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Effects of Apigenin on Glutamate-induced $[Ca^{2+}]_i$ Increases in Cultured Rat Hippocampal Neurons

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Flavonoids have been shown to affect calcium signaling in neurons. However, there are no reports on the effect of apigenin on glutamate-induced calcium signaling in neurons. We investigated whether apigenin affects glutamate-induced increase of free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured rat hippocampal neurons, using fura-2-based digital calcium imaging and microfluorimetry. The hippocampal neurons were used between 10 and 13 days in culture from embryonic day 18 rats. Pretreatment of the cells with apigenin (1 μM to 100 μM) for 5 min inhibited glutamate (100 μM, 1 min) induced $[Ca^{2+}]_i$ increase, concentration-dependently. Pretreatment with apigenin (30 μM) for 5 min significantly decreased the $[Ca^{2+}]_i$ responses induced by two ionotropic glutamate receptor agonists, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA, 10 μM, 1 min) and N-methyl-D-aspartate (NMDA, 100 μM, 1 min), and significantly inhibited the AMPA-induced peak currents. Treatment with apigenin also significantly inhibited the $[Ca^{2+}]_i$ response induced by 50 mM KCl solution, decreased the $[Ca^{2+}]_i$ responses induced by the metabotropic glutamate receptor agonist, (S)-3,5-dihydroxyphenylglycine (DHPG, 100 μM, 90 s), and inhibited the caffeine (10 mM, 2 min)-induced $[Ca^{2+}]_i$ responses. Furthermore, treatment with apigenin (30 μM) significantly inhibited the amplitude and frequency of 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. These data together suggest that apigenin inhibits glutamate-induced calcium signaling in cultured rat hippocampal neurons.

Key Words: Apigenin, Intracellular calcium, Hippocampal neuron, Glutamate, Flavonoid

INTRODUCTION

Glutamate is a major excitatory neurotransmitter in the central nervous system. Glutamate induces opening of ligand-gated non-N-methyl-D-aspartate (non-NMDA) receptor channels that are permeable to mainly Na^+, which leads to depolarize membranes in neurons. This depolarization induces Ca^{2+} influx from the extracellular space through activation of NMDA channel and voltage-gated Ca^{2+} channels. In addition, glutamate induces increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by the release of Ca^{2+} from inositol-1,4,5-trisphosphate (IP_3)-senstive intracellular stores through metabotropic glutamate receptor-induced activation of phospholipase C (PLC). The $[Ca^{2+}]_i$ can be further increased by Ca^{2+}-induced Ca^{2+} release through ryanodine receptors. Ca^{2+} acts as an intracellular messenger that regulates many processes in cells such as cell growth, differentiation, synaptic transmission, excitation, and so on. An excess $[Ca^{2+}]_i$ increase, resulting from a massive glutamate release, induces cell death through many processes such as activation of proteases, lipases, and nucleases, and increase in NO production and free radical, and so on (Sattler & Tymianski, 2000).

Apigenin, one of the most common flavones, is widely distributed in many fruits and vegetables, including Chinese cabbage, bell pepper, garlic, celery and guava. It has been used for anxiolytic, sedative and antidepressant effects (Markakis et al, 2004). Apigenin exhibits neuroprotection against oxidative stress-induced cell death by blocking the caspase-3 activity in SH-SY5Y cells (Wang et al, 2001), or glutamate-induced neurotoxicity by reduction of NMDA receptor-mediated responses in cultured cortical neurons (Losi et al, 2004). In addition, apigenin has been reported to inhibit the influx of extracellular Ca^{2+} and release of intracellular Ca^{2+} in rat thoracic aorta (Ko et al, 1991), and NMDA or GABA receptor channels (Losi et al, 2004). However, there are detailed reports on the effect of apigenin on glutamate- induced $[Ca^{2+}]_i$ signaling in cultured rat hippocampal neurons.

In this study, we examined whether apigenin inhibits glutamate-induced $[Ca^{2+}]_i$ signaling in cultured rat hippocampal neurons, and also whether apigenin has an effect on the synaptically induced $[Ca^{2+}]_i$ spikes induced by 0.1 mM $[Mg^{2+}]_o$.

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ABBREVIATIONS: AMPA, alpha-amino-3-hydroxy-5'-methy1-4-isoxazolepropicnic; DHPG, (S)-3,5-dihydroxyphenylglycine; DMEM, Dulbecco's modified Eagle's medium; HHSS, HEPES-buffered Hank's balanced salt solution; IP_3, Inositol-1,4,5-triphosphate; $[Ca2^+]_i$, intracellular Ca^{2+} concentration; NMDA, N-methyl-D-aspartate; PLC, phospholipase C.

METHODS

Materials

Materials were purchased from the following companies: apigenin; Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) from Invitrogen (Carlsbad, CA); fura-2 acetoxymethyl ester (AM) from Molecular Probes (Eugene, OR); apigenin, NMDA, (S)-alpha-amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), (S)-3,5-dihydroxyphenylglycine (DHPG) and all other reagents from Sigma (St. Louis, MO).

Primary hippocampal cell culture

Rat hippocampal neurons were grown in primary culture as previously described (Shim et al, 2006) with minor modifications. Sprague-Dawley maternal rats (300~400 g) were used in this study. All experimental procedures performed on the animals were conducted with the approval of the Catholic Ethics Committee of the Catholic University of Korea and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (revised 1996). Fetuses were removed on embryonic day 18 from maternal rats anesthetized with urethane (1.3 g/kg b.w., i.p.). Hippocampi were dissected and placed in Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution, pH 7.4. Cells were dissociated by trituration through a 5 ml pipette and then a flame- narrowed pasteur pipette. Cells were pelleted and resuspended in DMEM without glutamine and supplemented with 10% fetal bovine serum and penicillin/ streptomycin (100 U/ml and 100 μg/ml, respectively). Dissociated cells were then plated at a density of 50,000 cells/well onto 25 mm round cover glasses that were coated with poly-D-lysine (0.1 mg/ml) and washed with H_2O. The cells were grown in a humidified atmosphere of 10% CO_2/90% air (pH 7.4) at 37°C. The media were replaced after 72~90 h of plating with DMEM supplemented with 10% horse serum and penicillin/streptomycin and fed every 7 days by exchange of 25% of the media. The cells were cultured without mitotic inhibitors for a minimum of 14 days, and were used between 10 and 13 days in culture. During this period, neurons developed extensive neuritic networks and formed functional synapses.

