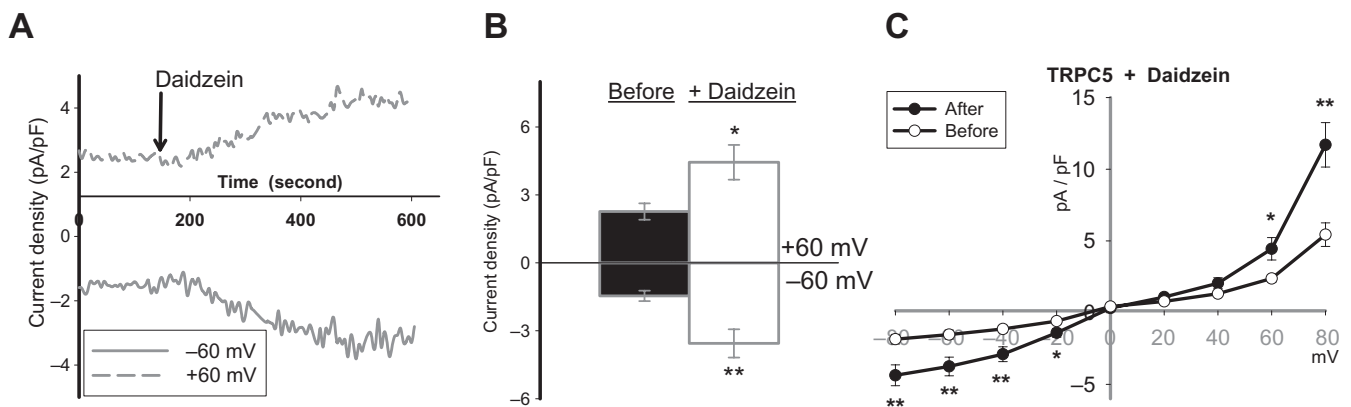


Figure 6 Effect of daidzein on whole-cell currents in TRPC5-over-expressing HEK cells. (A) Representative time-course of whole-cell currents. Currents were sampled every 5 s at +60 and –60 mV. Daidzein (50 μ M) was applied to the bath at the time-point indicated by arrow. (B) Summary of maximal whole-cell currents before (before) and after daidzein (+daidzein) addition at +60 and –60 mV. Mean \pm SEM ($n = 7$). (C) Current–voltage relationships of whole-cell currents immediately before and 5 min after bath application of 50 μ M daidzein. Voltage protocol is the same as in Figure 1E. Mean \pm SEM ($n = 7$). * $P < 0.05$; ** $P < 0.01$ compared to ‘before’.