Digital $[Ca^{2+}]_i$ imaging

To measure $[Ca^{2+}]_i$, hippocampal cells were incubated for 45 min at 37°C in 10 μM fura-2 AM in HEPES-buffered Hank's balanced salt solution (HHSS: 20 mM HEPES, 137 mM NaCl, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 0.4 mM KH_2PO_4, 0.6 mM $Na_2H_2PO_4$, 3.0 mM $NaHCO_3$, and 5.6 mM glucose) containing 0.5% bovine serum albumin. The cover glass was then mounted in a flow-through chamber (Thayer et al, 1988) that was superfused at a rate of 1.5 ml/min. Digital calcium imaging was performed as described by Rhie et al. (Rhie et al, 2003). The chamber containing the fura-2 loaded cells was mounted on the stage of an inverted microscope (Nikon TE300, Tokyo, Japan), and alternately excited at 340 or 380 nm by rapidly switching optical filters (10 nm band pass) mounted on a computer-controlled wheel (Lambda 10-2, Sutter Instruments, Novato, CA) which was placed between a 100 W Xe arc lamp and the epifluorescence port of the microscope. Excitation light was reflected from a dichroic mirror (400 nm for fura-2) through a 20× objective (Nikon; N.A. 0.5). Digital fluorescence images (510 nm, 40 nm band-pass) were collected with a cooled charge-coupled device camera (1,280×1,035 binned to 256×207 pixels, Quantix, Photometrics, Tucson, AZ.) controlled by a computer. Image pairs were collected every 3~60 s using an Axon Imaging Work Bench 2.2 (Axon Instruments, Forster City, CA.); exposure to excitation light was 120 ms per image. $[Ca^{2+}]_i$ was calculated from the ratio of the two background-subtracted digital images. $[Ca^{2+}]_i$ was calibrated by converting the ratios to $[Ca^{2+}]_i$ by using the equation $[Ca^{2+}]_I = K_d \beta (R - R_{min})/(R_{max} - R)$(Grynkiewicz et al., 1985), where R is the 340/380 nm fluorescence ratio. The K_d used for fura-2 was 224 nM, and β was the ratio of emitted fluorescence at 380 nm in the absence and presence of calcium. R_{min}, R_{max}, and β were determined in ionomycin-permeabilized cells in calcium free and saturated solution (R_{max}=8.207, R_{min}=0.999, β=8.52).

Recording of $[Ca^{2+}]_i$ spikes using fura-2 based photometry

$[Ca^{2+}]_i$ spikes were determined using fura-2 based microfluorimetry (Shim et al, 2006). Cells were loaded, and placed by the same methods described in calcium imaging study. The bath was mounted on an inverted microscope (Nikon S-100F, Nikon, Tokyo, Japan), and the cells were superfused with HHSS at a rate of 1.5 ml/min for 15 min before starting the experiment. $[Ca^{2+}]_i$ spikes were induced by HHSS containing 0.1 mM $MgCl_2$ and 0.01 mM glycine. For excitation of fura-2, light from a 75 W Xe arc lamp (LPS-220, Photon Technology International, NJ) was passed through band-pass filters (340/20 and 380/20 nm, respectively). Excitation light was reflected sequentially from a dichroic mirror (400 nm) through a 40× phase contrast oil immersion objective (Nikon, Tokyo, Japan). The emitted light was reflected through a 510/40 nm filter to photomultiplier tube (Model 710, Photon Technology International) operating in photon-counting mode. Recordings were spatially defined with a rectangular diaphragm of photometer (Model D-104C, Photon Technology International). $[Ca^{2+}]_i$ was calibrated by the same methods described in calcium imaging study. R_{min}, R_{max}, and β in ionomycin-permeabilized cells were determined in calcium free and saturated solution (R_{max}=15.85, R_{min}=0.88, β=7.50).

Electrophysiological recordings

Whole-cell patch clamp recordings were performed in 35mm culture dishes at room temperature (21~24°C) on the stage of an inverted microscope (TS-100, Nikon, Japan). Cells were continuously superfused with extracellular solution containing (in mM) : 150 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 10 HEPES and 10 D-glucose (pH adjusted to 7.4 with NaOH and osmolality adjusted to 340 mosm/kg with sucrose) at a rate of 2~3 ml/min. Patch pipettes were pulled from thin-walled borosilicate glass tubing with filament using a P-97 micropipette puller (Sutter Instruments, Novato, CA). Pipette tips had resistances of 3.0~5.0 MΩ when filled with internal solution containing (in mM) : 140 CsCl, 2 $MgCl_2$, 5 EGTA and 10 HEPES (pH adjusted to 7.4 with CsOH, osmolality adjusted to 310 mosm/kg with sucrose). Whole-cell currents were amplified using an EPC-7 patch-clamp amplified (HEKA Elektronik, Lambrecht, Germany) and saved on PC using DigiData1322

and pClamp 8.2 software (Axon Instruments). Currents were filtered at 10 kHz using a three pole Bessel filter and digitized at 10 kHz. Capacitive currents were compensated with analog compensation, however, leak subtraction was not used in this study.

After establishing whole-cell configuration, cells were placed in front of one side of θ-tubing pipette. The extracellular solution, which contained AMPA, flowed through different side of θ-tubing which was pulled to an inner diameter of $\sim 300\,\mu$m. Solutions were rapidly switched around the cell using a piezoelectric translator (P-287.70, Physik Instruments, Waldbronn, Germany), which displaced the θ-tubing laterally so that the cell could be exposed for a defined period of time in drug containing solution, and then rapidly returned to drug free solution. The piezoelectric translator was controlled by a high voltage amplifier (E-507, Physik Instruments) and triggered by pClamp 8.2 software (Axon Instruments). Solution flow was driven by gravity from wells placed above the preparation, and the application of the solution was controlled by a valve placed upstream of the drug containing tubing.

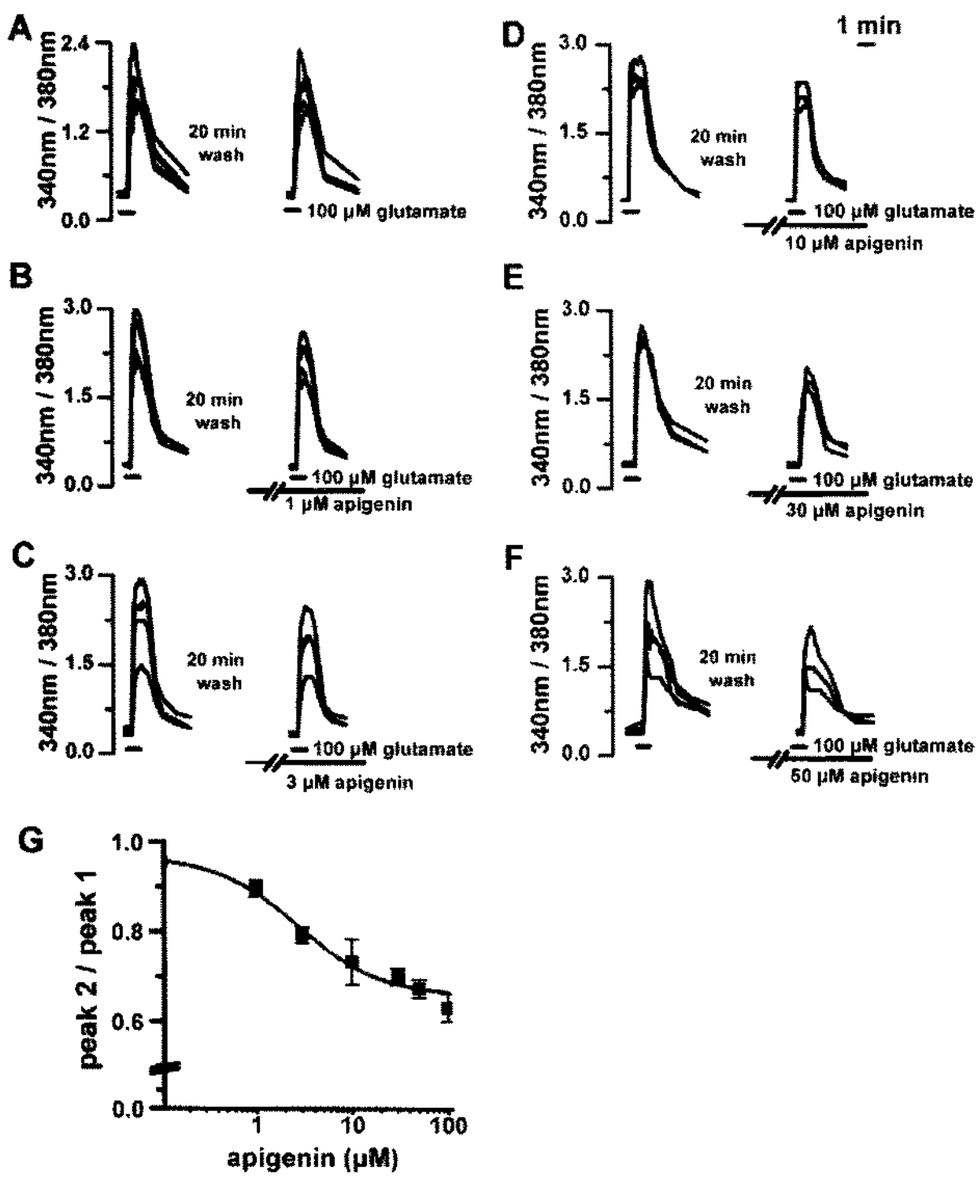

Fig. 1. Apigenin inhibits glutamate-induced $[Ca^{2+}]_i$ increases in cultured rat hippocampal neurons. **A**, Reproducible glutamate-induced $[Ca^{2+}]_i$ increases were induced by treatment with glutamate (100 μM) for 1 min at 20 min intervals. **B-F,** Pretreatment with apigenin for 5 min inhibited the glutamate-induced responses in a concentration dependent manner. **G**, Plot summarizes the inhibition of the glutamate-induced $[Ca^{2+}]_i$ increases by apigenin (1 μM, n =8; 3 μM, n=15; 10 μM, n=17; 30 μM, n=14; 50 μM, n=13; 100 μM, n=17). Glutamate-induced response is presented as a percentage of initial glutamate-induced $[Ca^{2+}]_i$ response (peak 2/peak 1) for apigenin pretreated cells. Data are expressed as means±SEM.

Data analysis

Data are expressed as means±SEM. Significance was determined with unpaired or paired Student's *t*-test, and one-way analysis of variance (ANOVA) followed by a Bonferroni's test.

RESULTS

Effects of apigenin on glutamate-induced $[Ca^{2+}]_i$ increases

Treatment with glutamate (100 μM) for 1 min evoked $[Ca^{2+}]_i$ increases in cultured rat hippocampal neurons. Reproducible responses could be elicited by applying 100 μM glutamate for 1 min at 20 min intervals (peak 2/peak 1 =97.5±1.4%, n=21) (Fig. 1A). Pretreatment with apigenin (1 μM) for 5 min did not significantly affect the glutamate-induced $[Ca^{2+}]_i$ responses. However, pretreatment with higher concentrations of apigenin (3~50 μM) inhibited the glutamate-induced responses in a concentration dependent manner (peak 2/peak 1 = 89.7±1.8% at 1 μM, n=8; 79.3±1.8% at 3 μM, n=15; 73.3±5.0% at 10 μM, n=17; 70.0±1.8% at 30 μM, n=14; 67.4±2.0% at 50 μM, n=13; 61.7±7.6% at 100 μM, n=17) (Fig. 1B~G).

Effects of apigenin on ionotropic glutamate receptor-induced $[Ca^{2+}]_i$ increases

In order to determine how apigenin inhibits the glutamate-induced $[Ca^{2+}]_i$ increases in hippocampal neurons, we first observed the effects of apigenin on non-NMDA receptors agonist, AMPA-induced $[Ca^{2+}]_i$ responses. Reproducible AMPA-induced $[Ca^{2+}]_i$ increase was induced by treatment with 10 μM (S)-AMPA for 1 min at 20 min intervals (peak 2/peak 1 = 92.1±4.5%, n=16) (Fig. 2A).