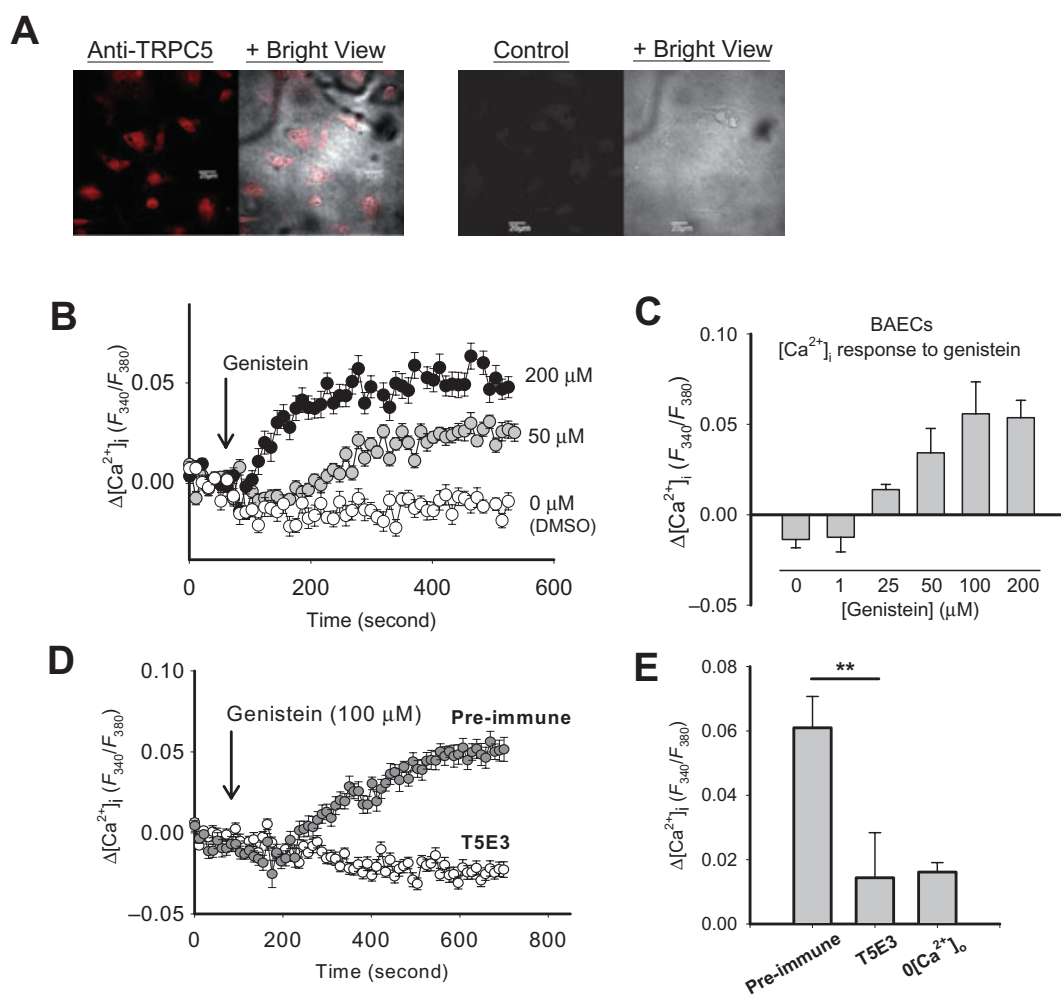


Figure 7 Effect of genistein on TRPC5-mediated $[\text{Ca}^{2+}]_i$ rise in native vascular endothelial cells. (A) Immunocytochemical detection of TRPC5 proteins in BAECs using confocal microscopy. Shown were the paired images of TRPC5 fluorescent signal (red, left panel) and the BrightView image merged with fluorescent signal. The control images (right panel) were from samples without addition of primary antibodies. Scale bar represents 20 μm . (B and D) Representative time-course of $[\text{Ca}^{2+}]_i$ change in response to vehicle (0.1% DMSO) or genistein in BAECs. (D) Pre-incubated with pre-immune IgG (4 $\mu\text{g}\cdot\text{mL}^{-1}$) or T5E3 (antibody to TRPC5 channels; 4 $\mu\text{g}\cdot\text{mL}^{-1}$) for 10 min. Each trace represents mean \pm SEM of at least 15 cells from one representative experiment ($n = 6-12$). (C) Summary of data showing the maximal $[\text{Ca}^{2+}]_i$ changes in response to 0.1% DMSO or various concentrations of genistein. Mean \pm SEM ($n = 6-12$). (E) Summary of data showing $[\text{Ca}^{2+}]_i$ changes 10 min after genistein application in the presence of pre-immune IgG (4 $\mu\text{g}\cdot\text{mL}^{-1}$) or T5E3 (4 $\mu\text{g}\cdot\text{mL}^{-1}$) or Ca^{2+} -free HBS ($0[\text{Ca}^{2+}]_o$). Mean \pm SEM ($n = 3-8$). ** $p < 0.01$.

most of other studies, we found that genistein stimulates TRPC5 channel activity. Furthermore, the effect of genistein was demonstrated in excised inside-out patches, suggesting that the action is relatively direct and did not require a cytosolic factor. Unfortunately, the present study cannot differentiate whether genistein acts on TRPC5 channels intracellularly or extracellularly. The patch clamp study showed that the genistein applied from either side of the membrane was able to stimulate TRPC5 activity (Figures 1–3), but this was expected because genistein is a membrane-permeable agent (Akiyama *et al.*, 1987). The detailed mechanism of activation of TRPC5 channels by genistein is still unclear, but it could be related to the alteration of lipid bilayer properties. Evidence shows that genistein can be adsorbed and partitioned onto the plasma membrane, thus altering elastic properties of lipid bilayer (Arora *et al.*, 2000; Hwang *et al.*, 2003; Whaley *et al.*, 2006). Such an alteration in lipid environment

may cause a change in gating properties of some ion channels. This model has been previously used to explain the genistein-induced activation of gramicidin A channels (Hwang *et al.*, 2003), which are a group of short antibiotic peptides produced by *Bacillus brevis*. We speculate that a similar mechanism may underlie the stimulating action of genistein on TRPC5 channels. In agreement, daidzein, which only differs from genistein by having one less $-\text{OH}$ and is also incorporated into lipid bilayers (Whaley *et al.*, 2006), exerted a similar stimulatory action on TRPC5 channels. Interestingly, several reports have shown that TRPC5 channel activity could be modulated by phosphatidylinositol-4,5-bisphosphate (Trebak *et al.*, 2009), lysophosphatidylcholine (Flemming *et al.*, 2006) and sphingosine 1-phosphate (Beech, 2007a), all of which are capable of inserting into lipid bilayers. Indeed, the TRPC5 channel has been proposed as a sensor for endogenous phospholipids (Flemming *et al.*, 2006; Beech, 2007a,b).

Therefore, it is likely that natural isoflavones, such as genistein and daidzein, may stimulate TRPC5 channels by altering the channel–lipid interactions. The present study is the first to demonstrate the ability of isoflavones to stimulate a TRP channel.

In conclusion, genistein stimulated the activity of TRPC5 channels. The action was neither related to tyrosine kinase inhibition nor to oestrogen receptors. Genistein and La^{3+} ions acted synergistically to stimulate TRPC5 channel activity. The stimulating action of genistein on TRPC5 was mimicked by daidzein, which can also be partitioned into lipid bilayers. Furthermore, the stimulation of TRPC5 channels could be recorded on excised inside-out patches. These data all suggest that the action of genistein was relatively direct. It is possible that genistein enhanced TRPC5 channel activity by modifying the channel–lipid interaction in the lipid bilayer.

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Conflict of interest

None.

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