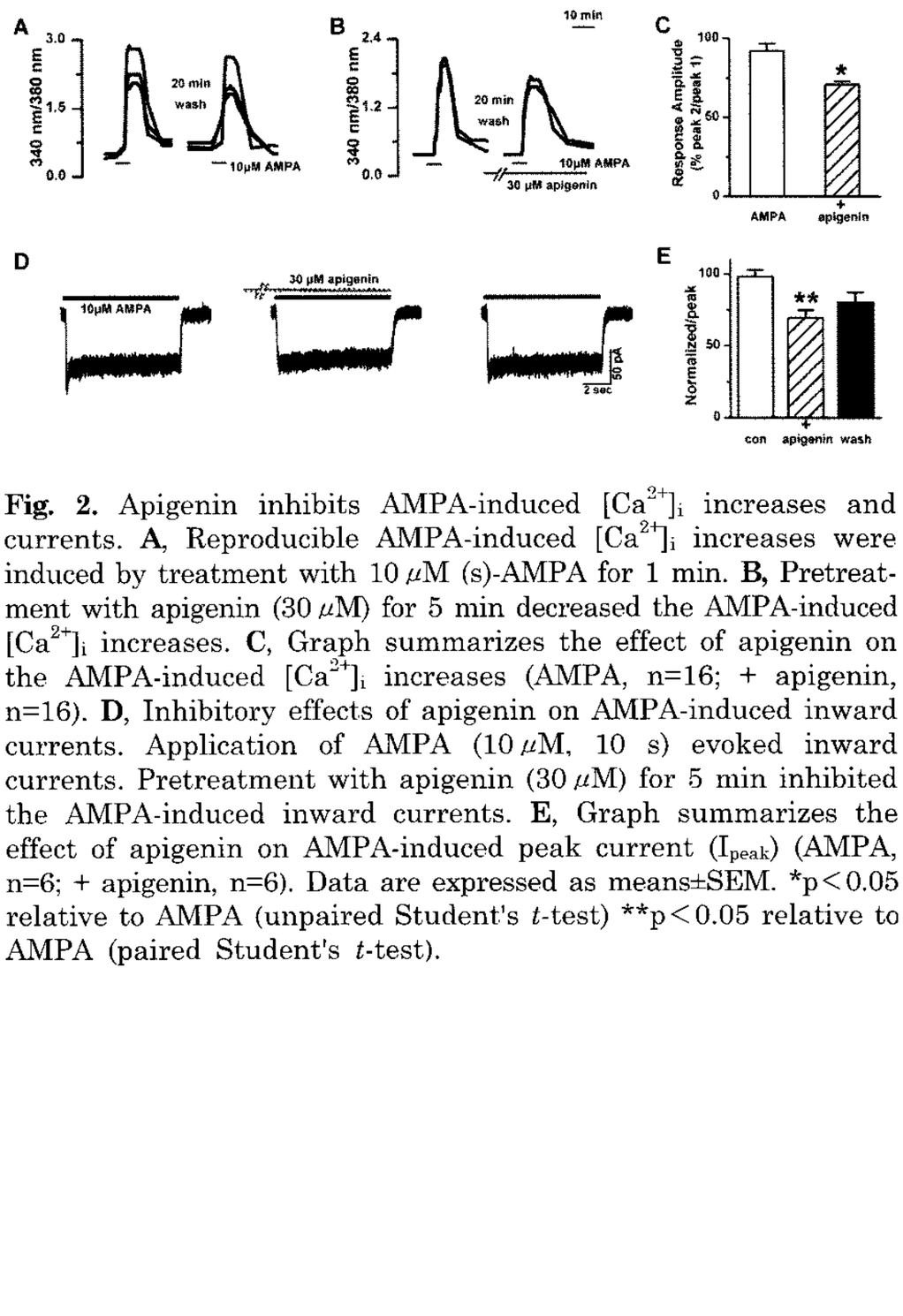

Fig. 2. Apigenin inhibits AMPA-induced $[Ca^{2+}]_i$ increases and currents. **A**, Reproducible AMPA-induced $[Ca^{2+}]_i$ increases were induced by treatment with 10 μM (s)-AMPA for 1 min. **B,** Pretreatment with apigenin (30 μM) for 5 min decreased the AMPA-induced $[Ca^{2+}]_i$ increases. **C**, Graph summarizes the effect of apigenin on the AMPA-induced $[Ca^{2+}]_i$ increases (AMPA, n=16; + apigenin, n=16). **D**, Inhibitory effects of apigenin on AMPA-induced inward currents. Application of AMPA (10 μM, 10 s) evoked inward currents. Pretreatment with apigenin (30 μM) for 5 min inhibited the AMPA-induced inward currents. **E**, Graph summarizes the effect of apigenin on AMPA-induced peak current (I_{peak}) (AMPA, n=6; + apigenin, n=6). Data are expressed as means±SEM. *$p<0.05$ relative to AMPA (unpaired Student's *t*-test) **$p<0.05$ relative to AMPA (paired Student's *t*-test).

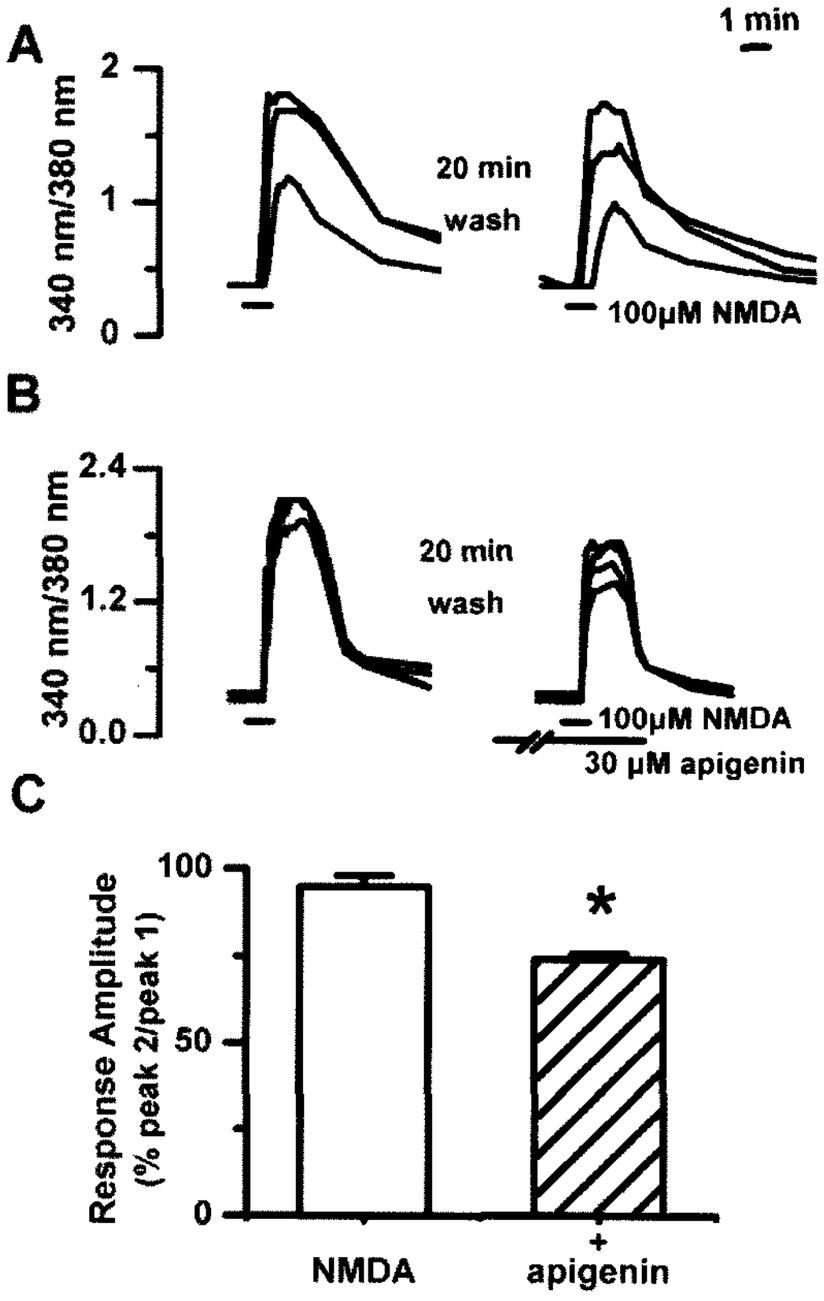

Fig. 3. Apigenin inhibits NMDA-induced $[Ca^{2+}]_i$ increases. **A**, Reproducible NMDA- induced $[Ca^{2+}]_i$ increases were induced by treatment with 100 μM NMDA for 1 min. **B**, Pretreatment with apigenin (30 μM) for 5 min decreased the NMDA-induced responses. **C**, Graph summarizes the effect of apigenin on the NMDA-induced responses (NMDA, n=17; + apigenin, n=13). Data are expressed as means±SEM. *$p<0.05$ relative to NMDA (unpaired Student's *t*-test).

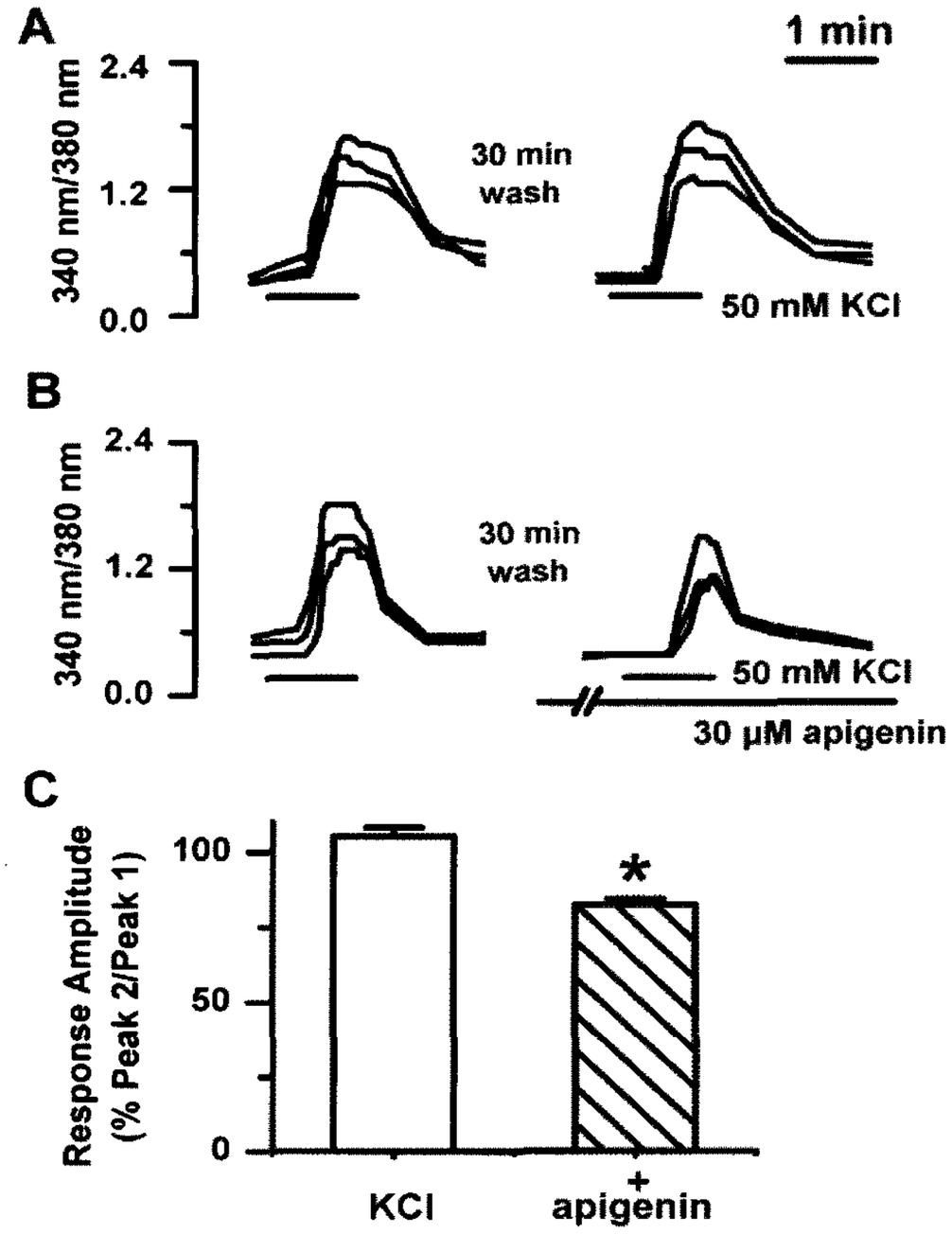

Fig. 4. Apigenin inhibits the high K^+-induced $[Ca^{2+}]_i$ increases. **A**, Reproducible high K^+-induced $[Ca^{2+}]_i$ increases were induced by treatment with HHSS containing 50 mM KCl for 1 min at 30 min intervals. **B**, Pretreatment with apigenin (30 μM) for 5 min decreased the high K^+-induced responses. **C**, Graph summarizes the effect of apigenin on the high K^+-induced responses (KCl, n = 63; + apigenin, n=17). Data are expressed as means±SEM. *$p<0.05$ relative to KCl (unpaired Student's *t*-test).

However, pretreatment with apigenin (30 μM) for 5 min significantly inhibited the AMPA-induced $[Ca^{2+}]_i$ responses (peak 2/peak 1 = 71.0±2.2%, n=16, $p<0.05$) (Fig. 2B and C).

Next, we confirmed whether apigenin inhibits AMPA-induced inward currents, using a whole-cell voltage- clamping technique. At a holding potential of −50 mV, reproducible AMPA-induced inward currents were elicited by treatment with 10 μM (S)-AMPA for 10 s at 5 min interval (peak current=98.12±4.8% of control responses, n =6). Pretreatment with apigenin (30 μM) for 5 min significantly inhibited the AMPA-induced peak currents (69.27±5.5% of control responses, n=6, $p<0.05$) (Fig. 2D and E).

In addition, we tested the effects of apigenin on another ionotropic glutamate receptor agonist, NMDA-induced $[Ca^{2+}]_i$ increases. Reproducible NMDA-induced $[Ca^{2+}]_i$ increases were induced by treatment with 100 μM NMDA for 1 min at 20 min intervals (peak 2/peak 1 = 96.7±3.3%, n=17) (Fig. 3A). Pretreatment with apigenin (30 μM) for 5 min also significantly inhibited the NMDA-induced $[Ca^{2+}]_i$ increases (peak 2/peak 1 = 74.2±1.7%, n=13, $p<0.05$) (Fig. 3B, C).

Effects of apigenin on high K^+-induced $[Ca^{2+}]_i$ increases

Glutamate-induced depolarization secondarily activates voltage-gated Ca^{2+} channels and induces $[Ca^{2+}]_i$ increase. To determine the effect of apigenin on the glutamate-induced secondary activation of Ca^{2+} channels, we examined whether apigenin affects the depolarization-induced $[Ca^{2+}]_i$ increases by 50 mM K^+ HHSS (Fig. 4). Reproducible $[Ca^{2+}]_i$ increases were induced by treatment with 50 mM K^+ HHSS for 1 min at 30 min intervals (peak 2/peak 1 = 95.7±1.5%, n=63) (Fig. 4A). Treatment with apigenin (30 μM) for 5 min inhibited the high K^+-induced $[Ca^{2+}]_i$ responses (peak 2/peak 1 = 83.6±1.8%, n=17, $p<0.05$) (Fig. 4B and C).

Effects of apigenin on metabotropic glutamate receptor agonist-induced $[Ca^{2+}]_i$ increases

Group I metabotropic glutamate receptors (mGluRs), composed of mGluR1 and mGluR5, are exclusively expressed in postsynaptic sites of the hippocampus (Lujan et al, 1996; Martin et al, 1992; Shigemoto et al, 1993). They can induce a release of Ca^{2+} from intracellular stores

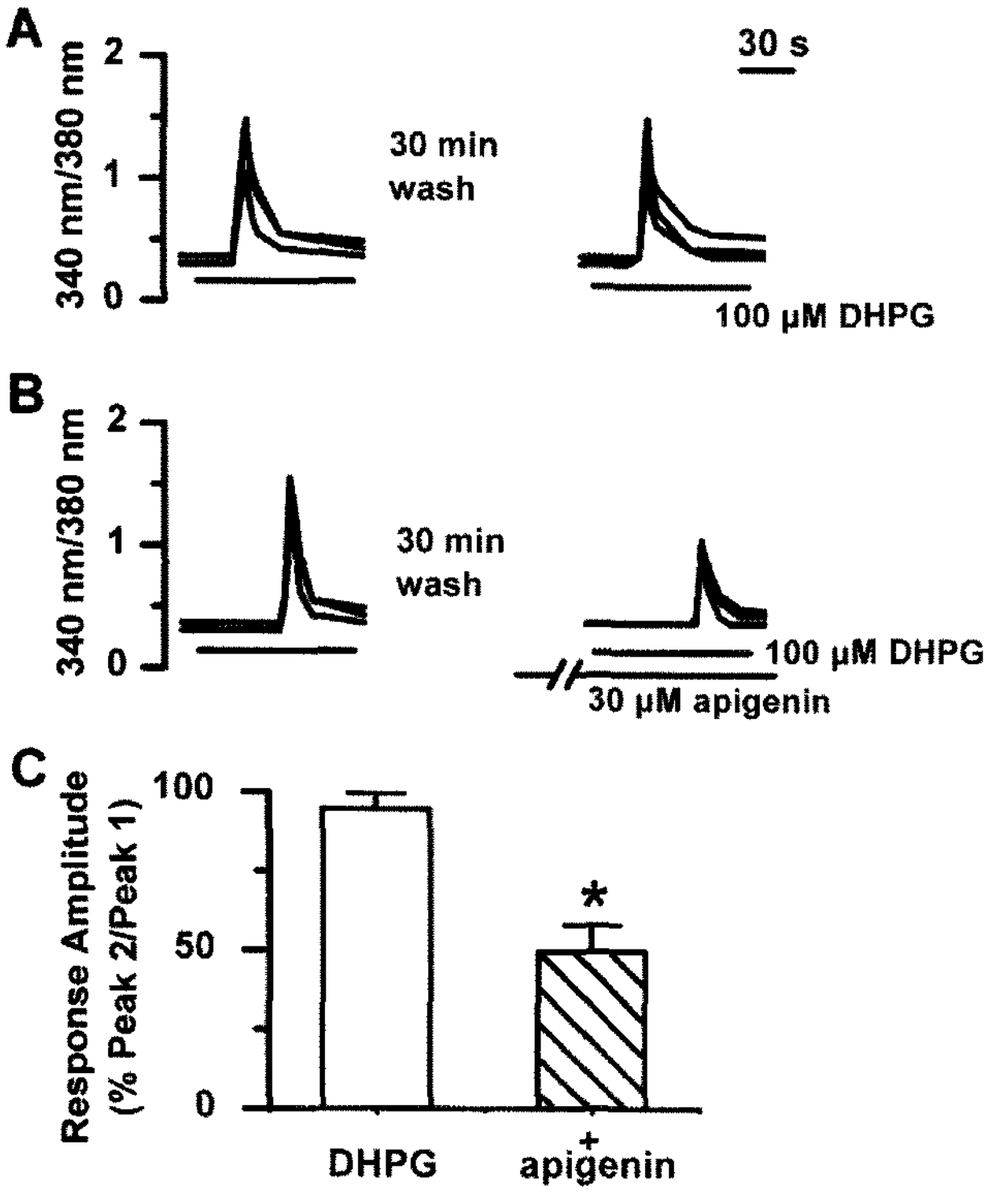

Fig. 5. Apigenin inhibits $[Ca^{2+}]_i$ increases induced by group I mGluR agonist DHPG. **A**, Reproducible DHPG-induced $[Ca^{2+}]_i$ increases were induced by treatment with 100 μM DHPG for 90 s at 30 min intervals. **B**, Pretreatment with apigenin (30 μM) for 5 min decreased the DHPG-induced responses. **C**, Graph summarizes the effect of apigenin on the DHPG-induced responses (DHPG, n=35; + apigenin, n=25). Data are expressed as means±SEM. *$p<0.05$ relative to DHPG (unpaired Student's *t*-test).

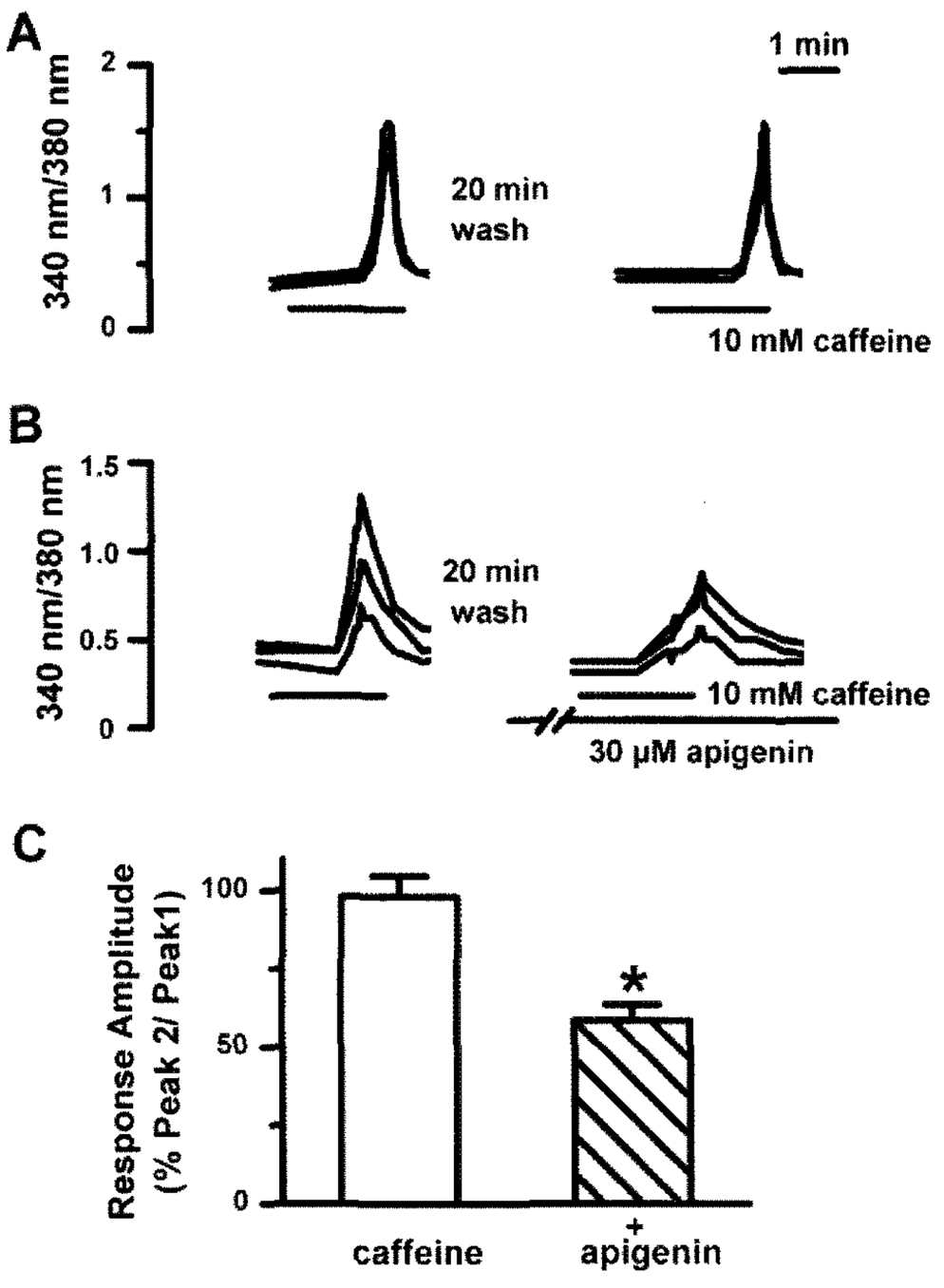

Fig. 6. Apigenin inhibits the caffeine-induced $[Ca^{2+}]_i$ increases. **A**, Reproducible caffeine-induced $[Ca^{2+}]_i$ increases were induced by treatment with 10 mM caffeine for 2 min at 20 min intervals. **B**, Pretreatment with apigenin (30 μM) for 5 min decreased the caffeine-induced responses. **C**, Graph summarizes the effect of apigenin on the caffeine-induced responses (10 mM caffeine, n=11; + apigenin, n=20). Data are expressed as means±SEM. *$p<0.05$ relative to caffeine (unpaired Student's *t*-test).

through an activation of PLC (Conn & Pin, 1997). Therefore, we examined whether apigenin affects the mGluRs-induced $[Ca^{2+}]_i$ increase. Reproducible $[Ca^{2+}]_i$ increases were induced by treatment with mGluRs agonist DHPG (100 μM) for 90 s at 30 min intervals (peak 2/peak 1 = 104.2±2.5%, n=35) (Fig. 5A). However, pretreatment with apigenin (30 μM) for 5 min significantly inhibited the DHPG-induced $[Ca^{2+}]_i$ responses (peak 2/peak 1 = 49.5± 8.3%, n=25, $p<0.05$) (Fig. 5B and C).

Effects of apigenin on caffeine induced $[Ca^{2+}]_i$ increases

In addition to IP_3 receptors, the increased $[Ca^{2+}]_i$ can further mobilize Ca^{2+} through Ca^{2+}-induced Ca^{2+} release from intracellular stores through ryanodine receptors following activation of glutamate receptors (Llano et al, 1994; Sandler & Barbara, 1999). Reproducible $[Ca^{2+}]_i$ increases were induced by treatment with the ryanodine receptor agonist caffeine (10 mM) for 2 min at 20 min interval (peak 2/peak 1 = 98.0±6.6%, n=11) (Fig. 6A). Pretreatment with apigenin (30 μM) for 5 min, however, inhibited the caffeine-induced $[Ca^{2+}]_i$ increases (peak 2/peak 1 = 58.4±5.2%, n=20, $p<0.05$) (Fig. 6B and C).

Effect of apigenin on the 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes

We examined whether apigenin affects the synaptically mediated $[Ca^{2+}]_i$ spikes induced by low $[Mg^{2+}]_o$. Reducing $[Mg^{2+}]_o$ to 0.1 mM elicited an intense pattern of $[Ca^{2+}]_i$ spikes (Fig. 7A), which depends upon synaptic transmission (Shim et al, 2006). Treatment with 0.1 mM $[Mg^{2+}]_o$ solution induced repetitive $[Ca^{2+}]_i$ spikes in the cultured rat hippocampal neurons. The low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes were significantly inhibited by treatment with apigenin (30 μM) (Fig. 7B). Also, treatment with apigenin (30 μM) significantly inhibited the amplitude and frequency of 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes.

DISCUSSION

In the present study, apigenin was found to decrease the glutamate-induced $[Ca^{2+}]_i$ increase by inhibiting AMPA-, NMDA- and high K^+,- and metabotropic glutamate receptor-induced $[Ca^{2+}]_i$ increase in cultured rat hippocampal neuron. Apigenin also inhibited caffeine- induced

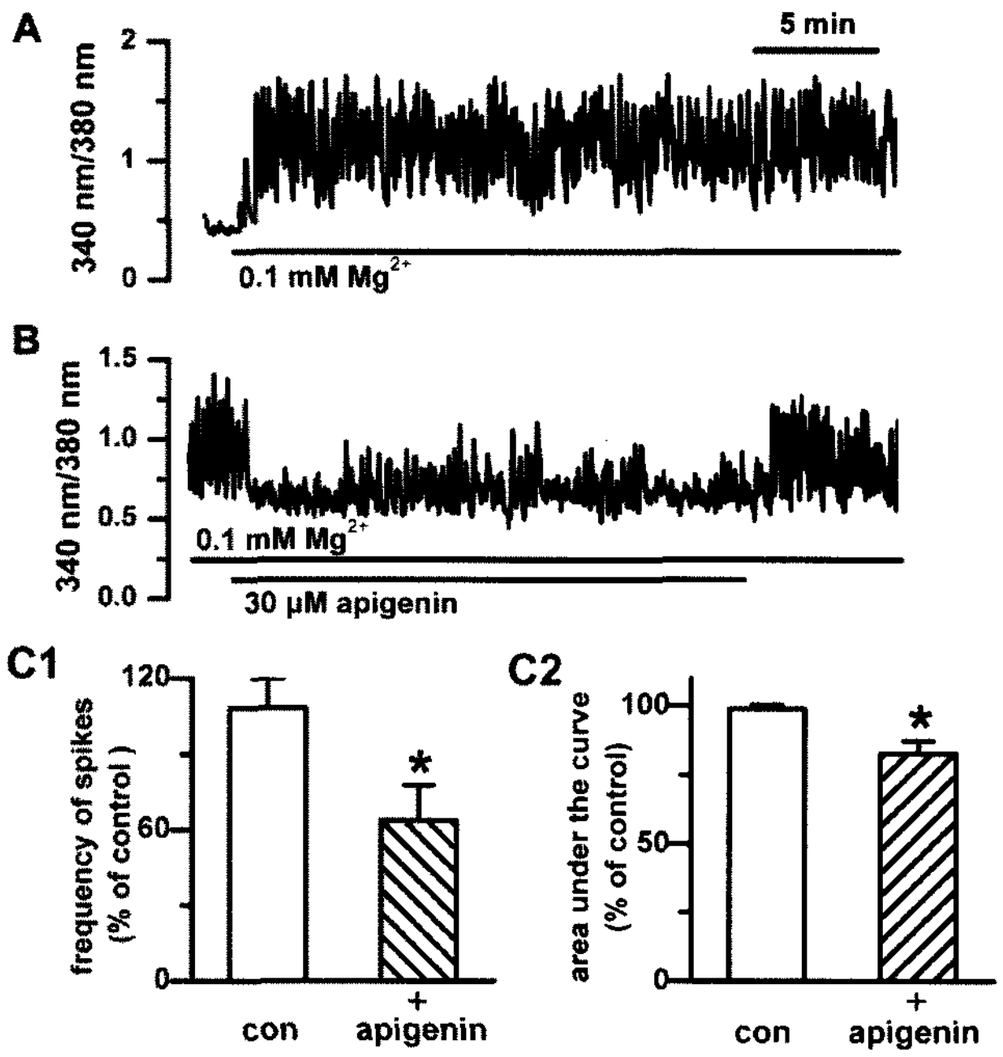

Fig. 7. Apigenin inhibits synaptically mediated Ca^{2+} spikes induced by treatment with 0.1 mM $[Mg^{2+}]_o$ in a cultured rat hippocampal neuron. **A**, Reducing the extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) to 0.1 mM induced $[Ca^{2+}]_i$ spikes. **B**, Treatment with apigenin (30 μM) inhibits the $[Ca^{2+}]_i$ spikes. **C1 & C2**, Graph summarizes the effect of apigenin on 0.1mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes (control, n=4; + apigenin, n=4) Data are expressed as means±SEM. *$p<0.05$ relative to 0.1mM $[Ma^{2+}]_o$ (unpaired Student's *t*-test).

Ca^{2+} release. In addition, apigenin inhibited the synaptically-mediated $[Ca^{2+}]_i$ increase spikes.

In this study, we observed that apigenin inhibited glutamate-induced $[Ca^{2+}]_i$ increases, AMPA-induced currents and AMPA-induced $[Ca^{2+}]_i$, suggesting that apigenin can inhibit the $[Ca^{2+}]_i$ increase by inhibiting the Ca^{2+} permeable AMPA receptors and the depolarization- induced $[Ca^{2+}]_i$ increases stimulated by activation of AMPA receptors. It has earlier been reported that Ca^{2+}-permeable AMPA receptors as well as Ca^{2+}-non permeable AMPA receptors are expressed in rat hippocampal neurons (Yin et al, 1999; Ogoshi & Weiss, 2003). In fact, apigenin decreased the NMDA-and high K^+-induced $[Ca^{2+}]_i$ increase. These data together suggest that apigenin inhibits Ca^{2+} influx from extracellular space by inhibiting AMPA channels, NMDA channels, and voltage-gated Ca^{2+} channels, in agreement with a report that apigenin inhibits NMDA channel (Losi et al, 2004). In addition, apigenin has been reported to inhibit an influx of extracellular Ca^{2+} and a release of intracellular Ca^{2+} in rat thoracic aorta (Ko et al, 1991).

The group I mGluR agonist, DHPG, induces a release of Ca^{2+} from IP_3-sensitive stores by activating PLC (Conn & Pin, 1997; Fagni et al, 2000). In this study, apigenin was found to inhibit the DHPG-induced $[Ca^{2+}]_i$ increase. However, it remains unclear whether apigenin inhibits Ca^{2+} release from IP_3-sensitive stores or DHPG-induced activation of PLC. Further study is needed to determine whether apigenin inhibits a release of Ca^{2+} from IP_3-sensitive stores or metabotropic glutamate receptor-induced activation of PLC. The ryanodine receptor agonist caffeine induces a release of Ca^{2+} from ryanodine-sensitive stores by Ca^{2+}-induced Ca^{2+} release (Bennett et al, 1998). In the present study, apigenin inhibited the caffeine-induced $[Ca^{2+}]_i$ increase, indicating that apigenin inhibits a release of Ca^{2+} from ryanodine-sensitive stores via Ca^{2+}-induced Ca^{2+} release as well as metabotropic glutamate receptor-induced release of Ca^{2+} from IP_3- sensitive Ca^{2+} stores.

In the present study, apigenin inhibited the glutamate-induced $[Ca^{2+}]_i$ increase by inhibiting AMPA-, NMDA-, and depolarization-induced Ca^{2+} influx. Apigenin also inhibited the metabotropic glutamate receptor-induced $[Ca^{2+}]_i$ increase and Ca^{2+}-induced Ca^{2+} release from intracellular stores. These data suggest a possibility that apigenin inhibits synaptic transmission. Reducing $[Mg^{2+}]_o$ bathing cultured CNS neurons elicits $[Ca^{2+}]_i$ spikes that depends upon glutaminergic synaptic transmission (Rose et al, 1990; Shen et al, 1996; Abel et al, 2000). In this study, apigenin inhibited the synaptically mediated low $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spikes. These data suggest that apigenin inhibits glutamatergic synaptic transmission in cultured rat hippocampal neurons by inhibiting AMPA-, NMDA-, and depolarization-induced Ca^{2+} influx as well as metabotropic glutamate receptor-induced release of Ca^{2+} from IP_3-sensitive intracellular stores and Ca^{2+}-induced Ca^{2+} release from ryanodine-sensitive stores. However, it has not been studied in the present study whether apigenin affects the release of glutamate in the presynaptic sites, although apigenin inhibited the high K^+-induced $[Ca^{2+}]_i$ increase in the soma.

Apigenin has neuroprotective effects against oxidative stress-induced cell death in SH-SY5Y cells (Wang et al, 2001), or glutamate-induced neurotoxicity in cultured cortical neurons (Losi et al, 2004). Our results showed that apigenin inhibits glutamate-induced calcium signaling. These results suggest a possibility that inhibitory effects of apigenin on glutamate-induced calcium signaling can partly be due to the neuroprotection against neuronal cell death, demonstrating a possibility that apigenin might be used as a neuroprotective agent against glutamate-induced neurotoxicity, partly through inhibition of calcium signaling.

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